CONTRIBUTION TO THE RISK ASSESSMENT OF CIGUATERA POISONING IN EUROPE (THE BALEARIC AND CANARY ISLANDS AND THE MADEIRA ARCHIPELAGO)

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DOCTORAL THESIS

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I STATE that the present study, **entitled "Contribution to the risk assessment of Ciguatera Poisoning in Europe (The Balearic and Canary Islands and the Madeira Archipelago)"**, presented by **Àngels Tudó Casanova** for the award of the degree of Doctor, has been carried out under my supervision at the Department of Basic Medical Sciences of this university, and at the IRTA Institute Sant Carles de la Ràpita, and that it fulfils all the requirements to be eligible for the International Doctor Award.

Reus, 25th May 2021,

Dr. Jorge Diogène **Dr. Francesc X. Sureda**

Dedicat a l'Anna Fusté

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ABREVIATIONS AND ACRONYMS

ATCC American Type Culture Collection

CBA cell-based assay

Ca^{2+} calcium

CP ciguatera poisoning

CTX ciguatoxin

EFSA European Food Safety Authority

FDA Food and Drug Administration

GA gambieric acid

HAB harmful algal bloom

HPLC high-performance liquid chromatography

HRMS high resolution accurate mass spectrometry

IC_{20} and **IC_{50}** concentrations producing 20 % and 50 % of cell viability

IUSA Institute of Animal Health and Food Safety

LC-MS/MS liquid chromatography-mass spectrometry

LC-HRMS liquid chromatography-high resolution accurate mass spectrometry

LOD limit of detection

LOQ limit of quantitation

MBA mouse bioassay

MRM Multiple reaction monitoring

MTT thiazolyl blue tetrazolium bromide

MTX maitotoxin

Neuro-2a CBA neuroblastoma cell-based assay

Neuro-2a neuroblastoma cell line

Na^{2+} sodium

O ouabain

RBA receptor binding assay

SPE solid-phase extraction

STT surface sea temperature

TRPV1 transient receptor potential cation channel subfamily V member 1

TTX tetrodotoxin

V veratridine

SUMMARY

The new climatic conditions, disturbances in the habitat and the exploitation of natural resources seem to stimulate the growth of harmful algal blooms (HABs). Given that these factors will not diminish in the coming years, the scientific community must make an effort to understand, prevent and mitigate the effects of HABs.

One of the concerns raised by HABs is Ciguatera Poisoning (CP), which occurs when humans consume fish or invertebrates that contain ciguatoxins (CTXs). These toxins are produced by benthic dinoflagellates of the *Gambierdiscus* and *Fukuyoa* genera and they are bioaccumulated through food webs. Apparently, the frequency and intensity of CP cases is tending to increase the world over. One of the reasons for this is the geographical expansion of the *Gambierdiscus* and *Fukuyoa* species.

The main goal of this thesis is to contribute to the risk assessment of CP in two zones in Europe: the West Mediterranean Sea (the Balearic Islands) which is a CP-free zone and the North East Atlantic Ocean (Madeira, Desertas, Selvagens, and the Canary Islands) where cases of CP have been detected since 2004. This risk assessment of CP is based on the geographical distribution of *Gambierdiscus* and *Fukuyoa* species, and the detection of the toxins in microalgae and in fish.

This thesis has contributed to the detection of two CP causative species: *G. australes* in the Balearic Islands and *G. belizeanus* in the Canary Islands. These findings have shown that the *Gambierdiscus* genus was not well reported in these areas. Although the *Gambierdiscus* and *Fukuyoa* strains from the Balearic Islands exhibited CTX-like compounds, no fish from this area have shown the presence of CTX-like toxins. In the Canary Islands, *G. excentricus* makes the greatest contribution to the existing cases of CP. In the Madeira archipelago, the positive CTX-like toxicity and the detection of CTX analogues in fish have shown that there is a need to extend the list of ciguateric species and focus on new ciguateric zones. All these findings are indicative of the little we know about CP in Europe.

RESUM

Les noves condicions climàtiques, l'alteració de l'hàbitat i l'explotació dels recursos naturals semblen afavorir les proliferacions d'algues nocives (PANs). Atès que aquests factors no disminuiran en els pròxims anys, la comunitat científica ha d'esforçar-se per comprendre, prevenir i mitigar els efectes dels PANs.

Una de les preocupacions relacionades amb les PANs és la *Ciguatera Poisoning* (CP), que és una intoxicació alimentària que es produeix quan els humans consumeixen peixos o invertebrats marins que contenen ciguatoxines (CTXs). Aquestes toxines són produïdes per les dinoflagel·lades bentòniques dels gèneres *Gambierdiscus* i *Fukuyoa* i s'acumulen al llarg de la xarxa tròfica. Aparentment, hi ha una tendència global en l'increment de la freqüència i la intensitat dels casos de CP. Entre les causes suggerides d'aquest augment hi ha l'expansió geogràfica de les espècies del gènere *Gambierdiscus* i *Fukuyoa*.

L'objectiu principal d'aquesta tesi és contribuir en l'evaluació el risc de CP en dues zones d'Europa: el Mediterrani occidental (les Illes Balears) que és una zona lliure de CP, i el nord-est de l'oceà Atlàntic (les Illes Canàries i l'Arxipèl·lag de Madeira) on s'han detectat casos de CP des del 2004. L'evaluació del risc es basa amb la identificació de la distribució geogràfica de les espècies dels gèneres *Gambierdiscus* i *Fukuyoa*, i amb la detecció de les toxines a les microalgues i en peixos.

Aquesta tesi ha contribuït a la detecció de dues espècies per primera vegada de *G. australes* a les Illes Balears i de *G. belizeanus* a les Illes Canàries. Aquestes troballes han demostrat que el gènere *Gambierdiscus* no estava ben registrat en aquestes zones. A més, malgrat que hi ha toxicitat positiva del tipus CTX de les soques de microalgues *Gambierdiscus* i *Fukuyoa* de les Illes Balears, cap peix d'aquesta zona ha mostrat la presència de toxines del tipus CTX. A les Canàries, *G. excentricus* s'ha mostrat com el màxim contribuent als casos existents de CP. A l'arxipèlag de Madeira, la toxicitat positiva del tipus CTX i els anàlegs de CTX confirmats en els peixos han demostrat la necessitat d'ampliar la llista de les espècies relacionades amb la CP i de centrar l'atenció en noves zones de l'arxipèlag. Tots aquests descobriments són l'evidència del poc coneixement que tenim de la CP a Europa.

CHAPTER 1

GENERAL INTRODUCTION

1. GENERAL INTRODUCTION

1.1 THE IMPORTANCE OF MICROALGAE

Microalgae are single-celled organisms with sizes that range between 0.2 and 200 µm and an extremely diverse morphology (Fig.1). They mainly live in the euphotic zone of seas and freshwaters worldwide and can live individually or form chains or groups. Microalgae constitute a paraphyletic group of various prokaryote and eukaryote evolutionary lineages. Recent insights into genetic diversity have revealed a greater number of species and added new lineages showing that the group is extremely branching [1]. Most of these lineages are autotrophic, meaning that they have the capacity to convert inorganic carbon to organic carbon by photosynthesis. However, obligate heterotrophs and mixotrophs also exist, which use only organic carbon, and a combination of autotrophic and heterotrophic nutrition, respectively.

The discovery of fossilized cyanobacteria from 2.4 billion years ago showed that these photosynthetic microorganisms created our oxygen-rich atmosphere, which has been essential to the evolution of life [2]. In fact, microalgae have essential roles in numerous biogeochemical systems. They generate 50 % of the atmospheric oxygen and primary production on the planet [3]. Besides, their ability to fix the inorganic carbon (CO_2) to produce organic carbon via photosynthesis plays an essential role in the global carbon cycle. Organic carbon is produced and can be transferred to other trophic levels or can be sequestered towards deeper levels of the sea thus controlling the global Earth's climate [4]. Moreover, microalgae are essential in aquatic food webs. A further role is that they regulate the general biochemistry, taking up, transforming and recycling nutrients, such as nitrogen, iron, and phosphorus, which are crucial for other organisms [5].

In recent years, interest in microalgae has increased because of the role they play in regulating CO_2 in climate change but also because they are a renewable, sustainable source of biofuels [6], food [7], and bioactive compounds [8,9].

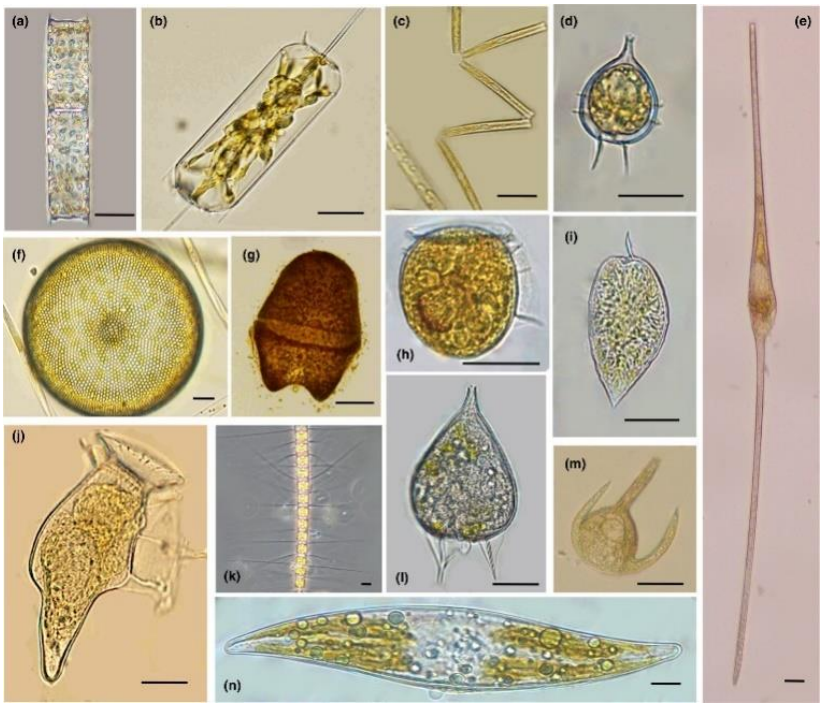

Figure 1. Examples of phytoplankton cells (a) *Cerataulina pelagica* (Cylinder); (b) *Ditylum brightwellii* (prism on triangular base); (c) *Thalassionema nitzschioides* (parallelepiped); (d) *Protoperidinium* sp. (cone + half sphere); (e) *Tripos fusus* (double cone); (f) *Coscinidiscus* sp. (cylinder); (g) *Akashiwo sanguinea* (ellipsoid); (h) *Phalacroma* sp. (ellipsoid); (i) *Prorocentrum micans* (cone + half sphere); (j) *Dinophysis caudata* (ellipsoid + cone); (k) *Chaetoceros didymus* (prism on elliptic base); (l) *Podolampas bipes* (cone); (m) *Tripos* sp. (ellipsoid + 2 cones + cylinder); (n) *Pleurosigma* sp. (prism on parallelogram base). Scale bar = 20 µm. Source: Ryabov et al. (2021) [10].

Normally, these chemical compounds are secondary metabolites, which are the molecules that originate from the metabolic pathways that are not directly involved in the growth, development, or reproduction of the organism and therefore are not essential for the life of the producing organisms. The chemical structures and physiological activities of secondary metabolites from microalgae are extremely diverse. Some advantages of these molecules are that microalgae are not consumed by grazers [11,12], they colonize niches (competing with other species) and, in mixotrophic species, they can act as a venom to immobilize prey [13]. Therefore, they can play a significant role in ecological processes. However, the evolution and functional role of most secondary metabolites remains unclear.

1.2. MICROALGAL BLOOMS

In favourable conditions, microalgae can proliferate and cause algal blooms. This is a natural phenomenon. The high density of microalgal cells can cause an innocuous discoloration of water, which is typically known as “red tide”. However, these microalgal proliferations can sometimes cause problems, which are known as “harmful algal blooms” (HABs).

Historically, the term HABs has been used to describe massive algal proliferations but it has also been used to refer to blooms of species that can be very dangerous at very low densities. Besides, the term includes the proliferation of both microalgae and macroalgae or seaweeds [14]. Some proliferations can be so thick that they prevent light from reaching deeper levels or create a hypoxic environment affecting other communities [15,16]. Moreover, microalgae can produce toxins as secondary metabolites, which can or can not be released into the environment. Toxins can have fatal effects on other organisms. It is estimated that approximately 80 species of the approximately 5000 microalgae can produce toxins, so only 2-3 % of species can produce HABs [17].

The increase in coastal uses and scientific research means that humans are more aware of HABs. Apparently, blooms are more frequent, more intense and more widely distributed all over the world [14,18]. Some of the plausible reasons for this are the increase in aquaculture and fisheries, the degradation of the coast, changes in nutrient loads, translocation by ballast water and climate change [19]. The demand for fish and the overexploitation of the sea has promoted aquaculture in recent decades. Fisheries often change the surrounding waters by, for example, enriching the nutrients, which can favour the growth of some microalgal species. Furthermore, new structures such as ports, hotels and oil platforms have clearly degraded the coast. The natural disturbances caused by climate change such as the increase in the number of extreme events (hurricanes and storms), coral bleaching and global warming seem to play a role in the biogeographical expansion of microalgae [19]. It seems that non-indigenous microalgal species now exist in areas where before they were absent [14,20] or they are more abundant, and they have started to create emerging problems [21, 22].

Some factors such as surface sea temperature (SST) or thermal stratification are well known to influence HAB dynamics [24]. However, HABs are not easy to predict. One of the main problems is to discern the most important factors that are involved in microalgal proliferation [25]. Moreover, HAB response to factors can be non-linear, and regions have different chemical characteristics which vary over seasons and

years and in response to anthropogenic activities. All these conditions make difficult to rely on the past to solve future HAB concerns.

1.2.1. Impacts of HABs

Economic impact

The high biomass of microalgae can create of hypoxia or anoxia events that affect other marine organisms. In fisheries, non-toxic microalgal cells can kill fish by obstructing or damaging their gills [26], while toxic cells can release haemolytic or neurotoxic compounds [27]. HABs can cause high economic losses in fisheries or aquaculture. Even though the economic impact is evident, few studies have evaluated this aspect. In the US, for instance, annual losses between 1987 and 2000 were estimated to be about 75 million USD [28]. In the European Union (EU), it was estimated that the annual cost of monitoring and losses at 800 million USD [28]. Currently, seafood companies have specific management strategies to anticipate or prevent HABs [29]. Some of these strategies are monitoring microalgal toxic species or their preys (for mixotrophs), detecting toxins in the water column, building blocks to separate the harvesting areas and the blooms, or diminishing the external inputs that are favourable for bloom growth. Companies also have measures to control HABs when they have already appeared. Some use grazers or viruses to limit the microalgal population or block regions to prevent the bloom from expanding. Nonetheless, measures for preventing and mitigating HABs are often limited so companies basically focus on reducing their impact. For instance, seafood harvesting is reduced, or seafood is towed in net pens to sites that are less affected by HABs.

HABs can also have an impact on the tourism sector. They can make changes in water coloration and foam and sometimes leave residues. If this happens in recreational areas, they may be less appealing to tourists, which will have a clear impact on the economy [30].

Ecological impact

Both toxic and non-toxic HABs can cause ecological damage. Generally, the ecological consequences of HABs are not as evident as the economic or health impacts and, for a long time, they have been ignored. HABs create unusual environmental conditions: for example, they can cause less light to reach the depths below the bloom. Besides, when blooms decline, and microalgal cells start to die, the bacterioplankton, viruses and protozoa increase, which can create hypoxia (Fig. 2). This situation may displace some species and encourage other opportunistic species. On the other hand, toxins can create episodes of non-grazing, perhaps because of the nutritional inadequacy of the zooplankton, and modulate the population of the higher levels of grazers [31].

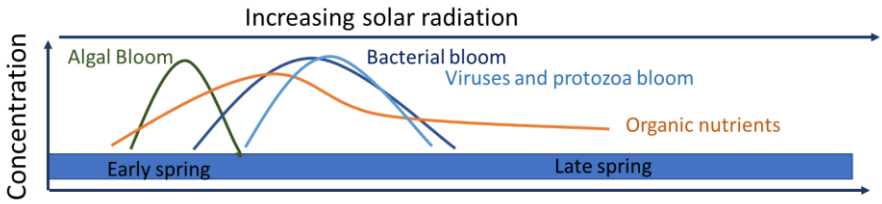

Figure. 2 Scheme depicting the algae-bacteria interactions.

Social impact

Like ecological impacts, the social impacts of HABs are often only noticeable after a long time. Nonetheless, there are clear examples of changes after HAB events: for example, the diet. The diet of humans from the Pacific islands basically consists of seafood, but when HABs cause poisoning, people are forced to substitute seafood for other food alternatives [32]. Moreover, Rongo et al. [33] suggested that the search for alternative food sources when there is a high prevalence of ciguatera poisoning has probably caused extensive migrations from the present French Polynesia. Furthermore, the increase in poisoning events has forced fishermen from Rarotonga (Cook Islands, Pacific Ocean) to change their fishing activities [32].

Impact on health

Most HABs are composed largely of non-toxic microalgae, but there is a small fraction that can harm or kill seabirds [34], marine mammals [35], humans [36] and other organisms [37]. For example, in the Mediterranean Sea, proliferations of *Ostreopsis* sp. produce toxic aerosols that cause respiratory and cutaneous irritation

in humans [22]. Moreover, there is a significant health impact when secondary metabolites that enter the food web and accumulate in fish or shellfish are consumed by humans or marine organisms. There are several types of toxin-driven seafood poisonings: paralytic shellfish poisoning (PSP), diarrhetic shellfish poisoning (DSP), ciguatera poisoning (CP), neurotoxic shellfish poisoning (NSP), azaspiracid shellfish poisoning (AZP), palytoxicosis, cyanobacterial poisoning, amnesic shellfish poisoning (ASP) and tetrodotoxin poisoning. Except for the last one, these poisonings are caused by biotoxins produced by microalgae. Table 1 shows the principal marine poisonings.

The present thesis focuses on the risk hazard assessment of one type of marine poisoning: ciguatera (CP). CP is caused by the consumption of fish or seafood that contain ciguatoxins (CTXs). *Gambierdiscus* [38] and *Fukuyoa* [39], two very close genera of dinoflagellates that produce CTXs. Historically, CP afflicted only populations in tropical regions, in the Pacific and Indian Ocean and the Caribbean Sea (35°N and 35°S) [40]. CP now seems to be expanding to temperate areas, outside the latitudes of classical CP endemic regions [41,42]. Among the reasons for this are the increasing numbers of travellers returning from tropical areas [42,43] and the increase in the global seafood trade [45]. Other suspected reasons are the biogeographical expansion of CTX-producing species, the increasing abundance of the populations of CTX-producing species and changes in the migration patterns of fish [46]. These suspected reasons are influenced by new climate change conditions and anthropogenic factors. The expansion of CP has revealed a clear need for international collaboration to monitor and manage the disease. To this end, several fields of study such as cell and molecular biology, toxicology, chemistry, IT sciences and modelling need to be integrated.

Table. 1 Main marine HAB toxic syndromes. Adapted from Berdalet et al. (2016).

	Amnesic shellfish poisoning	**Azaspiracid shellfish poisoning**	**Paralytic shellfish poisoning**	**Diarrheic shellfish poisoning**	**Neurotoxic shellfish poisoning**	**Ciguatera poisoning**	**Palytoxicosis**
Biotoxins	Domoic acid and isomers	Azaspiracid and its derivatives	Saxitoxin and its derivatives	Okadaic acid and its derivatives	Brevetoxins	Ciguatoxins	Palytoxin, ostreocin, ovatoxin
Causative agent	*Pseudo-nitzschia* spp. *Nitzschia*	Amphidomataceae (*Amphidoma* spp. *Azadinium* spp.)	*Alexandrium* spp. *Gymnodinium catenatum, Pyrodynium bahamense* var. *compressum* Some calcareous red macroalgae	*Dinophysis* spp. *Prorocentrum lima*	*Karenia brevis* *K. papilionacea* *K. selliformis* *K. bicuneiformis*	*Gambierdiscus* spp. Probably *Fukuyoa* spp.	*Ostreopsis* spp.
Symptoms	Nausea, vomiting, diarrhoea, headache, dizziness, confusion, disorientation, memory deficits, arrythmia, respiratory distress, coma and possibly death	Nausea, vomiting, diarrhoea, abdominal cramps, probably damage in liver, intestine and spleen	Tingling, sensation or numbness around lips, face and neck; prickly sensation in fingertips and toes; headache, dizziness, vomiting	Diarrhoea, nausea, vomiting, abdominal cramps, respiratory distress	Chills, temperature sensation reversals, muscle weakness, muscle and joint pain, headache, diarrhoea, nausea, and vomiting. The aerosols can cause respiratory and eye irritation	Cold allodynia, paresthesia, nausea, vomiting, diarrhoea, numbness of mouth and extremities. Cardiac and neurological symptoms can persist during months.	Nausea, vomiting, diarrhoea, abdominal cramps, lethargy, tingling of the lips mouth, face and neck, lack of sensation, myalgia, weakness, hypersalivation, difficulty of breathing. Aerosols cause, eye, and nose irritation, rhinorrhoea, malaise fever and cutaneous irritations
Route of exposoure	Shellfish, probably fish	Shellfish	Shellfish crustaceans and fish	Shellfish	Shellfish, and inhalation of the aerosols from blooms	Consumption of reef fish, gastropods, molluscs, sea urchins	Seafood, and inhalation of aerosols, contact with water
Main affected areas	Worldwide	Europe, Africa and North America	Worldwide	Worldwide	Gulf of Mexico, Japan, China, Korea and New Zealand.	Tropics, subtropics, and some temperate areas	Tropics and subtropics, respiratory problems in the Mediterranean Sea.

1.3. CIGUATERA POISONING (CP)

Ciguatera poisoning occurs when humans consume fish or invertebrates (sea urchins, gastropods, bivalves and echinoderms) contaminated by ciguatoxins (CTXs) [47,48]. CTXs are microalgal toxins produced by two genera of benthic dinoflagellates, *Gambierdiscus* and *Fukuyoa* [49]. These toxins are transferred, metabolized and bioaccumulated in the upper levels of food webs [40]. CP is an ancient disease. The first report of ciguatera poisoning (CP) was in 1521. The Spanish army were in the Gulf of Guinea where they ate a barracuda (*Sphyraena* sp.) which gave them diarrhoea [50].

The first symptoms of CP are typically gastrointestinal (vomiting, diarrhoea, abdominal pain), which can last a few hours or days, followed by cardiac (hypotension and bradycardia), systemic (fatigue, dizziness) and neurological disorders (e.g. cold allodynia and generalized itching). Some symptoms such as fatigue, myalgia and headaches can recur for months or even become chronic [36,51,52]. Fatal cases are rare [53,54].

CP is diagnosed basically by the symptomatology and the patient's recent fish-eating history since there are no biochemical markers [55–57]. At present, more than 175 symptoms have been described [46]. As a consequence, the variability of symptoms can lead to confusion with other types of poisoning [58], and this makes it more difficult to diagnose and estimate the precise number of cases. In fact, it is estimated that only 2-10 % of cases are reported to the health authorities [59,60]. One reason for this may be the difficulty of diagnosing CP cases. In addition, the fact that patients may not attach any importance to the poisoning they experience may also explain why CP cases are underreported. Nevertheless, some studies claim that the number of cases range from 25,000 to 500,000 per year [61,62], which makes CP the most common non-bacterial seafood poisoning in the world [46]. Despite this, at present, there is no validated treatment for CP cases.

Currently, preventing CP is a difficult task, since CTXs are thermostable, so no cooking, salting, or freezing method can eliminate or diminish them. In addition, CTXS are odourless, tasteless and cannot be detected from the appearance of seafood. Furthermore, more than 400 fish species have been related to the disease and other organisms such as sea urchins [63] and molluscs [64] can also be involved.

1.3.1. Dinoflagellates, CP causative agents

The first association between dinoflagellates and CP was when Yasumoto and colleagues [65] were in the Gambier Islands (French Polynesia) during an outbreak of CP in 1976. They linked the toxic dinoflagellates from dead coral to the toxicity of the content in the stomachs of some fish. The dinoflagellates were erroneously identified as *Diplopsalis* [65], and later they gave rise to the new genus *Gambierdiscus* [38] (within the order *Peridinales* and the family *Heteraulaceae*). For a long time, the only species of the genus was *Gambierdiscus toxicus* [40,66,67] and the genus was thought to be confined to tropical and subtropical areas [68]. Moreover, the *Gambierdiscus* genus contained species exhibiting either globular or anterior-posteriorly compressed morphologies.

After a taxonomic re-evaluation of the genus *Gambierdiscus*, the globular species gave rise to the new genus *Fukuyoa* [39] and *G. toxicus*, which had a compressed morphology, gave rise to a large number of species [68]. Figure 3 shows an example of the two types of morphology and figure 4 shows the topology of rDNA-based phylogenetic tree for *Gambierdiscus* and *Fukuyoa* species. At present, the *Gambierdiscus* genus comprises eighteen species while the genus *Fukuyoa* comprises three species, many of which co-occur and both genera have now been reported in other areas [69,70]. Table 2 reports the *Gambierdiscus* and the *Fukuyoa* species and their geographical distribution.

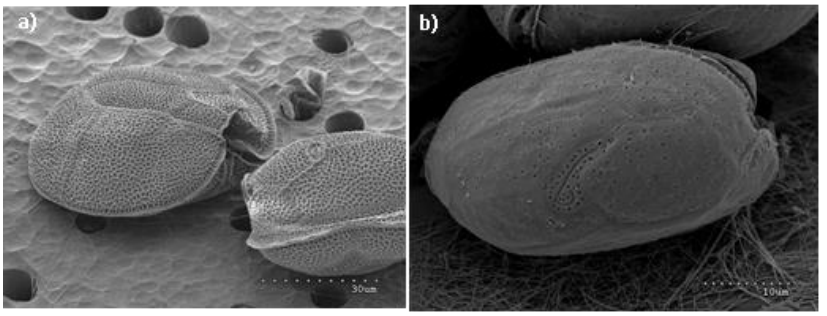

Figure 3. a) *Gambierdiscus belizeanus* and b) *Fukuyoa paulensis*. Scale bar: a) 30 µm and b) 10 µm, . Source: Tudó, À.

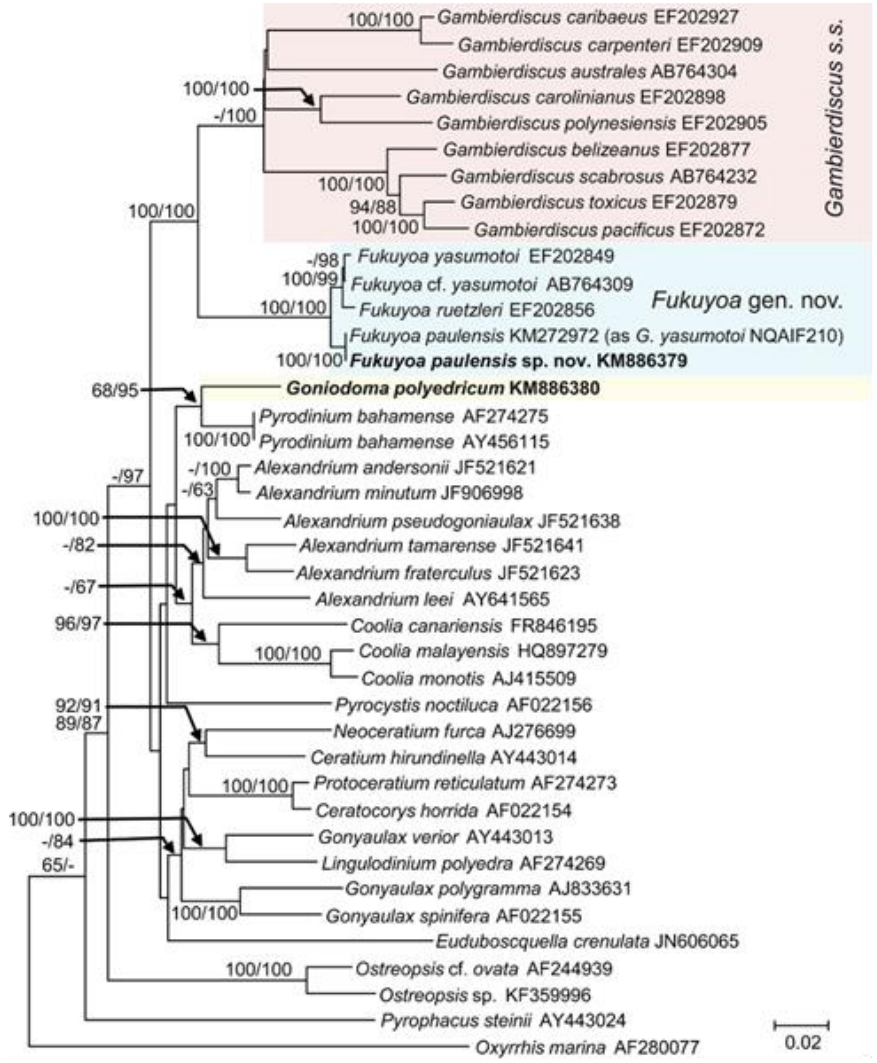

Figure 4. SSU rDNA-based phylogeny including the *Gambierdiscus* and *Fukuyoa* genera with some gonyaulacoid dinoflagellates. Support of nodes is based on bootstrap values of ML/NJ with 1000 and 500 resamplings, respectively. Only values greater than 60 are shown. *Oxyrrhis marina* was used as outgroup. Source: Gómez et al. (2015) [39].

Table 2. Geographical distribution of the *Gambierdiscus* and *Fukuyoa* species.

Species	Geographical distribution	References
F. paulensis	Brazil (West Atlantic Ocean) Balearic Islands (West Mediterranean Sea)	[39, 71]
F. ruetzleri	Belize, Gulf of Mexico and North Carolina (North West Atlantic Ocean)	[72]
F. yasumotoi	New Zealand (Pacific Ocean) Australia (Pacific Ocean) Japan (Sea of Japan)	[74, 75, 76]
Fukuyoa ribotype HK Type 1	China, Pacific Ocean	[77]
G. australes	Pacific Islands (Pacific Ocean) Canary Islands and Madeira Archipelago (North East Atlantic Ocean) Balearic Islands (West Mediterranean Sea) China (Pacific Ocean) Japan (Sea of Japan)	[78, 79, 80, 81, 76]
G. balechii	Indonesia, Celebes Sea (Pacific Ocean)	[82]
G. belizeanus	(USA), Bahamas, Bermuda, Cancun (North West Atlantic) Canary Islands (North East Atlantic Ocean) Eastern Mediterranean Sea Red Sea	[72, 80, 84, 85]
G. caribaeus	Belize, Central America, (Caribbean Sea) Canary Islands China (Pacific Ocean)	[72, 21, 81]

G. carolinianus	North Carolina, (West Atlantic Ocean) Canary Islands (Est Atlantic Ocean) Eastern Mediterranean Sea	[72, 21, 86]
G. carpenteri	Belize, Central America, Caribbean Sea Guam, Mariana Islands (Pacific Ocean)	[72]
G. cheloniae	Pacific Islands (Pacific Ocean)	[87]
G. excentricus	Canary Islands, Madeira (North East Atlantic Ocean)	[79, 88]
G. holmesii	Australia (Pacific Ocean)	[89]
G. honu	Pacific Islands (Pacific Ocean)	[95]
G. jejuensis (Gambierdiscus ribotype 2)	Jeju Island, Korea, East China Sea	[91]
G. lapillus	Australia (Pacific Ocean)	[92]
G. lewisii	Australia (Pacific Ocean)	[89]
G. polynesiensis	Pacific Islands (Pacific Ocean)	[78]
G. pacificus	Pacific Islands (Pacific Ocean) China (Pacific Ocean)	[78] [81]
G. scabrosus	Japan (Sea of Japan)	[93]
G. silvae	Canary Islands (North East Atlantic Ocean)	[94]
G. toxicus	Australia (Pacific Ocean) Pacific Islands (Pacific Ocean)	[40, 66]
Gambierdiscus ribotype 2	Belize, Central America (Caribbean Sea)	[69]

Gambierdiscus sp.* type *3	Japan (Pacific Ocean)	[76]
Gambierdiscus sp. type 4	Marakei, Republic of Kiribati (Pacific Ocean)	[96]
Gambierdiscus sp. type 5	Marakei, Republic of Kiribati (Pacific Ocean)	[96]

1.3.2. Which metabolites play a role in CP?

It is well established that CP occurs after the consumption of seafood containing CTXs [37,47,56,71]. CTXs are polyethers with 13 or 14 ether rings and molecular weights of ~1000–1150 Da. So far, at least 37 congeners of CTXs have been described [73]. Table 3 shows the congeners and analogues of CTXs. At first, CTX classification was based on the geographic origin of the fish in which CTX analogues had been found: P-CTXs (Pacific Ocean), C-CTXs (Caribbean Ocean) and I-CTXs (Indian Ocean) [97,98,99]. At present, the above-mentioned classification is controversial since different types of CTX can be found in several zones. For instance, the CTXs classified as Caribbean (C-CTXs) are also found in the Atlantic Ocean [98,99]. Presently, even though CTX classification based on geographic origin is still used, experts recommend classifying CTXs in terms of chemical structure [46]. Within the group of P-CTXs (13 ether rings) there are two types: P-CTX1B or CTX1B and CTX3C. Figure 5 shows the most common CTXs detected in matrix fish.

The classical Caribbean CTXs (C-CTXs) and Indian CTXs (I-CTXs) have 14 ether rings. CTXs have been isolated from fish and shellfish matrixes [100] but also from the producers, *Gambierdiscus* and *Fukuyoa* cultures [101]. The complexity of their structure could explain part of the huge variety of CP symptoms. CTX1B (also known as P-CTX-1B) seems to be responsible for neurological symptoms, whereas C-CTX is responsible for gastrointestinal ones. It is thought that the oxidation of CTXs by fish metabolic pathways increases the toxicity of CTXs [102]. Nonetheless, symptoms also vary depending on the dose and the physical condition of the individuals affected [103].

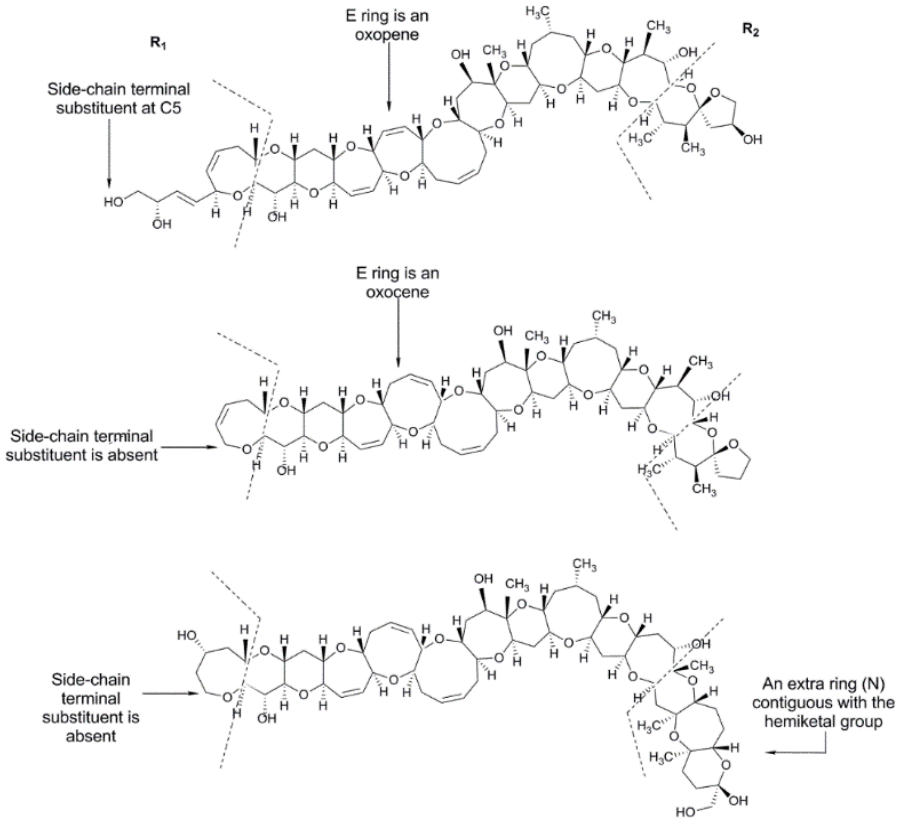

Figure 5. Structure of the most common ciguatoxins detected in fish: a) CTX1B, b) CTX3C and c) C-CTX-1.

Table 3. Known CTX congeners. Source: Soliño and Costa (2018) [100].

CTX Type	CTX congener	Other names	Formula	Molecular Weight
P-CTX-1	CTX4B	Scaritoxin1, SG-1, SCTX, Gambierotoxin 4B, GT4B, GTX4B, P-CTX4B	$C_{60}H_{84}O_{16}$	1060.6
	CTX4A	Scaritoxin1, SG-1, SCTX, Gambierotoxin 4A, GT4A, GTX4A, P-CTX4A, 52-epi CTX4B	$C_{60}H_{84}O_{16}$	1060.6
	M-seco CTX4B	-	$C_{60}H_{86}O_{17}$	1078.6
	M-seco CTX4A	M-seco 52-epi-ciguatoxin-4B	$C_{60}H_{86}O_{17}$	1078.6
	CTX1B	Scaritoxin2, SG-2, ST-2, CTX, P-CTX, P-CTX-1, P-CTX1B	$C_{60}H_{86}O_{19}$	1110.6
	52-epi CTX1B	CTX-4, 52-epi CTX	$C_{60}H_{86}O_{19}$	1110.6
	54-epi CTX1B	54-epi CTX	$C_{60}H_{86}O_{19}$	1110.6
	54-epi 52-epi CTX1B	54-epi 52-epi CTX	$C_{60}H_{86}O_{19}$	1110.6

	7-oxo CTX1B	7-oxo CTX	$C_{60}H_{86}O_{20}$	1126.6
	7-hydroxy CTX1B	7-hydroxy CTX	$C_{60}H_{88}O_{20}$	1128.6
	4-hydroxy-7-oxo CTX1B	4-hydroxy-7-oxo CTX	$C_{60}H_{88}O_{21}$	1144.6
	54-deoxy-50-hydroxy CTX1B	54-deoxy-50-hydroxy CTX	$C_{60}H_{86}O_{19}$	1110.6
	52-epi-54-Deoxy CTX1B	P-CTX-2, CTX-2, 52-epi-54-Deoxy CTX	$C_{60}H_{86}O_{18}$	1094.6
	54-Deoxy CTX1B	P-CTX3, CTX-3, 54-Deoxy CTX	$C_{60}H_{86}O_{18}$	1094.6
P-CTX-II	CTX3C	-	$C_{57}H_{84}O_{16}$	1022.6
	CTX3B	49-epi CTX3C	$C_{57}H_{84}O_{16}$	1022.6
	M-seco CTX3C	-	$C_{57}H_{86}O_{17}$	1040.6
	M-seco CTX3C methyl acetal	-	$C_{58}H_{88}O_{17}$	1054.6
	51-hydroxy CTX3C	-	$C_{57}H_{84}O_{17}$	1038.6
	51-hydroxy -3-oxo-CTX3C	-	$C_{57}H_{84}O_{18}$	1054.6
	A-seco-51-hydroxy CTX3C	-	$C_{57}H_{88}O_{17}$	1042.6
	2,3-dihydroxy CTX3C	-	$C_{57}H_{84}O_{18}$	1056.6
	2,3-dihydro-2-	-	$C_{57}H_{84}O_{17}$	1038.6

	hydroxyCTX 3C			
	2 3-dihydro-51-hydroxy-2-oxoCTX3C	-	$C_{57}H_{84}O_{18}$	1054.6
	2,3-Dihydro-2,3-dihyroxyCTX3C	-	$C_{57}H_{85}O_{19}$	1071.6
	2,3-dihydro-2,3,51-trihydroxyCTX3C	-	$C_{57}H_{86}O_{20}$	1088.6
	A-seco-2,3-dihydro-51-hydroxyCTX 3C	-	$C_{57}H_{90}O_{20}$	1092.6
	2,3,51-Trihydroxy CTX3C	-	$C_{57}H_{86}O_{19}$	1072.6
	2-hydroxy CTX3C	-	$C_{57}H_{86}O_{17}$	1040.6
C-CTX	C-CTX-1	-	$C_{62}H_{92}O_{19}$	1140.6
	C-CTX-2	56 epi-C-CTX-1	$C_{62}H_{92}O_{19}$	1140.6
I-CTX	I-CTX-1	-	$C_{62}H_{92}O_{19}$	1140.6
	I-CTX-2	-	$C_{62}H_{92}O_{19}$	1140.6
	I-CTX-3	-	$C_{62}H_{92}O_{20}$	1156.6
	I-CTX-4	-	$C_{62}H_{92}O_{20}$	1156.6
	I-CTX-5	-	$C_{62}H_{90}O_{19}$	1138.6
	I-CTX-6	-	$C_{62}H_{90}O_{20}$	1154.6

1.3.3. Mechanism of action

All CTX congeners seem to share the same cellular mechanism of action. They bind to the alpha subunit of the voltage-gated sodium channels (VGSCs) [104], particularly to what has been called the site 5 (Fig. 6).

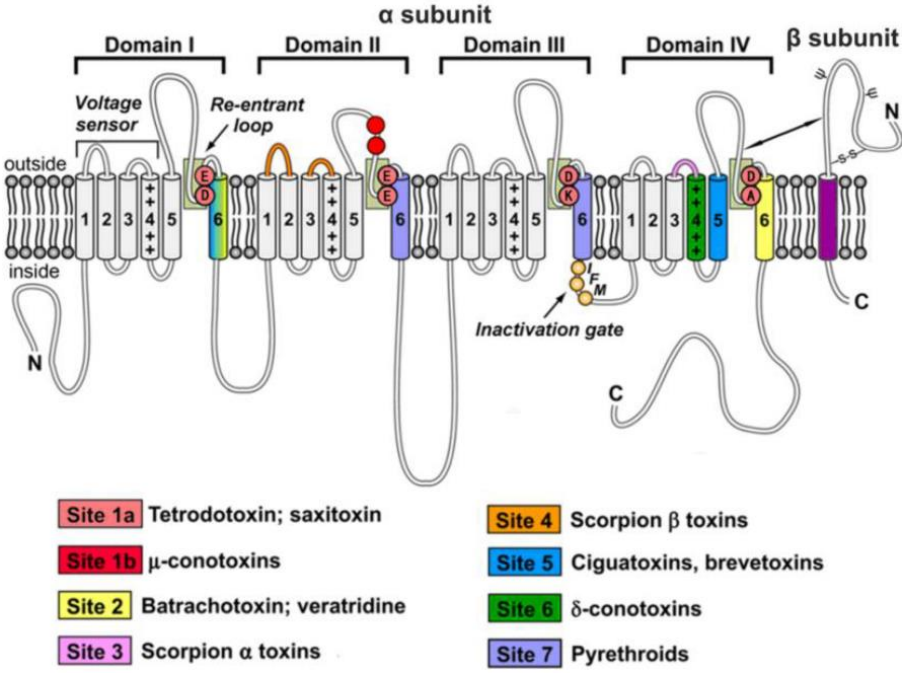

Figure 6. Binding sites for toxins in voltage-gated sodium channels. Source: Zhang et al. (2013) [85].

This binding persistently activates the channel causing the hyperpolarization of the membrane followed by repetitive action potentials [104] (Fig. 7). Besides, CTXs can block neuronal potassium channels (K^+ channels), and which exacerbates the effect of the sodium activation [104,106]. Moreover, the cascade of effects mediated by the elevation of intracellular Ca^{2+} due to the alteration of the cellular Na^+ gradient and subsequent recruitment of the Na^+/Ca^{2+} exchanger induce repetitive, synchronous and asynchronous neurotransmitter release. This produces a transient increase and decrease in the quantal content of synaptic responses and impairs synaptic vesicle recycling, which exhausts the pool of neurotransmitters available for release [107]. Besides, the expression of transient receptor potential ankyrin 1 (TRPA-1) increases in the peripheral sensory neurons [108]. This protein is involved in sensing noxious. Its increasing causes cold allodynia, a common ciguatera

symptom and the repetitive firing potentials cause swelling in the nodes of Ranvier, nerve terminals, and the perisynaptic Schwann cells of neurons [109–111].

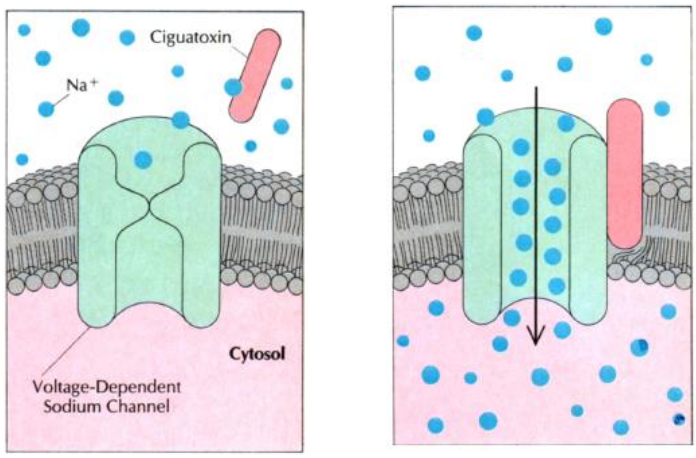

Fig. 7. Representation of the mechanism of action of ciguatoxin on voltage-gated sodium channels. Ciguatoxin binds to the channel and causes permanent activation. Source: Schatz (1989) [112].

CTX analogues have different affinities to different types of VGSC and K^+ channels [113]. CTXs affect the central and peripheral nervous systems [114] as well as the cardiac myocytes [115]. In cardiomyocytes, CTXs reduce the effectiveness of the Na^+/Ca^{2+} exchanger, releasing intracellular Ca^{2+} and increasing cardiac contractility [116]. In addition, CTXs affect beta-1 adrenoreceptor stimulation by releasing the noradrenaline in cardiomyocytes not, as was first thought, by directly affecting the myocardial sodium channels and stimulating the release of acetylcholine (ACh) [116]. Studies evaluating biological activity are scarce, mainly because of the difficulty of obtaining pure CTXs.

1.3.4. Other secondary metabolites

CTXs are not the only active molecules produced by *Gambierdiscus* and *Fukuyoa* genera. In *Gambierdiscus* species, gambieric acid (GA), maitotoxins (MTXs), gambierol, gambierone and gambieroxide have also been detected. While in the *Fukuyoa* genus gambierone has also been detected. Although it has been confirmed that CTXs play a role in CP, other metabolites have also been discussed to take part. But these have never been confirmed, either because there is no evidence that these compounds can cause CP symptoms or because a significant accumulation of the metabolite has not been observed in viscera or flesh [54,117].

44-methylgambierone (previously reported as MTX3) acts like CTXs and increases the intracellular sodium concentration, but its potency is very low and it has never been detected in fish; therefore, it is unlikely that it contributes to CP [118]. Among other toxins, gambierol does not affect sodium channels although, like CTXs, inhibits the potassium channels [119], which induces calcium oscillations in cerebrocortical neurons [120] and cerebellar granule cells [121]. Further, gambierol has a potent effect on TRPV1 channels (transient receptor potential cation channel subfamily V member 1), also known as the capsaicin receptor [119], which is a non- selective cation channel that has been related to typical CP neurological symptoms like burning mouth syndrome and unusual temperature sensation. However, the involvement of gambierol in CP has not been confirmed because CTXs have no effect on TRPV1 channels [122]. Another metabolite GA was reported in shark, which shows that GA accumulates until high trophic levels [54] but GA exhibits antifungal properties [123]. To date, no biological effects have been reported for gambieroxide.

The role of MTXs in CP is the most controversial. MTXs are rings of polyether with one or two sulphate ester groups. Six MTX analogues have currently been identified: maitotoxin-1 (MTX1), maitotoxin-2 (MTX2), maitotoxin-4 (MTX4), desulfo-51 MTX1 and didehydro-demethyl-desulfo-MTX1. MTX-like activity is associated to a massive calcium influx and rapid cell death [124–126]. It is assumed that the presence of sulphate groups is responsible for the bioactivity of the MTX congeners [127].

Again, as in the case of CTXs, the lack of standards of these metabolites, hampers the evaluation of the biological activity. Therefore, despite not being confirmed, some biological effects of these substances could be related to CP symptoms or could explain the variability of CP symptoms.

1.4. RISK ANALYSIS OF CIGUATERA POISONING

Risk is the probability or chance that an event will occur. Although risk can refer to both positive and negative events, in health sciences, risk is mainly used to refer to events with negative consequences (hazards). Under this premise, risk analysis is the process of recognizing and estimating the negative or adverse consequences of a hazard to human health and safety, identifying and implementing appropriate measures to control negative events, and communicating them to the general public. Risk analysis comprises three blocks: 1) risk assessment, which is the process of defining the problem and estimating the risk, describing all the vectors and mechanisms, and providing possible management strategies. 2) risk management, which is the process of deciding and implementing the regulations that can mitigate the hazard, and 3) risk communication, which is the process of disseminating information about risk, and advice and instructions to diminish risk for the general public.

The present thesis focuses on CP risk assessment (step 1). Risk is assessed by scientists who obtain information and evidence, and assess the risk level to be communicated to governments and health authorities. After a careful examination of the risk, scientists should propose possible actions to the authorities who will implement the decisions to decrease it. In order to estimate the risk in one area, four steps are needed: identification of the hazard (step 1), characterization of the hazard or assessment of toxicity (step 2), assessment of exposure (step 3) and characterization of risk (step 4) (Fig. 8).

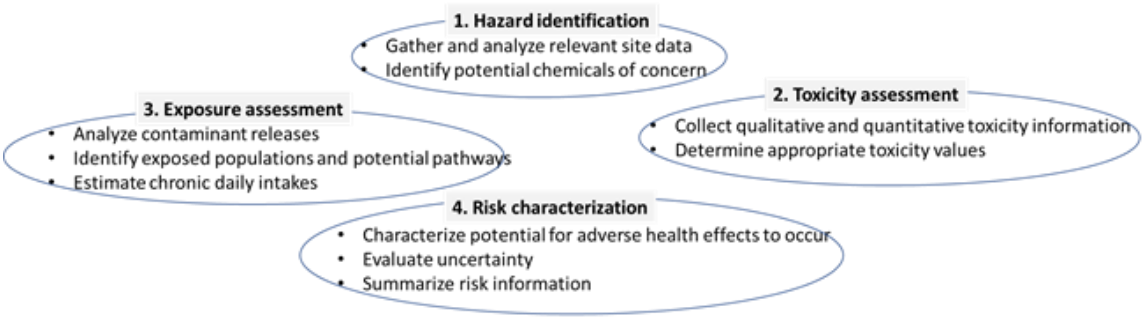

Figure 8. The four steps of risk assessment: hazard identification, toxicity assessment, exposure assessment, and risk characterization.

The present thesis has dealt with three steps of CP risk assessment. The first step, the identification of the hazard, is the qualitative process used to discover potential adverse health effects (e.g potential toxic microalgae related to CP or potential CTXs in fish). The second step is hazard characterization, which assesses the variants of the hazard under different conditions and scenarios: the dose-response under acute, chronic exposures, high-dose, different routes of exposure, different species, progression, regression, or the persistence of changes. In this case, the evaluation of the effect of toxins at the cellular level provides information about the hazard they represent. For example, differences have been identified between the toxic effects of microalgae, including *Gambierdiscus* and *Fukuyoa*. The third step is exposure assessment, which determines the source, the amount, and the duration of exposure to the potential hazard. This step includes quantifying CTXs in microalgal strains and fish and estimating the abundance of microalgae in the islands and sampling stations. Finally, risk characterization (step 4), which was not specifically dealt with in this thesis, summarises all the risk data of the previous phases, and evaluates the uncertainties and gaps in the scientific understanding of risk.

CP is now considered to be an emerging risk in Europe by the EFSA (European Food Safety Authority). Commonly, cases of CP in mainland Europe have been reported in travellers returning from the tropics [128–130] or by consumers of imported tropical fish [130,131]. In the present thesis, risk assessment focuses on the Balearic Islands (West Mediterranean Sea), the Canary Islands and Madeira Archipelago (Macaronesia, East Atlantic).

Currently, in the Balearic Islands, as will be demonstrated here, ciguatera is at the stage that populations of microalgae are producing CTX-like toxins and have been doing so for some years, but CTX-like toxins have not yet been identified in fish and no poisonings have been reported. Nonetheless, recent reports of *Gambierdiscus* and *Fukuyoa* in the Mediterranean Sea have increased the need to identify and anticipate the potential presence of CTX-like toxins in seafood and, eventually, cases of CP in the islands. Macaronesia is the only place where CP is autochthonous in temperate Europe. This area needs to be studied so that the population can be protected from the consumption of ciguateric seafood. It is also interesting to study why CP appeared in Macaronesia for the first time in these last two decades and to study the future trend.

1.4.1 Hazard identification based on the detection of CTX-producing species

Historically, *Gambierdiscus* and *Fukuyoa* species have been identified by morphological examinations using conventional microscopy. Conventional microscopy is crucial for the first assessment of microalgal communities and, when possible, the identification of microalgae. Nonetheless, this methodology has some limitations and in the past taxonomists have misdiagnosed some species, so some bioactive compounds have been attributed to the wrong species [108,109]. Molecular tools have improved identification and, hence, the quality of studies on biodiversity, identifying cryptic and pseudocryptic species [68]. In order to estimate the risk, it is important to consider that not all species produce the same amounts of metabolites or type of compounds [134,135]. Therefore, the presence of some toxic species may be more problematic than that of other. Figure 9 shows an example of CTX-like toxicity for some *Gambierdiscus* and *Fukuyoa* species from the Caribbean and the Gulf of Mexico. At present, *G. polynesiensis* is considered to be the most toxic producer of CTXs in the Pacific Ocean [68] and *G. excentricu*s, which has been reported in Macaronesia, is the second highest producer [135, 136].

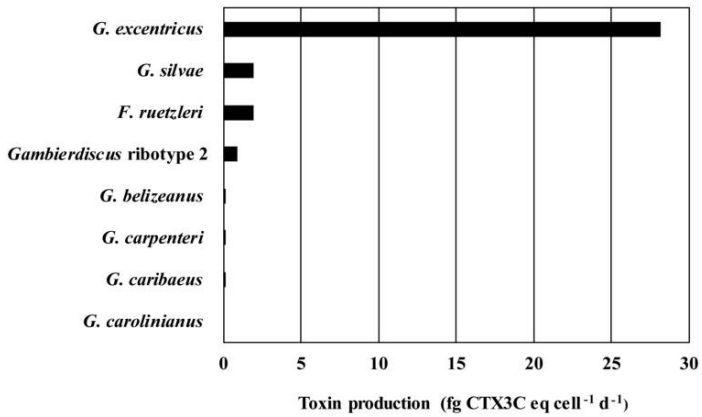

Fig. 9 Estimate CTX-like toxicity for different species of *Gambierdiscus* and *Fukuyoa* from the Caribbean and the Gulf of Mexico. Source: Litaker et al. (2017) [136].

Detection of CTXs and related toxins

As has been mentioned above, CTXS are odourless and tasteless, and they cannot be detected from the appearance of seafood. One pivotal step in CP management is to discern between toxic and non-toxic microalgal blooms and between toxic and non-toxic seafood. To do this, various methods for detecting CTXs have been developed. The structural variability of CTXs and the various biological activities and

potencies harmper their confirmation and quantitation. In addition, the fact that the lack of reliable standards means that it is difficult to detect them and confirm by instrumental analysis techniques. Other alternative methods such as, cell-based assays (CBAs) and immunoassays have been developed [137], which are based on toxicological effects or the recognition of specific structures. The main drawback is that these methods do not quantify individual toxins. They provide only an overall toxin content.

Most countries affected by CP have drawn up regulations and recommendations regarding the consumption of certain fish species which have been related to cases of CP. For instance, Polynesia and New Caledonia (Pacific Ocean), Reunion Island (Indian Ocean), Guadeloupe and Martinique (Caribbean Sea) have local regulations that list the reef fish species that cannot be placed on the market. In Europe, the European Commission asked the EFSA for their scientific opinion about the regulatory levels of CTXs in food products. The EFSA and the United States Food and Drug Administration (US FDA) estimate that doses under 0.01 µg of CTX1B kg^{-1} of fish cause no symptoms. Another equivalence is 0.1 µg of C-CTX1 kg^{-1}. To date, safety guidance levels have not been reported for I-CTXs. These regulatory levels require methods to detect and quantify the toxins. As mentioned, obtaining certified standards is difficult and there is no validated methodology for CTX detection. This may explain why there are no regulatory levels or an official method of monitoring CTXs in Europe. Regulation in the EU only bans placing food contaminated with CTXs on the market (Commission Delegated Regulation (EU) 2019/624, 2019). However, the Government of the Canary Islands has developed a specific monitoring program to prevent cases of CP in which fish above a minimum weigh for a particular species are evaluated for CTX-like toxicity using the neuroblastoma cell-based assay (neuro-2a CBA) before being put on sale. Individual fish over the assigned weights are retained, a sample is obtained, and the fish is frozen. If the fish presents CTX-like toxicity, it is not placed on the market. It allows to determine overall toxicity for CTXs for each species or groups of individuals with particular characteristics and perform statistical analysis for modelling to predict CTX concentration.

Sample preparation

Sample preparation is a necessary previous step for CTX detection. It consists of extracting CTXs from the biological matrixes (microalgae or seafood) before they are detected. The method of toxin extraction plays an important role in detection so it must be ensured that the extract obtained is appropriate for the analytical method selected. In the detection of CTXs, other co-eluting compounds should not interfere. To this end, sample preparation may include steps to reduce interferences in the assays. For example, in toxicological assays, the interaction of other compounds with CTXs has already been described [138].

Extraction methods should be selected bearing in mind the matrix of the sample, and the level of purification desired for the detection method. Other factors that affect the selection are that CTXs have to be extracted with the minimum time and cost possible. Currently, detection often requires complicated extraction protocols, which are non-automated and time-consuming. For instance, numerous extraction methods from fish tissue have been reported. These include a first step of methanolic or acetanolic extraction, followed by liquid-liquid partitions. Other steps such as solid-phase extraction (SPE) can be performed to clean and purify the extract [139]. Extraction protocols vary, for example, in terms of the solvents used, the sample-to-solvent ratio, the number of extraction cycles, and the number of SPE steps.

1.5. METHODS OF TOXIN DETECTION

1.5.1. Bioassays

Bioassays focus on the effects on living organisms, tissues or cells that have been exposed to a substance. Among the tests using animals, the effects can be evaluated on invertebrates such as mosquitos [140], brine shrimps [141], dipteran larvae [142] or vertebrates as mongoose [143], cats [144] and mice [143]. The main drawbacks when implementing bioassays in animals are that ethical requirements make the process time-consuming, the response among the individuals tested is highly variable and expertise is required to handle the animals. Further, bioassays are nonspecific: the toxic effect cannot be attributed to a specific molecule. The only bioassay that is still used to evaluate marine toxins in shellfish monitoring programmes is the mouse bioassay (MBA).

Mouse bioassays (MBA)

Mouse bioassays (MBA) were developed according to Banner et al. (1960) [143]. They can be performed by intraperitoneal injection or oral gavage of the extract in mice. During the assay, symptoms are observed in mice. The LD_{50} of a toxin in an extract can be determined from the dose-curve to death and is the dose of extract that causes the death of 50 % of animals in a particular group at a given time (normally 24h). Signs of poisoning can be ataxia, loss of body weight, salivation and death. The limit of quantitation (LOQ) of the MBA is approximately 0.56 ng g^{-1} for CTX1B [143], which is higher than the recommended safety levels for EFSA and FDA of 0.01 ng g^{-1} for CTX1B. Like all bioassays, the MBA has a high number of false positives and poor sensitivity. It is time-consuming, cannot be automated, and requires following specific regulations for the care of mice. In addition, its poor specificity does not allow the type of toxin to be determined or the mechanism of action of the toxin to be evaluated. The European Commission encourages researchers to find alternative methods to replace the MBA [146].

Cell-based assays (CBAs)

Cell-based assays (CBAs) detect the effect of substances on cells. The effects can be evaluated and measured by the changes in morphology, cell viability, ions and metabolites. For ethical reasons, CBAs can be used instead of animal bioassays. Cell cultures can be obtained from primary sources or from immortalized cells. In primary cultures, cells are isolated directly from the tissues by mechanical or enzymatic methods. Primary cell lines have morphological and functional characteristics very similar to those of the original tissue, and they reflect the conditions of the cells in the organisms better than immortal cell lines. However, their survival is limited. By contrast, immortalized cell lines are also isolated with the same mechanical or enzymatic methods, but they are immortal because they are isolated from tumoral tissues or transfected. Therefore, fewer organisms are killed or hurt. They can be kept in laboratories for a long time. Immortal cell lines are more homogeneous than primary cell lines and are therefore less variable in their growth and response to xenobiotics, and are easier to manage. For all these reasons, immortalized cell cultures have been used more than primary cells. Nonetheless, gene expression can vary in tumorous cell lines through culture passages.

As has been mentioned above, CTXs activate sodium channels, inhibit their closure and cause firing potentials. The exposure of the cell lines tested so far to CTX at nanomolar levels does not appear to affect the cells [147]. The effect of toxins on cells can be potentiated or suppressed using agonists or antagonists. Therefore, to

observe changes in cells that may be caused by CTXs, other drugs are used to enhance their toxic effect. It has to be noted that CTXs are not the only toxins that can bind to VGSCs. Toxins such as brevetoxins can activate VGSCs while others such as saxitoxins (STXs) or tetrodotoxins (TTXs) can block them. Therefore, during assays on these cells, the toxic effect can be caused by any molecule that affects VGSCs.

Neuroblastoma cells have historically been used to detect marine toxins that target sodium channels. A typical assay to detect CTX-like toxicity is the neuroblastoma cell-based assay (neuro-2a CBA), which uses neuroblastoma murine cells (neuro-2a cells). This assay derives from the original method used to detect TTXs [148] and was adapted by Manger et al. (1993) [149] for the detection of CTXs. The neuro-2a CBA is based on the effect of two drugs on neuro-2a cells: veratridine, a sodium channel activator [127], and ouabain which is a Na^+/K^+ ATPase inhibitor [151]. After the neuro-2a cells have been exposed to extracts with ouabain and veratridine treatment (O/V+), the cell viability is reduced when CTXs or other toxins that increase intracellular sodium are present. Figure 10 shows how cell viability is reduced in presence of CTX and O/V treatment. Cell viability can be easily assessed by a colorimetric assay such as MTT, which estimates cell viability through mitochondrial dehydrogenase activity. The assay is relatively simple, can be partially automated and does not require highly specialized personnel. In addition, it is more sensitive than the MBA and the limit of detection (LOD) can be lower than the EFSA recommendations for safety levels of CTXs.

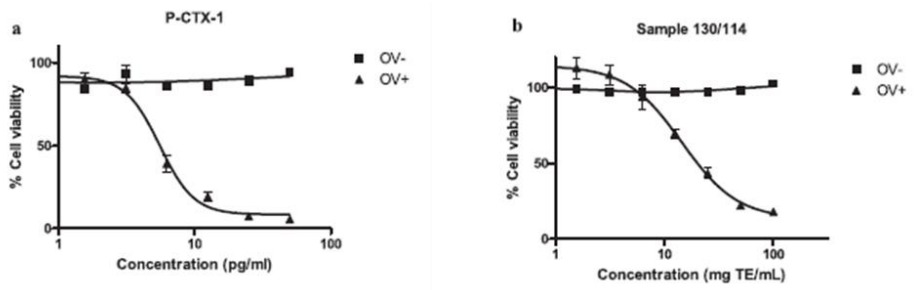

Figure 10. Concentration-response curves of neuro-2a cells exposed to a) P-CTX-1 or CTX1B standard and b) fish extract (code: 130/114). O/V- (absence of ouabain and veratridine); O/V+ (presence of ouabain and veratridine). TE (tissue equivalent). Error bars represent the standard deviation from three replicates. Source: Soliño et al. (2015) [152].

In recent years, new models of cell-based assays have been developed. For instance, Coccini et al. (2017) [153] developed the SH-SY5Y assay based on the neuroblastoma cell line which can explore the effects of CTXs in human brain, particularly the mechanisms of neurotransmission and nociception under CP intoxication. This cell line is more sensitive than neuro-2a cells to treatment with O/V+, which reduces the time of the assays. Other cell-based assays have been developed to detect CTXs, such as NG108-15 cells [151] or a fluorescent assay using the neuroblastoma cell line SH-SY5Y [154].

It is important to remember that CBAs for marine toxin detection are always not specific. In other words, it is not possible to confirm that a particular toxin structure is present by evaluating its toxic effect [149]. Nonetheless, it is plausible to give global levels of toxicity in comparison to other molecules. To that, the cytotoxicity of extract, the level of toxicity of the tested extract is compared to the level toxicity of one purified molecule (standard). As mentioned above, obtaining CTX purified standards is limited to only two CTXs (CTX1B and CTX3C) which are very expensive and scarce. Consequently, the laboratories purify their own standards from samples. Thus, the standars used in studies are often varied and toxicological effects are not always comparable.

Cell-based assays have also been used to detect MTX-like activity. Perhaps the most used method is the neuroblastoma cell-based assay with neuro-2a cells using SKF96365, the antagonist of MTX developed by Caillaud et al. (2010) [155] on the basis of previous work by Soergel and collaborators (1992) [124]. Another assay to test the MTX-like activity is the erythrocyte lysis assay (ELA). This test is based on the haemolytic activity of MTXs. In the presence of MTX, haemoglobin is released, and the amount can be measured. However, variability can exist depending on the origin of erythrocytes (species, population and individual).

1.5.2. Biochemical assays

Receptor Binding Assays (RBA)

Receptor Binding Assays (RBA) are based on the affinity of the toxins for cellular receptors, in the case of CTXs the case VGSCs. The method is basically a competitive binding experiment, which the extract CTX-containing competes for site 5 in the VGSC with the labelled brevetoxin [156]. One method restriction is the labelling of brevetoxin molecules. They were often radioactively labelled; at present, new mechanisms allow fluorescent or chemiluminescent labelling [157] (fig. 11). This assay has been used for samples from fish and microalgae [156,158].

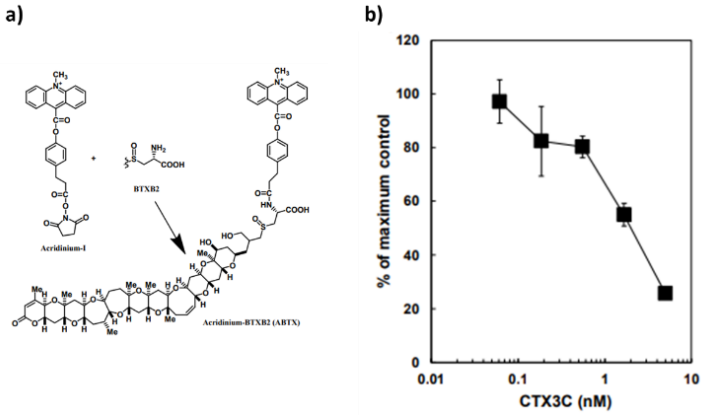

Fig. 11 a) Preparation of labelled brevetoxin (ABTX) for chemiluminiscence experiment. b) Competitive binding assay between ABTX and CTX3C. Source: Murata et al. (2019) [157].

Immunoassays

Immunoassays are based on the recognition of an antigen (in this case CTXs) and the affinity of an antibody (Ab) for the antigen. Therefore, instead of the toxic effect, like RBA, detection is based on the structure of the CTX. These assays prevent interferences from the matrixes [159]. Figure 12 shows CTX-detection strategy by the immunoassay is schematically depicted. On the one side, two different mouse monoclonal antibodies (mAbs) immobilised on magnetic beads are able to recognise the left wing of CTX1B and 54-deoxyCTX1B (3G8 mAb) or the left wing of CTX3C and 51-hydroxyCTX3C (10C9 mAb). Once the CTX analogue is bound to the capture antibody, a biotinylated antibody able to recognise the right wing of these four

analogues (CTX1B, CTX3C, 54-deoxyCTX1B, and 51-hydrox-yCTX3C) is added and polyHRP-streptavidin is used for signal reporting in the presence of an enzyme substrate. Antibodies can bind to several targets within a structural group of toxins. Immunoassays are quick and easy to use, and the reduction of interferences means that the toxin extraction protocol is simpler. Besides, the high specificity of the antibodies means that several have to be used to determine the CTX profile. The main problem of this approach is that the production of antibodies is not easy. Several approaches have combined different antibodies to simplify the detection of CTXs, which led to Leonardo et al. (2020) [159] developing one of the first electrochemical immunosensors. Like CBAs, the LOD can be lower than the safety levels [159].

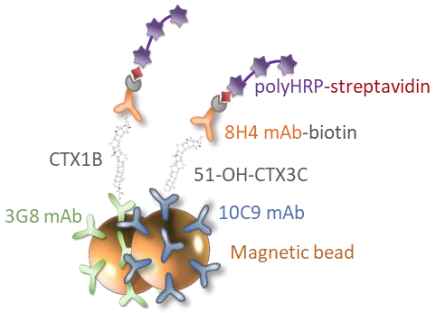

Figure. 12 Schematic representation of the immunoassay.

1.5.3. Analytical assays

CTX congeners differ chemically but they have very similar molecular weights. Analytical methods can identify the different CTX variants with high precision and sensitivity. These methods are required if CTXs are to be identified and quantified unambiguously. For instance, High Performance Liquid Chromatography (HPLC) is based on the solubility of CTXs in a mobile phase (liquid) under pressure. It is the technique of choice for separating CTX congeners. HPLC has been applied with fluorescence detection or UV detectors, which has always resulted in an LOQ that is higher than the safety level of 0.01 ng g^{-1} for CTX1B.

Other techniques such as HPLC coupled with tandem mass spectrometry (HPLC-MS/MS) and HPLC coupled with high resolution mass spectrometry LC-HRMS identify and quantify CTXs. In combination with extra purification steps and the optimization of liquid chromatography, ionization sources, ion choices and acquisition modes, these methods can reach safe detection levels. However, a

standard molecule is required. The scarcity of standards makes it difficult to detect as many toxic compounds in the extracts as toxicology-based functional assays do. Therefore, risk assessment based solely on analytical methods can lead to errors in the estimation of CP risk.

This thesis uses a combination of detection techniques. For instance, the functional assay using mammalian cells is used to detect CTX-like and MTX-like toxicity and the biochemical or chemical instrumental analysis is used to confirm the presence of CTXs.

CHAPTER 2

OBJECTIVES AND HYPOTHESES

2. OBJECTIVES AND HYPOTHESES

2.1 Thesis statement and objectives

Ciguatera poisoning is a significant illness, mainly in tropical and subtropical areas of the world, that can restrict people's lives and, in severe cases, cause death. Having CP in a region involves a medical economic investment but also makes fishing regulatory policies necessary. At present, the increasing and expansion of the number of CP cases and the unprecedented recent findings of ciguatoxin producer species and toxic fish in new temperate areas show the necessity to evaluate the risk of ciguatera so authorities can have a strategic plant to prevent it. Understanding and managing the CP in the region implies establishing relations between CP and the environment, understanding the exposure routes, types and levels of toxins that produce CP. For that, the identification of the potential CTX-producing species and their geographical distribution and the detection of CTX-like toxins in microalgal species and seafood are crucial.

Within this context, the general objective is to increase the knowledge about the ciguatera phenomenon and contribute to its risk assessment in two regions in Europe: the Balearic Islands (Western Mediterranean Sea) and the Canary Islands and the Madeira Archipelago (North-East Atlantic Ocean).

To that end, the specific objectives of this thesis are:

In the Balearic Islands (chapter I):

- Determine which species within *Gambierdiscus* and *Fukuyoa* genera live in the islands.
- Determine the geographical distribution and the recurrence in several years of *Gambierdiscus* and *Fukuyoa* species.
- Assess if these species can produce compounds with CTX-like and MTX-like activity.
- Characterize their toxic profiles further than CTX and MTX compounds.
- Assess the toxicity of fish by CTX-like compounds that was caught in the Balearic Islands.
- Estimate the CP of risk in the Balearic Islands based on the points that have been mentioned above.

In the Canary Islands (chapter II):

- Determine which species within *Gambierdiscus* and *Fukuyoa* genera live in the islands.
- Determine the geographical distribution of *Gambierdiscus* and *Fukuyoa* species.
- Assess if these species can produce compounds with CTX-like and MTX-like activity and list the most toxic species.
- Characterize their toxic profiles further than CTX and MTX compounds.

In the Madeira Archipelago (chapter III):

- Assess the toxicity of fish by CTX-like compounds that was caught in the Archipelago.
- Examine and compare two methods of toxin extraction in for fish.
- Compare results of toxin analysis of flesh and liver extracts from the same fish.
- Characterize the toxic profiles of toxic fish by CTX-like compounds from the archipelago.

2.2 Hypotheses

In this thesis, it has been hypothized that:

1. CTX-producing species are present in the Balearic Islands, however, their toxicity is low and their geographical distribution is still restricted.
2. The presence of CTX-producing species present in the Canary Islands is higher than in the Balearic Islands, with higher toxicity and widespread distribution.
3. The toxicity analysis of flesh and liver extracts from CTX-containing fish have significative differences and are influenced by the extraction method.

CHAPTER 3

RESULTS

3. RESULTS

3.1. CP HAZARD IDENTIFICATION IN THE BALEARIC ISLANDS

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

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Gambierdiscus (Class *Dinophyceae*) species are benthic dinoflagellates living in marine littoral zones of circumtropical areas and have recently been described in temperate waters [1]. Some species are producers of potent neurotoxins: ciguatoxins (CTXs) and maitotoxins (MTXs). Ciguatoxins are linked to ciguatera poisoning (CP). Ciguatera used to be restricted to tropical and subtropical areas, but since the last decade, it appears to be expanding to more temperate latitudes. For example, outbreaks of ciguatera have been reported in the Canary Islands and Madeira Archipelago (eastern Atlantic Ocean), where several species of the genus *Gambierdiscus* have been identified [2].

In the Mediterranean Sea, no thorough evidence of cases of ciguatera exists. The only reports of CTX-like toxins in fish, which are not confirmed, were based on the Cigua-Check Fish Poison Test kit (Oceanit, Hawaii), a method that has proved to be unreliable [3]. Nonetheless, *Gambierdiscus* species have been reported in the last decade in Crete and Cyprus (eastern Mediterranen Sea) [4-6]. One species of *Fukuyoa* (*F. paulensis*), a genus that includes species previously included in the genus *Gambierdiscus*, was reported in the Balearic Islands in 2015 [7]. Little is known about diversity, distribution and toxicity of *Gambierdiscus* spp. in the Mediterranean Sea. The present study confirms the presence of *G. australes* in the two Balearic Islands of Majorca and Minorca, and this constitutes the first report of the *Gambierdiscus* genus in the western Mediterranean Sea.

In this study, microalgal samples were collected from macroalgae and rocky substrates in 19 stations in Majorca and Minorca in September 2017. Water temperatures ranged from 24 to 27 °C and salinity from 36.2 to 38.0. In the

laboratory, samples were observed under the microscope and individual cells were isolated with micropipettes to establish cultures for morphological and molecular analysis. Calcofluor white stain was used for morphological identifications. Cells were observed with a compound microscope equipped with epifluorescence at 630X (Leica DMLB). The *Gambierdiscus* cells observed were anterior-posteriorly compressed. Morphology of the epitheca and the hypotheca is shown in Fig. 1 in which the plate terminology employed follows Fraga and collaborators [8].

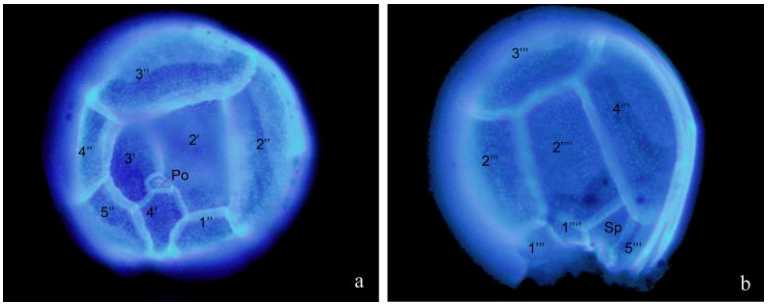

Fig. 1. Epitheca (a) and hypotheca (b) of *Gambierdiscus australes* cells stained with Calcofluor White.

The epitheca has a rectangular-shaped 2' apical plate and the Po plate is ventrally oriented; the hypotheca has a narrow 2'''' plate equivalent to 1p plate in Chinain [9]. The cell surface is smooth. The cell length and width of 62 individuals were measured. Length ranged from 60.9 to 92.3 µm (mean of 75.6 µm) and width ranged from 64.1 to 90.8 µm (mean of 78.6 µm). The original description [9] described a length range of 76-93 µm and a cell width of 65-84 µm. Further morphological analysis will be performed using electron microscopy. To facilitate molecular identification to species level, DNA was extracted from individual or a few clonal cells using the ArcturusTM PicoPureTM DNA Extraction Kit (Applied Biosystems, CA, USA). Afterwards, the domain D8-D10 of the LSU rRNA gene was amplified by a Polymerase Chain Reaction (PCR) using the pair of primers FD8 and RB [9], and products were sequenced. The D8-D10 sequences obtained in this study were deposited in GenBank under accession numbers: MG708117- MG708130. DNA sequence analysis of amplified rDNA fragments confirmed that all *Gambierdiscus* spp. corresponded to *G. australes*, which was in accordance with the morphological identification. *G. australes* was present in 10 out of the 19 sampling stations in Majorca and Minorca indicating that this species is well established at different locations around the coasts of both islands. Figure 2 shows sampling stations where *Gambierdiscus* cells were isolated.

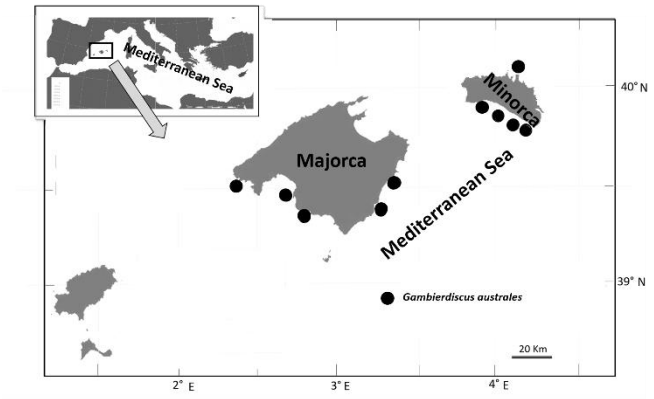

Fig. 2 Locations where *G. australes* was recorded in the Balearic Islands (39° 30'N, 3° 00' E), Spain.

It will be important to evaluate the temporal distribution of this species. The first report of *G. australes* was in the Australes archipelago (French Polynesia). This species is also widely distributed in areas such as New Zealand and the Canary Islands, but it had not been reported yet in the Mediterranean Sea. Some studies mentioned that the spatial expansion of *Gambierdiscus* and CP may be related to the increase of temperatures caused by climate change. [10]. The Mediterranean Sea, which is a semi-enclosed sea, seems to be one of the regions strongly affected by the rising of temperatures, and this makes this region more suitable for tropical species [11]. A recent study describes a high diversity of Gambierdiscus species in the Canary Islands which would suggest that this genus is not a recently introduced taxon in that area, although climate change may contribute to increase the populations density [2]. It will be important to understand the origin of *Gambierdiscus* in the Mediterranean and the effect that climate change may have on *Gambierdiscus* populations. Moreover, it will be necessary to study whether the Balearic Islands could be a new spot of ciguatera.

Improving our knowledge about diversity and toxicity of these benthic dinoflagellates will provide a better characterization of health risks taking into consideration climate change trends.

Acknowledgements

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ii. *Gambierdiscus* and *Fukuyoa* as potencial indicators of Ciguatera Risk in the Balearic Islands

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ABSTRACT

Gambierdiscus and *Fukuyoa* are genera of toxic dinoflagellates which were mainly considered as endemic to marine intertropical areas, and that are well known as producers of ciguatoxins (CTXs) and maitotoxins (MTXs). Ciguatera poisoning (CP) is a human poisoning occurring after the consumption of fish or more rarely, shellfish containing CTXs. The presence of these microalgae in a coastal area is an indication of potential risk of CP. This study assesses the risk of CP in the Balearic Islands (Western Mediterranean Sea) according to the distribution of both microalgae genera, and the presence of CTX-like and MTX-like toxicity in microalgal cultures as determined by neuroblastoma cell based-assay (neuro-2a CBA). Genetic identification of forty-three cultured microalgal strains isolated from 2016 to 2019 revealed that all of them belong to the species *G. australes* and *F. paulensis*. Both species were widely distributed in Formentera, Majorca and Minorca. Additionally, all strains of *G. australes* and two of *F. paulensis* exhibited signals of CTX-like toxicity ranging respectively between 1 and 380 and 8–16 fg CTX1B equivalents (equiv.) cell^{-1}. Four extracts of *F. paulensis* exhibited a novel toxicity response in neuro-2a cells consisting of the recovery of the cell viability in the presence of ouabain and veratridine. In addition, *G. australes* showed MTX-like toxicity while *F. paulensis* strains did not. Overall, the low CTX-like toxicities detected indicate that the potential risk of CP in the Balearic Islands is low, although, the presence of CTX-like

and MTX-like toxicity in those strains reveal the necessity to monitor these genera in the Mediterranean Sea.

1. Introduction

Gambierdiscus [1] and *Fukuyoa* [2] (*Dinophyceae*) are marine benthic dinoflagellates that live attached to different substrates such as macroalgae, corals, rocks and sands in well-illuminated habitats but also at very low light levels (>45 m depth) [3]. Historically, the genera *Gambierdiscus* and *Fukuyoa* were known to be distributed primarily in tropical and subtropical areas of the Caribbean Sea, the Pacific and Indian Ocean. However, in recent decades, both genera have been reported in warm-temperate areas. The genus *Gambierdiscus* was recently recorded in the North East Atlantic Ocean [4–6], North West Atlantic Ocean [7], South West Atlantic [8], the Mediterranean Sea [9,10], the Red Sea [11], Sea of Japan [12] and the South Pacific Ocean [13,14]. In contrast species of the genus *Fukuyoa* (formerly within the genus *Gambierdiscus*), have been reported in the Atlantic Ocean [2], the Mediterranean Sea [15,16], the South Pacific Ocean [17], the China Sea and the Asia Pacific region [14,18,19].

Gambierdiscus and *Fukuyoa* produce multiple secondary metabolites, among which are included ciguatoxins (CTXs) and maitotoxins (MTXs) [20–24]. CTXs are lipophilic polyethers, that bind to voltage-gated sodium channels (VGSCs), thereby inhibiting the inactivation process of VGSCs resulting in intracellular sodium increase [25–28]. Moreover, CTXs are potassium channel inhibitors [29]. MTXs are amphiphilic polyethers that bind to Ca^{2+} independent voltage gated channels and non-selective ion channels causing an increase of intracellular Ca^{2+} [30].

CTXs in fish or invertebrates are responsible for the human intoxication known as ciguatera poisoning (CP) [31,32]. CTXs enter marine food webs through invertebrates and herbivorous fish, where they may be biotransformed along the food webs and bioaccumulated at different trophic levels, eventually reaching humans [32,33]. Regarding MTXs, their implication in CP is unlikely. Although its intraperitoneal administration in mice is more toxic than CTXs, their bioaccumulation along the food webs is low [34–36] and they have not been found in the tissue of seafood involved in CP cases. However, snapper (*Chrysophrys auratus*) (previously *Pagrus auratus*), that had been experimentally fed with *G. australes* contained MTXs in their viscera, liver and muscle [37].

Although, epidemiological records of CP are not available at a global level, it is estimated that CP affects between 25,000 – 500,000 people per year [38–40]. CP effects include gastrointestinal, neurological, and cardiovascular symptoms, and the latter two can last for months or years [40]. Fatal cases of CP are rare [41,42]. CP occurs mainly in tropical and subtropical areas (35 °N - 35 °S), but in more recent decades, CP cases have been reported in temperate areas, previously free of CP [43–45]. In the Mediterranean Sea, the presence of CTXs in fish, or confirmed CP cases have not been demonstrated. Follow-up investigations of previous descriptions of CP cases in the eastern Mediterranean did not find CTXs in fish tissue [46–50]. The detection of possible CTX-compounds in *Siganus* sp. by Bentur and Spanier (2007) was performed using a CiguaCheck strip test, which was later considered unreliable test [51]. In addition, the clinical symptoms described for mediterranean cases of CP, including hallucinations, are rare in CP [44] and they are indicative of ichthyoallyeinotoxism, which is often mistaken for cases of CP [52].

At present, five confirmed species of the genus *Gambierdiscus* and *Fukuyoa* live in the Mediterranean Sea [7,10,15,16]. The presence of certain CTX-producing species in the area can be indicative of a higher risk of CP in comparison to areas where they are absent [40,44,53]. Nonetheless, evaluating CTX-production by these species is important to estimate the risk, since CTX production varies according to species, and high and low CTX-producers species have been characterized [54,55]. For the estimation of CTX production in *Gambierdiscus*, growth phases and strain variability among isolates of the same species have to be taken into account [56,57].

The goal of this study was to assess the potential risk of CP based on the presence in the Balearic Islands of the genera *Gambierdiscus* and *Fukuyoa* (Western Mediterranean Sea), and their potential production of compounds with CTX-like and MTX-like activity. This is the first study that provides information about the risk of CP in the Balearic Islands, according to the presence of the genera *Gambierdiscus* and *Fukuyoa* in several sampling locations, and their evaluation of toxin production of several strains.

2. Materials and methods

2.1. Reagents and equipment

CTX1B was provided by Dr. Lewis, University of Queensland. Neuroblastoma murine cells (neuro-2a) were purchased from ATCC LGC standards (USA). Poly-L-lysine, foetal bovine serum (FBS), L-glutamine solution, ouabain, veratridine, phosphate buffered saline (PBS), penicillin, streptomycin, RPMI-1640 medium, sodium pyruvate, thiazolyl blue tetrazolium bromide (MTT) and SKF96365 were purchased

from Merck KGaA (Germany). Dimethyl sulfoxide (DMSO) and absolute methanol were purchased from Honeywell (Spain) and Chemlab (Spain) respectively. Taq Polymerase was purchased from Invitrogen (Spain). QIAquick PCR Purification Kit was obtained from Qiagen (Germany).

2.2. Sampling, cell isolation and initial culturing

The Balearic Archipelago (North West Mediterranean Sea) is located at 170 km distance from the Iberian Peninsula (Fig. 1). It is characterized by a narrow continental shelf surrounding a rocky coast, with occasional sea grass meadows over a biogenic muddy bottom. Samples from the Balearic Islands were collected at different islands, specifically, in Formentera in late September 2016, in Majorca and Minorca in early September 2017 and in early October 2018. In Minorca an additional sampling was performed in late September 2019. At each sampling point, two different types of samples were collected: 1) epilithic, which were obtained by scraping of the substrate (rocks) with a plastic bottle (Nalgene, HDPE, 1 L), and 2) epiphytic, which were obtained from macroalgae that were collected using plastic bottles under water. Macroalgae were identified morphologically at the genus level. Each sample was kept in the container and was intensively shaken by hand to release the dinoflagellates from the substrates. Samples were sieved through a 300 µm nylon mesh. The filtered water was stored in two plastic bottles (Nalgene, HDPE, 125 mL), one with 125 mL was kept untreated to isolate live cells and another was preserved in 3 % Lugol's iodine solution for further observation in the laboratory. Coordinates of each sampling station were recorded by GPS. Salinity, oxygen (% and mg L^{-1}), temperature and pH were recorded in situ using a multiparametric probe (YSI 556 MPS).

Samples were observed under an inverted light microscope Leica DMIL (Leica Microsystems GmbH, Germany) and individual microalgal cells were isolated by capillary method [58] to establish clonal cultures. Each cell was inoculated in a well of an untreated Nunc 24 well plate (Thermo Fisher Scientific) with 1 mL of modified ES medium [59]. Medium was prepared from sterile aged seawater from L'Ametlla de Mar (Spain), Mediterranean Sea (40.8465° N; 0.77243° E) and salinity was adjusted to 36. After 2–3 weeks, when cell abundance of cultures reached 20–30 cells mL^{-1}, cells were transferred to 28 mL round bottom glass tubes (Thermo Fisher Scientific) containing 10 mL of medium. Cultures were maintained in a culture chamber at a temperature of 24 ± 0.5 °C, which is the average of the range of the optimal temperatures of growth for *G. australes* [60] and in coherence with our previous studies, Reverté et al. (2018) [56], Caillaud et al. (2010)[61]. Illumination in a 12:12 light:dark cycle was provided by fluorescent tubes with white light and with photon irradiance of 100 µmol photons m^{-2} s^{-1} measured by an irradiometer (QSL-

2100 Radiometer, Biospherical Instruments, San Diego, USA). Preserved field samples were settled in 10 mL sedimentation chambers and observed under an inverted light microscope for microalgal identification.

2.3. Molecular identification

Molecular identification at species level was performed by sequencing the D8-D10 region of the 28S ribosomal large subunit gene (LSU rDNA). Molecular identification was conducted for 34 *Gambierdiscus* strains and 9 *Fukuyoa* strains. To that purpose, strains were inoculated in 50 mL of medium at 50 cells mL^{-1} in 25 cm^2 sterile Nunclon™ culture flasks (Thermo Fisher Scientific), and when cultures achieved the exponential phase, they were harvested by centrifugation at 4300 g for 20 min (Allegra X-15R, Beckman Coulter). Genomic DNA was extracted by phenol/chloroform/isoamylalcohol (PCI) extraction following Toldrà et al. (2018) [62].

After DNA extraction, genomic DNA was quantified and checked for its purity using a NanoDrop 2000 spectrophotometer (Thermo Fisher Scientific) and stored at −20 °C. Afterwards, the region D8-D10 was amplified by PCR using the primers FD8 and RB [80]. Each 25 µL reaction mixture contained 600 µM dNTP, 2 mM $MgCl_2$, 0.2 µM of each primer, 1 U of Taq polymerase, 5 % DMSO, and 0.4 - 2 ng ul^{-1} of DNA template. Amplifications were carried out in a Mastercycler nexus gradient thermal cycler (Eppendorf, Spain) as follows: an initial denaturation step of 5 min at 95 °C, 40 cycles of 30 s at 95 °C, 45 s at 60 °C, and 30 s at 72 °C and a final extension step of 10 min at 72 °C. Each PCR reaction was verified by agarose gel electrophoresis and visualized with ethidium bromide stain. The resulting PCR products of ~ 840–910 bp were purified with the QIAquick PCR Purification Kit. Purified products were bi-directionally sequenced by an external company (Sistemas Genómicos, LLC, Valencia, Spain).

Consensus sequences obtained from both reads for each strain were manually edited and deposited in GenBank. Sequences were aligned using MAFFT v.7 [63] with G-INS-1 progressive method. The final alignment consisted of 617 positions. The evolutionary model of data was estimated using jModelTest 2.1.10 [64] and the phylogenetic relationships were inferred by Maximum likelihood (ML) using RaxML v.8 [65] and Bayesian inference (BI) using Mr. Bayes v.3.2.2 [66]. In the BI approach two analyses were run in parallel, 10^6 generations, and four chains in each run. The parameters used for analysis were nst=mixed and rates=gamma. By default, 25 % of the trees were discarded. Stability of the chains were checked using Tracer v.1.7.1 [67].

2.4. Morphological characterization

For morphological characterization strains were acclimated at least one year to avoid stress-induced variance during the adaptation period to laboratory conditions [68].

2.4.1. Light microscopy (LM)

Seven monoclonal cultures of *G. australes* and two of *F. paulensis* were inoculated at 20–30 cells mL^{-1} in 28 mL round bottom glass tubes. When cultures arrived at final exponential phase (after ± 20 days) cells were stained with Calcofluor White M2R (Sigma Aldrich, Spain) according to Fritz and Triemer (1985) [69]. Calcofluor-stained cells were observed using an epifluorescence microscope (LEICA DMLB and NIKON eclipse 80i) equipped with an Olympus camera (Olympus DP70), and they were measured using the software Olympus DP controller (Olympus Corporation). Morphological characteristics of microalgal cells were based on the tabulation system described in Fraga et al. (2011) [5]. Cell length was determined as the apical to antapical distance dimensions, depth as the dorso-ventral distance and width as the transdiameter distance that is the longest distance between opposed sides of the cingulum (Balech, 1989) [70]. Cell dimensions were expressed as mean ± standard deviation (SD).

2.4.2. Scanning electron microscopy (SEM)

SEM was used to study two monoclonal cultures of *G. australes* (IRTA-SMM-17–253 and IRTA-SMM-17–164) and one of *F. paulensis* (IRTA-SMM-17–211). For that, 10 mL samples of cultures at the initial exponential growth phase were fixed with glutaraldehyde at a final concentration of 4 % during 2 h at room temperature. After that, 3 mL of culture were collected with a syringe by applying a low pressure on 5 µm Nuclepore Track-Etch Membrane (Thermo Fisher Scientific) coated by poly-L-lysine and held in a plastic filter mold 13 mm (PALL, life Science). Filters were rinsed twice. Once with seawater (autoclaved and filtered by active carbon 0.2 µm) and a second time with filtered seawater/MilliQ water (50:50, v:v). Afterwards, filters were rinsed in a graded EtOH series of 30, 50, 70, 80, 90 and twice with 96 % (v:v). Later, filters were kept in a recipient with absolute EtOH and they were sent to the Scanning Electron Microscopy Service in the Institute of Marine Science (ICM-CSIC).

In the facilities, filters were submitted to critical-point drying with liquid carbon dioxide in a BAL-TEC CPD030 unit (Leica Microsystems, Austria). Dried filters were mounted on stubs with colloidal silver, then sputter-coated with gold in a Q150R S (Quorum Technologies Ltd). Cells were observed with a Hitachi S3500N scanning electron microscope (Hitachi High Technologies Co., Ltd, Japan) at an accelerating

voltage of 5 kV. Length and width of the Po plate and the second antapical plate, 2''' plate [5], were measured and the number of pores of the Po plate were counted. Measurements were made using ImageJ software [71].

2.5. Growth dynamics analysis

Before the growth dynamics analysis strains were first acclimated to laboratory conditions for approximately 1 year. To evaluate growth dynamics, three strains of *G. australes* (IRTA-SMM-17–162, IRTA-SMM-17–189, IRTA-SMM-17–271) and one strain of *F. paulensis* (IRTA-SMM17–209) were randomly selected from the algal collection. For each strain, 500 mL of medium were inoculated into 1.5 L Fernbach flasks at an initial concentration of 50 cells mL^{-1} in triplicate. Every 2–3 days, at the same time of the day, each culture was vigorously manually homogenized and 3 mL samples from each replicate were collected and preserved with 3% Lugol's iodine solution. Three countings of each sample were conducted under observation in an inverted light microscope using a 0.5 mL Kolkwitz counting chamber (Plankton Chamber acc. to Kolkwitz-Hydro-bios). For each day and replicate, average of the cell abundance (cells mL^{-1}) and SD were estimated. The growth rate (r) of each replicate was estimated by the equation of a linear regression by the least square fit after logarithmic transformation of the cell abundance vs time considering at least 3 points of the exponential phase. The growth rate was expressed in units of divisions (div.) day^{-1}. Moreover, the doublings per day (K) were calculated as $K = r/\ln(2)$ (Eq. 1) and expressed as doublings day^{-1} [72]. Besides, the time of division or doubling time (Td) was calculated as $Td = \ln(2)/ r$ (Eq. 2) and expressed as day^{-1} [72]. Also, growth phases were defined as Wood et al. (2005) [73] where the exponential phase (log phase) was defined as the period when the slope of the regression line between elapsed time and log cell concentration was maximum. Late exponential (late log)-early stationary phase was defined when the slope of the regression line between elapsed time and log cell concentration is reduced in comparison to the slope from the log phase. The negative growth was defined by constituent decrease of cells, which was assessed by observation of microalgal cells by light microscope and confirmed by observation of empty thecae. However, cultures did not arrive at significantly negative growth.

2.6. Toxin analysis

2.6.1. Culture, harvesting and algal extraction

The CTX-like activity was evaluated for 21 strains of *G. australes* (11 strains from Majorca and 10 from Minorca), and 6 strains of *F. paulensis* (2 strains from Majorca

and 4 from Minorca) harvested at late log - early stationary phases of culture. For this purpose, strains were inoculated in 500 mL of medium in 1.5 L Fernbach flasks at an initial concentration of 50 cells mL^{-1}. When culture arrived at late-exponential phase (after 20 ± 3 days), cultures were vigorously shaken, and 15 mL aliquots were fixed using Lugol's iodine solution (3 %) to estimate the cell concentration (cell mL^{-1}) in the culture. Subsequently, the remaining volume was collected in sterile 50 mL Falcon tubes and centrifuged at 4300 g for 20 min. Supernatants were discarded, and pellets were pooled in one 50 mL Falcon tube. Centrifugation was repeated, the supernatant was discarded, and the pellet was kept at −20 °C with absolute methanol (10 mL for 10^6 cells) until toxin extraction.

To prepare microalgal extracts, cell pellets of approximately 5×10^5 to 10^6 cells with methanol were sonicated using an ultrasonic cell disrupter (Watt ultrasonic processor VCX750, USA). The tip amplitude was set at 37 % and 3 s on/2 s off for 15 min. The sample was then centrifuged at 600 g for 5 min at 4 °C. Supernatant was transferred to a glass vial. Procedure was repeated twice, one with methanol and another with aqueous methanol (50:50; v:v) (10 mL for 10^6 cells). The methanol extracts were then evaporated to dryness with a rotary evaporator (Büchi Syncore, Switzerland) or dried under N_2 gas (Turbovap, Caliper, Hopkinton, USA) at 40 °C. The aqueous methanol was evaporated at 70 °C. When dryness was achieved, absolute methanol was added to the glass vials, then extracts were pooled, filtered with PTFE filters (0.2 µm) and stored at −20 °C.

2.6.2. CTX-like toxicity evaluation

The evaluation was conducted using the neuro-2a CBA. This assay is used to detect bioactive compounds which target the voltage gated sodium channels (VGSCs) [74–77]. Ouabain blocks the sodium efflux through the inhibition of the Na^+/K^+-ATPase pump whereas, the veratridine blocks the sodium voltage-gate channel in an open position [78]. The cell viability of the neuro-2a cells is affected when the extract contains CTXs or CTX-like compounds (molecules that activates to VGSC) after the ouabain and veratridine treatment [74–77].

Exposure of neuro-2a cells to CTX1B standard (reference) or microalgal extracts was performed following the protocol described in Reverté et al. (2018) [56]. Briefly, neuro-2a cells were seeded at a density of 1.4×10^5 cells mL^{-1} in 96-well plates. After 24 h, ouabain and veratridine (O/V) were added to a final concentration at 140 µM and 14 µM respectively, then, 10 µL of each sample (serial dilutions of extract or standard) was added to each well in triplicate. Concentrations of CTX1B ranged between 0.2 to 25 pg mL^{-1} and concentrations of microalgal extracts ranged between 0.3 to 1000 cells equiv. mL^{-1} for *G. australes* and 10 to 4000 cells equiv.

mL^{-1} for *F. paulensis.* After 24 h, cell viability was measured using a colorimetric assay using 3-(4,5-dimethylthiazol-2-yl)−2,5 diphenyltetrazolium (MTT) [77]. Absorbance was measured at 570 nm using an automated plate spectrophotometer (Synergy HT, Biotek, USA). Hence, for every assay a calibration curve of cell viability with the standard was obtained. Curves were adjusted to a sigmoidal logistic 4- parameter regression using SigmaPlot software 12.0 (Systat Software Inc., USA). Limit of detection (LOD) was calculated as the necessary concentration of standard to inhibit the cell viability by 20 % (IC_{20}) [74]. Concentrations of CTX-like compounds in microalgal extracts were estimated inferring the concentration from the standard curve based on the viability of neuro-2a cells. The amounts of CTX-like compounds were expressed as femtograms (fg) of CTX1B equiv. per cell. The limit of quantitation (LOQ) was calculated as the ratio of the LOD obtained with standard to the maximum concentration of microalgal extract used in the assay with no matrix effect being observed. A matrix effect or unspecific toxicity was considered when toxicity was recorded in the neuro-2a cells after exposure to microalgal extracts without ouabain and veratridine treatment (O/V-).

2.6.3. MTX-like toxicity evaluation

The MTX-like toxicity was evaluated qualitatively for 15 *G. australes* strains following the protocol described by Caillaud et al. (2010) [61]. This assay is based on the inhibition of the toxic effect by the addition of SKF96365, which is the 1-[2-(4-methoxyphenyl)−2-[3-(4-methoxyphenyl)propoxy]ethyl-1H-imidazolhydrochloride to neuro-2a cells. SKF96365 blocks the voltage-gated calcium channels (VGCCs) [79] counteracting the increase of intracellular calcium levels caused by compounds that target VGCCs.

3. Results

3.1. Presence of *Gambierdiscus* and *Fukuyoa* genera in the Western Mediterranean Sea

Presence of *Gambierdiscus* and *Fukuyoa* genera was assessed using samples from live and Lugol's iodine preserved samples collected during 2016 to 2019. A total of 110 isolates from the genera *Gambierdiscus* and *Fukuyoa* were obtained from the epiphytic samples and 26 isolates from the epilithic samples. Epiphytic samples were obtained from macrophytes of the genera *Lobophora, Cystoceira, Jania, Padina*, and *Dictyota*. Furthermore, other dinoflagellates co-occurred with the *Gambierdiscus* and *Fukuyoa* genera, such as the genera *Prorocentrum*, *Coolia*, *Amphidinium* and *Ostreopsis*. Fig. 1 shows stations where the presence of the genera *Gambierdiscus* and *Fukuyoa* were recorded in the Balearic Islands during the entire sampling period. Results of the presence of the genera *Gambierdiscus* and *Fukuyoa* by sampling point and the environmental data (temperature, pH, oxygen and salinity) are provided in supplementary Table 1.

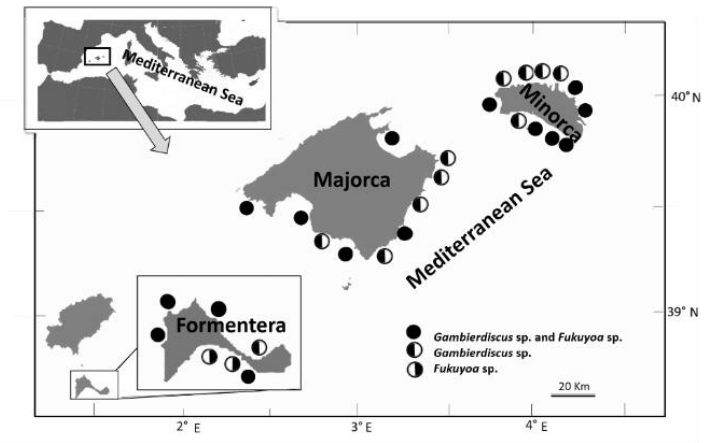

Fig. 1. Presence of the *Gambierdiscus* and *Fukuyoa* genera in the sampling stations in the Balearic Islands (Mediterranean Sea) during 2016-2019.

In Formentera, *Gambierdiscus* cells were present in 5 out of 9 sampling stations, in low amounts in both samples (epilithic and epiphytic). The presence was confirmed only in Lugol's iodine preserved samples; therefore, no live cells could be isolated. In Majorca, *Gambierdiscus* cells were found both in epiphytic and epilithic samples, although in Minorca cells were primarily found in the epiphytic samples. In Majorca and Minorca, in 2017 *Gambierdiscus* cells were present in all sampling stations with the exception of one site. Similar results for both islands were obtained in 2018. In

2019 only Minorca was sampled, and in 2 out of 4 sampling stations *Gambierdiscus* cells were present.

The genus *Fukuyoa* was present in epiphytic and epilithic samples, in 6 out of 9 stations of Formentera. *Fukuyoa* cells were found at very low amounts and only in preserved samples, therefore as with the genus *Gambierdiscus*, no cells could be isolated from Formentera. In 2017 in Majorca and Minorca, *Fukuyoa* cells were present in 5 out of 10 stations and 6 out of 9 stations respectively; while in 2018 cells were found in fewer stations: 4 out of 10 stations and 2 out of 9 stations, respectively. In 2019 in Minorca, cells were not observed in any of 4 sampling stations. *Fukuyoa* isolates from Majorca and Minorca were obtained only from epiphytic samples. During the entire study period, *Fukuyoa* cells were concomitant with *Gambierdiscus* in 4 stations in Formentera, and in all stations in Majorca and Minorca.

3.2. Molecular identification

Species level identification was performed for thirty-four *Gambierdiscus* and nine *Fukuyoa* isolates using the D8-D10 region (LSU) rDNA [7,80]. Sequences were matched in GenBank using the BLAST sequence similarity searches (National Center for Biotechnology Information) and they scored the highest identity and similarity with *Gambierdiscus australes* and *Fukuyoa paulensis.* Moreover, further phylogenetic analyses confirmed the identifications (Fig. 2).

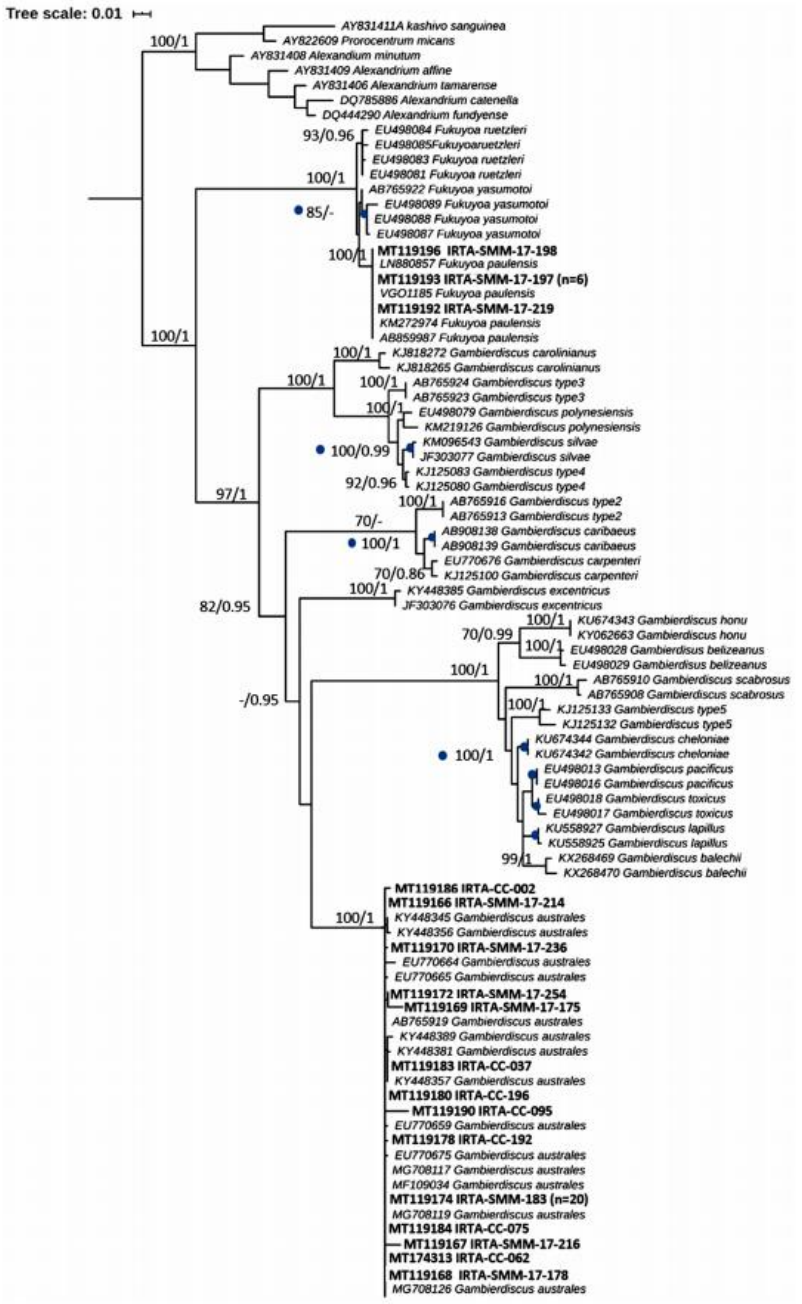

Fig. 2. Maximum likelihood (ML) phylogeny of D8-D10 LSU (rDNA) of *Gambierdiscus* and *Fukuyoa* species. The GenBank code accession numbers and species names are shown for each downloaded sequence. Strains isolated from samples from this study are shown in bold. Values at nodes represent the bootstrap values /bayesian posterior probability.

3.3. Morphological characterization

In the present study, cells of *G. australes* were anterior-posteriorly compressed showing a lenticular shape. Table 1 shows the measurements for *G. australes* and *F. paulensis* cells from the Balearic Islands in comparison to the measurements for these species retrieved from the literature. Measurements are shown in Table 1. The thecal plate formula was: Po, 4', 0a, 6''', 6c, ?s, 5''', 0p, 2''''. Figure 3 shows a representative SEM images for *G. australes*. *F. paulensis* cells were globular presenting a lateral compression. The thecal plate formula was Po, 4', 6'', 6c, ? s, 5''', 2''''. Figure 4 shows a representative SEM images for *F. paulensis*.

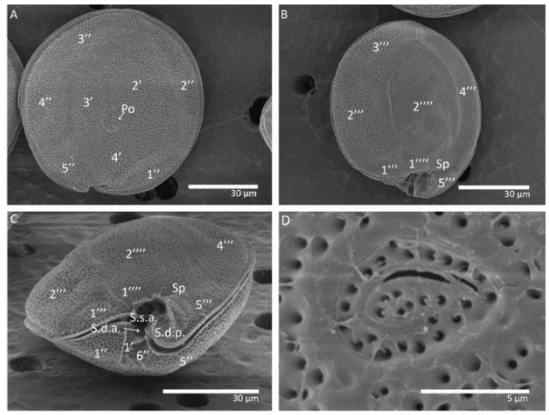

Fig. 3. SEM images of *G. australes* (IRTA-SMM-17–253): apical (A), antapical (B), ventral (C) views, detail of Po plate and pores (D).

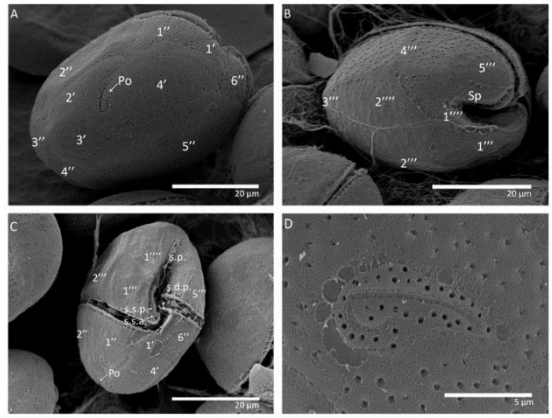

Fig. 4. SEM images of *F. paulensis* (IRTA-SMM-17–211): apical (A), antapical (B) and ventral (C) views and detail of Po plate and pores (D). (s. p.: sulcal posterior, s.s.p.: sulcal left posterior plate, s.d.p.: sulcal right posterior, s.s.a: sulcal left anterior plate).

3.4. Growth dynamics

All the studied strains (three *G. australes* and one of *F. paulensis* strains) displayed a typical growth curve in batch culture conditions. No significant differences were observed among the replicates of the strains. Strains of *G. australes*: IRTA-SMM-17–162, IRTA-SMM-17–189 and IRTA-SMM-17–271 arrived at the stationary phase at the 25th, 22nd and 21st days. For *F. paulensis*, strain IRTA-SMM-17–209 reached the stationary phase at day 21st of culture. The growth curves are provided in supplementary material figure S1 and results of growth rates are shown in Table 2.

Table. 2. Growth parameters of *G. australes* (n=3) and *F. paulensis* (n=1) from the Balearic Islands. Averages of the three replicates of: Max. conc.= maximum cell yield (cells mL^{-1}); r = growth rate (div. day^{-1}) ± standard deviation (SD), the period when r was calculated (days) is showed in brackets; K= doublings per day (doublings day^{-1}) ± SD; Td = doubling time ($days^{-1}$) ± SD.

	IRTA-SMM-17-162 ***G. australes***	**IRTA-SMM-17-189** ***G. australes***	**IRTA-SMM-17-271** ***G. australes***	**IRTA-SMM-17-209** ***F. paulensis***
Max. conc.	2288	1451	1244	1004
r	0.12 ± 0.04 (13-20)	0.15 ± 0.04 (13-20)	0.16 ± 0.04 (12-19)	0.24 ± 0.06 (7-14)
K (Eq. 1)	0.17 ± 0.05	0.21 ± 0.06	0.24 ± 0.06	0.34 ± 0.09
Td (Eq. 2)	6.25 ± 1.80	5.85 ± 1.07	4.36 ± 1.01	1.30 ± 1.64

Table. 1. Morphometric comparison of *G. australes and F. paulensis* strains of this study with published measurements for those species. Average and standard deviation of depth (D), length (L), width (W), ratio of depth and width (D:W), ratio of length and width (L:W); length of apical pore plate (Po) and surrounding pore numbers (No) and diameter (Ø), size of 2'''' plate. Data are expressed as the arithmetic mean, standard deviation (±SD) and number of measured cells (n).

		G. australes Chinain et al. 1999	*G. australes* Litaker et al., 2009	*G. australes* Rhodes et al., 2014b	*G. australes* Rhodes et al., 2014b	*G. australes* Bravo et al. 2019	*G. australes* This study	*F. paulensis* Rhodes et al., 2014a (*G. yasumotoi*)	*F. paulensis* Gómez et al. 2015	*F. paulensis* Laza-Martínez et al. 2016	*F. paulensis* This study
Isolates		RAV-92	RAV-92/NOAA24	CADW149	CAWD216			CAWD210	VGO1185	Dn35EHU	
Cell size	**L µm**	-	38.7 ± 3.8 (33.4 - 47.3)	32.0 (26.0 - 39.0)	39.0 (32.5 - 45.5)		-	59.8 ± 7.5 (54.3 - 67.3) (n=20)	56.0 ± 3.0 (51- 62)	48.9 ± 10.9 (35- 76) (n=100)	46.6 ± 8.7 (32.0-64.1) (n=21)
	D µm	86.0 ± 5.1 (76.0 - 93.0)	72.5 ± 3.8 (63.8 - 77.4)	44.5 (32.5 - 52.0)	58.5 (45.5 - 65.0)	81 ± 6.3 (68-95)	75.7 ± 6.0 (60.9 - 92.3) (n= 112)	54.8 ± 5.7 (49.1 - 60.5) (n=20)	50.0 ± 3.0 (45- 56)	40.8 ± 8.2 (31-67) (n=123)	40.5 ± 4.8 (36.4 - 51.1) (n=14)
	W µm	77.0 ± 3.7 (65.0 - 84.0)	63.4 ± 5 (55.2–73.8)	38.5 (32.5–52.0)	48.0 (40.0–52.0)	78 ± 7.5 (60-95)	78.7 ± 6.6 (54.7 - 90.8) (n= 112)	42.5 ± 4.1 (38.4 - 46.6) (n=20)	45.0 ± 2.0 (41- 48)	30.5 ± 6.6 (24- 38) (n=60)	41.1 ± 11.9 (11.9 - 41.1) (n=21)
	L: W		0.61	0.83	0.81			1.41	n. d	1.28 (n=10)	1.44 ± 0.21 (1.1 - 1.7) (n=21)
	D: W	1.12	1.14	1.16	1.22		1.02 ± 0.09 (0.8 – 1.2) (n= 112)	1.29	1.2	1.29 (n=48)	
Po plate	**L µm**	7.1 ± 0.8 (6.3–8.6)					7.2 ± 0.7 (6.2 – 8.4) (n=14)	9.9 (Laza-Martínez et al., 2016)	10-12	7.6	8.2 ± 1.4 (5.9 - 11.4) (n=13)
	W µm	6.1 ± 0.4 (5.7–6.8)					5.7 ± 0.7 (4.8 - 7.8) (n=14)	4.6 (Laza-Martínez et al., 2016)	6-7	4.1	3.0 ± 0.7 (2.2–4.4) (n=13)
	L: W	1.18					1.23 ± 0.1 (1.0- 1.5) (n=14)		n. d		3.1 ± 0.9 (1.8–4.4) (n=13)
	Number pores	31 ± 4.1					29 ± 1.6 (27– 33) (n=14)		23-39	29-39	35.3 ± 1.6 (32– 37) (n=13)
	Diameter pores µm	0.45 ± 0.03					0.39 ± 0.09 (0.2– 0.6) (n=197)			0.35 (n=150)	0.31 ± 0.08 (0.16 - 0.51) (n=52)
2^{nnn} antapical	**L 2^{nnn} µm**	54 ± 3.1					41.5 ± 4.8 (33.5 - 48.8) (n=14)		33-39		45.3 ± 2.9 (41.7– 48.8)
	W 2^{nnn} µm	27 ± 2.7					21.6 ± 3.3 (17.7 - 29.6) (n=14)		19-23		23.7 ± 3.4 (19.4 - 29.6)
	L: W 2^{nnn}	2.10					1.95 ± 0.28 (1.6 - 2.5) (n=14)				1.95 ± 0.32 (1.7 - 2.5) (n=7)

3.5. Evaluation of CTX-like and MTX-like toxicity

Exposure of neuro-2a cells to CTX1B standard was nontoxic. As expected, addition of ouabain/veratridine (O/V+) showed a typical curve of CTX-like toxicity in neuro-2a cells with an average LOD of 0.45 ± 0.24 pg CTX1B equiv. mL^{-1} and IC_{50} of 1.21 ± 0.48 pg CTX1B equiv. mL^{-1}. The maximum concentration of microalgal extract that did not cause any toxicity in the absence of ouabain and veratridine (O/V-) ranged from 10 to 220 and from 40 to 450 cells equiv. mL^{-1}, for *G. australes* and *F. paulensis*, respectively. All *G. australes* extracts (n = 21) presented CTX-like toxicity and toxicities ranged from 1.38 to 381 fg CTX1B equiv. $cell^{-1}$ (Table 3). Figure 5 shows representative dose-response curves of the types of neuro2a cell viability response for *G. australes*. Figure 5A corresponds to *G. australes* extract with low toxicity (IRTA-SMM-17–216). In the O/V+ conditions, the curve showed the typical dose-response curve of CTXlike toxicity with an estimated IC_{50} of 150 cell equiv. mL^{-1}. Figure 5B represents *G. australes* with high toxicity (IRTA-SMM-17–168) with an estimated IC_{50} of 2 cell equiv. mL^{-1}.

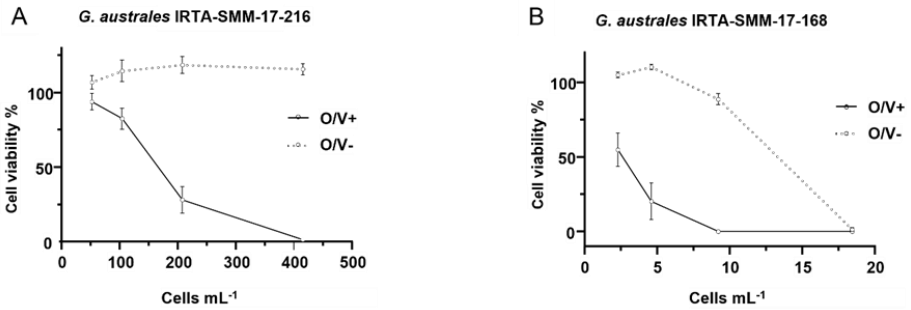

Fig. 5. Dose response curves obtained using neuro-2a CBA for *G. australes* extracts: IRTA-SMM-17-216 (A), IRTA-SMM-17-168 (B). O/V+: neuro-2a cells exposed to microalgal extract with the ouabain and veratridine treatment. O/V-: neuro-2a cells exposed to microalgal extract without the ouabain and veratridine treatment. Each point is the mean of triplicates and bars represent the SD.

Two strains of *F. paulensis* IRTA-SMM-17–209 and IRTA-SMM17–211 showed CTX-like compounds and the remaining four extracts did not show CTX-like toxicity (Table 3). Figure 6A corresponds to *F. paulensis* extract (IRTA-SMM-17–209). Cell exposure to this extract at 120 cells equiv. mL^{-1} in the O/V+ conditions significant toxicity was recorded, indicating a CTX-like effect. Figure 6B corresponds to *F. paulensis* extract (IRTA-SMM-17–198). Under both conditions with and without (O/V), the cell inhibition was significant, therefore no conclusion could be drawn in

reference to CTX-like toxicity. Two *F. paulensis* extracts (IRTA-SMM-17–206 and IRTA-SMM-17–220) caused cell mortality of neuro-2a in the absence of O/V (Fig. 6C, 6D). Nonetheless, under O/V+ conditions, the toxicity of these extracts was decreased, and this is a novel toxicity pattern described for this genus.

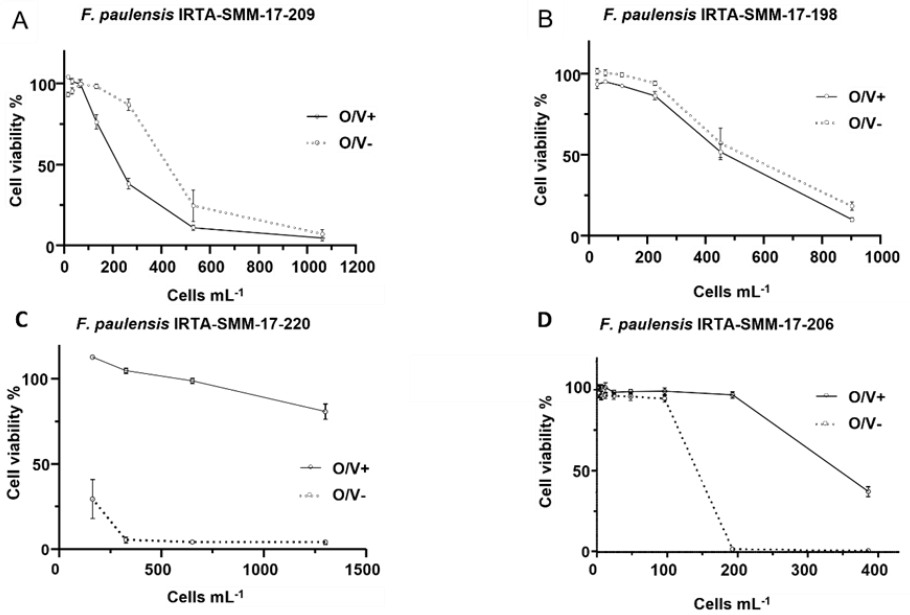

Fig. 6. Dose response curves obtained using neuro-2a CBA for *F. paulensis* extracts: IRTA-SMM-17-209 (A), IRTA-SMM-17-198 (B), IRTA-SMM-17-220 (C), IRTA-SMM-17-206 (D). O/V+: neuro-2a cells exposed to microalgal extract with the ouabain and veratridine treatment. O/V-: neuro-2a cells exposed to microalgal extract without the ouabain and veratridine treatment. Each point is the mean of triplicates and bars represent the SD.

In order to confirm the presence of MTX-like toxicity, neuro-2a cells were exposed to microalgal extracts in the presence of SKF96365. Twelve out of fifteen Gambierdiscus strains showed recovery of the cell viability when SKF96365 was added (Table 3). Figure 7 shows representative dose response curve of a *G. australes* extract with and without SKF96365. On the contrary, two *Fukuyoa* strains did not show recovery of the cell viability when SKF96365 was added.

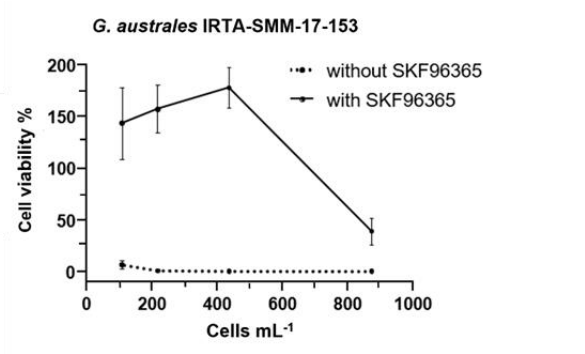

Fig 7. Dose response curve obtained using neuro-2a CBA with the *G. australes* extract (IRTA-SMM-17-153). Without SKF96365: neuro-2a cells exposed to extract in the absence of SKF96365; with SKF96365: neuro-2a cells exposed to toxin extracts in the presence of SKF96365. Each point is the mean of triplicates and the bars represent the SD.

Table 3. Evaluation of the presence of CTX-like and MTX-like toxicity by neuro-2a CBA. Species, code of strain, origin, cell concentration of cultures at harvesting time (cell mL^{-1}), values of CTX-like toxicity expressed in femtograms (fg) of CTX1B equiv. $cell^{-1}$ ± SD. n.s.: nonspecific toxicity; +: recovery of the cell viability in the presence of SKF96365; -: non-recovery of the cell viability in the presence of SKF96365; NT: not tested.

Species	Code	Island	Cell abundance (cells mL^{-1})	CTX-like toxicity (fg CTX1B equiv. $cell^{-1}$)	MTX-like Toxicity
G. australes	IRTA-SMM-17-153	Majorca	1750	1.38 ± 0.66	+
G. australes	IRTA-SMM-17-238	Majorca	1632	3.52 ± 0.18	NT
G. australes	IRTA-SMM-17-180	Minorca	613	5.25 ± 0.59	NT
G. australes	IRTA-SMM-17-218	Majorca	1686	9.47 ± 3.18	+
G. australes	IRTA-SMM-17-216[a]	Majorca	1476	13.14 ± 4.50	+
G. australes	IRTA-SMM-17-254	Majorca	1273	13.16 ± 1.34	+
G. australes	IRTA-SMM-17-253	Majorca	1935	13.45 ± 0.97	+
G. australes	IRTA-SMM-17-181	Minorca	1464	13.50 ± 0.80	+
G. australes	IRTA-SMM-17-178	Minorca	2040	14.52 ± 4.31	-
G. australes	IRTA-SMM-17-223	Majorca	1183	14.93 ± 4.69	+
G. australes	IRTA-SMM-17-155	Minorca	332	17.33 ± 1.60	-
G. australes	IRTA-SMM-17-173	Majorca	2087	21.89 ± 9.20	+
G. australes	IRTA-SMM-17-244	Majorca	924	34.33 ± 4.18	+
G. australes	IRTA-SMM-17-256	Majorca	1004	39.17 ± 16.44	NT
G. australes	IRTA-SMM-17-175	Minorca	1498	62.00 ± 0.66	-
G. australes	IRTA-SMM-17-164	Minorca	1022	72.60 ± 43.20	+
G. australes	IRTA-SMM-17-214	Majorca	1694	76.67 ± 29,86	+
G. australes	IRTA-SMM-17-189	Minorca	869	83.39 ± 12.14	NT
G. australes	IRTA-SMM-17-162	Minorca	1390	105.67 ± 18.27	NT
G. australes	IRTA-SMM-17-271	Minorca	843	172.63 ± 5.57	+
G. australes	IRTA-SMM-17-168[a]	Majorca	2274	381.83 ± 91.84	NT
F. paulensis	IRTA-SMM-17-209[a]	Minorca	782	16.30 ± 1.67	NT
F. paulensis	IRTA-SMM-17-211	Minorca	3250	7.96 ± 0.14	NT
F. paulensis	IRTA-SMM-17-198[a]	Majorca	4825	n.s	NT
F. paulensis	IRTA-SMM-17-206	Majorca	2053	n.s	-
F. paulensis	IRTA-SMM-17-220	Minorca	2128	n.s	-
F. paulensis	IRTA-SMM-17-221	Minorca	6636	n.s	NT

[a] response curves of CTX-like evaluation of these strains are shown in Fig. 5 and 6.

4. Discussion

The presence of the genus *Gambierdiscus* in the Eastern Mediterranean Sea was reported in 2003 by Aligizaki & Nikolaidis (2008) [81]. Reported species include *G. carolinianus* [82], *Gambierdiscus* sp., *G.* cf. *belizeanus* and *G. silvae* [16]. *Gambierdiscus australes* was detected later in the Balearic Islands, as presented in a brief communication [10]. The first detection of the genus *Fukuyoa* was in 2016 in the Western Mediterranean Sea [15] and in 2018 in the Eastern Mediterranean Sea [16].

To the best of our knowledge, the Balearic Islands is the location with the highest latitude worldwide, where the *Gambierdiscus* genus has been detected, specifically at 40.06 ° N. In the present study, the presence of the genus *Gambierdiscus* over large areas along the coasts of the Balearic Islands and the recurrence at some stations over three years suggests that this genus is well-established in the archipelago. However, the genus *Fukuyoa* was identified in 2017 and 2018, but not in 2019. Though the absence in 2019 should take into account that only four stations were sampled that year.

Water temperature influences on the growth and cell abundance of microalgae and can predict latitudinal distribution. *Gambierdiscus* species show different thermal limits, and distinct optimal temperatures [83]. Other variables such as salinity and irradiance can play an important role in the species distribution, though, their limits may be common for several species [83]. During the sampling dates, for Formentera, Minorca and Majorca water temperatures (22.8 and 27.2 °C) were close to optimal temperatures for *Gambierdiscus* species. Generally, for *Gambierdiscus* spp. the optimal temperature range is between 23 and 29 °C and the survival below 15 °C in laboratory conditions is rare [83,84]. *G. australes* is one of the most cryo-tolerant species in the genus; its optimal temperature for growth is relatively low, at 25 °C [85]. Besides, *G. australes* cells from the Canary Islands showed the ability to stay alive with no growth for six months at 15 °C, and they resumed growth when the temperature arose to 17 °C (personal communication by Dr. Isabel Bravo https://ciguateravgo.es/). This thermo-physiologic characteristic of *G. australes* could confer upon this species the ability to persist in the Balearic Islands in wintertime when the water temperature drops at 13 °C.

Regarding *Fukuyoa* spp., literature of the optimal temperature for growth is scarce. Nonetheless, *F. paulensis* (classified previously as *G. yasumotoi*) was recorded in New Zealand [86], where the water temperature oscillates between 14 and 23 °C. Additionally, one strain (Dn135EHU) from the Balearic Islands showed the formation

of cysts [15], and this could favour the species survival for long periods at low temperatures.

In previous studies of benthic microalgae samples collected in Majorca in 1997-1998 [87], 2001 and 2011 [15] no cells of the genera *Gambierdiscus* or *Fukuyoa* had been detected. In addition, during 2005-2006 an exhaustive sampling was conducted to characterize the phytoplankton communities from 1 to 15 m depth in the entire Balearic Archipelago [88] and cells of the genera *Gambierdiscus* or *Fukuyoa* were not detected. Although *Gambierdiscus* and *Fukuyoa* are mainly benthic, and Puigserver et al. (2008) [88] focused on phytoplankton, free-swimming cells could have been observed as was described in Parsons et al. (2011) [89]. The recent findings of cells of these genera in the Balearic Islands could be explained by an intense and specific sampling design for benthic species. Although cell abundance was not evaluated in the present work, the recent detection of these genera might be a result of an increase in abundance of endemic populations. The populations could be influenced by climate change [9,18,90,91]. In the Mediterranean Sea, it is expected to cause an increase in abundance of thermo-tolerant species and a decrease or disappearance of cold-tolerant stenothermal species [92]. In addition to regional temperature increase as potential cause to changes in microalgal populations, other factors such as storms or anthropogenic activities in coastal regions could be involved. It has been suggested that expansion of benthic dinoflagellates could be attributed to an increase of turf algal mats covering substrates due to environmental changes (storms, currents, acidification) [93–95], but also to the degradation of the marine environment directly associated to human activities such as bottom dredging for the creation of port structures and other forms of coastal embayments, drag-netting, pollution and over-exploitation of natural resources [96–99]. The environment of the Balearic Islands, most specifically the coastal areas, has suffered extreme pressures from tourism since the 1960s [100]. The impact of tourism has caused a clear degradation of the coast by increasing port structures, disturbing the coastal sediments and increasing the eutrophication [101]. In addition, their meadows of *Posidonia oceanica* are in decline in favour of colonisation of turf algal mats [102,103]. The reduction of these disturbances should not be expected in the coming years [100,103], so these factors could favour further increase in *Gambierdiscus* populations.

Another explanation for the presence of *Gambierdiscus* cells, may be new colonisations from other regions. For some benthic species of toxic dinoflagellates, the new colonisations may be associated with translocations of organisms by ballast waters [104]. In fact, it has been suggested that *Alexandrium pacificum* (previously identified as *Alexandrium catenella*), a species described as non-native in the

Mediterranean Sea, has been introduced by ballast waters of cargo vessels [98,105]. It is well reported that the Eastern Mediterranean Basin is suffering a large-scale invasion of tropical and subtropical species. At the moment, more than 700 species of organisms have been identified as having come from the Red Sea through the Suez channel [106]. However, from the genera *Gambierdiscus* and *Fukuyoa*, species reported in the Red Sea are *G. belizeanus* [11] and *F. yasumotoi* [107]. Therefore, the phenomenon of species translocation from the Red Sea to the Mediterranean Sea may not explain the current situation for these species. Considering the possibility that *Gambierdiscus* cells reached the Balearic Islands from the Atlantic Ocean, the genetic information provided by the D8-D10 region (LSU rDNA) shows that *G. australes* populations from the Balearic Islands and the Canary Archipelago are identical. Thus, more molecular markers should be analysed to determine the relationship between populations in these two areas. Population genetics and phylogeographic studies of these species have to be considered in future studies because they can help to identify the source of populations and reveal expansion patterns, and mechanisms of transfer [108].

Gambierdiscus australes cells from the Balearic Islands show morphological similarities to other *G. australes* described in previous studies (Table 1). Cell size (D and W) from the present study is partially consistent with the range of the first description of *G. australes* for the strain RAV-92 in the Pacific Ocean by Chinain et al. (1999) [80]. Later, strains RAV-92 and NOAA2 were measured by Litaker et al., (2009) [7] and their minimum extreme values of D and W were almost the same as in the current study. Cell morphology can change by natural factors, but also, over time of cultures in laboratory conditions [109]. The maximum values of D and W for *G. australes* were described for strains isolated from the Canary Islands (Atlantic Ocean) in Bravo et al. (2019) [110]. Values of Bravo et al. (2019) and the present work show larger sizes than those of Rhodes et al. (2014) [109], Chinain et al. (1999) [80] and Litaker et al. (2009) [7].

Regarding *Fukuyoa* isolates, the average L and D of cell size in the current study are inside the ranges of previous studies performed in the Mediterranean Sea, the Atlantic and the Pacific Ocean [2,86,111] (Table 1). However, the lowest values for L and W in the present work are smaller than in the previous studies.

The maximum cell yield for *G. australes* cultures in the present study of growth dynamics and toxicity was 2288 to 2274 cells mL^{-1}, respectively. These values are lower than for *G. australes* strains from the North Atlantic Ocean [56], where the maximum cell yield was 4470 cells mL^{-1}. Such differences may be attributed to the differences in the culturing conditions. In both works, cells were cultivated at the same temperature and medium, but in Reverté et al. (2018) [56] photon irradiance

was lower, a pump supplied the aeration and the vessel was a 3L round-bottom flask.

In the current work, growth rates for *G. australes* were lower than the rates of *G. australes* strains from the Atlantic Ocean reported by Reverté et al. (2018) [56], which ranged from 0.20 to 0.39 div. day^{-1}, and they are similar to the rates for *G. australes* strains, from the Pacific Ocean described before: 0.12 - 0.19 div. day^{-1} in Chinain et al. (2010) [53] and 0.149 ± 0.006 div. day^{-1} in Pisapia et al. (2017) [54]. The growth rate in the *Gambierdiscus* genus has been reported to be in the range of 0.01 to 0.55 div. day^{-1} [84,112]. Some studies for the genus *Gambierdiscus* link high division rates to high toxin production per cell [53–55], but in the present study, this relation was not studied.

Regarding *F. paulensis* growth, there was high variability of maximum cell yield among the strains from the current study. The maximum cell yield in the growth dynamics study was 1004 cells mL^{-1} (Table 2), and for the CTX-like toxicity study, values ranged between 333 and 6636 cells mL^{-1} (Table 3). These yields were much lower than those achieved at the stationary phase by Laza-Martínez et al. (2016) [15] of 14,800 cells mL^{-1}. In Laza-Martínez et al. (2016) [15], strains were cultured in culture plastic flasks, with f/4 medium with selenium that was previously reported by Guillard and Ryther (1962)[113] and salinity was adjusted at 35. Besides, cells were maintained at 25 °C and irradiance of 50-100 µmol m^{-2} s^{-1}. Regarding growth rates, the present study provides the first data for *F. paulensis* with 0.24 ± 0.06 div. $cell^{-1}$. Within the *Fukuyoa* genus, *F. ruetzleri* (previously *G. ruetzleri*) showed growth rates of 0.17, 0.18 and 0.35 div $cell^{-1}$ in Litaker et al. (2017) [55], Pisapia et al. (2017) [54], and Kibler et al. (2012) [83], respectively.

In the current paper, *G. australes* strains presented CTX-like activity with quantitations ranging between 1.4 and 380 fg CTX1B equiv. $cell^{-1}$. These quantitations are low compared to values for *G. australes* from the Atlantic Ocean reported by Reverté et al. (2018) [56], where values ranged from 200 to 697 fg CTX1B equiv. $cell^{-1}$. In both works, strains were acclimated for one year, but as it has been mentioned above, they were cultured in different culturing conditions. Therefore, dissimilar toxin production could be caused by distinct culturing conditions. In contrast, the CTX-like activity was similar to other *G. australes* strains from the Atlantic Ocean (31-107 fg CTX1B equiv. $cell^{-1}$) and from the Pacific Ocean (40 fg CTX1B equiv. $cell^{-1}$) reported by Rossignoli et al. (2020) [57] and Rhodes et al. (2017)[17], respectively.

Among *Gambierdiscus* species, *G. australes* has intermediate CTX-like toxicity. For instance, by standard mouse bioassay (MBA), *G. australes* extracts presented lower

toxicity than *G. pacificus* and *G. polynesiensis* [80], with the latter being the most toxic species in the genus. Furthermore, in Chinain et al. (2010) [53], the CTX-like response for strains from the Pacific Ocean was similar to *G. toxicus*, and 100-fold lower than in *G. polynesiensis.* Moreover, in Pisapia et al. (2017)[54], the CTX-like toxicity of ten strains was evaluated by neuro-2a CBA, and three *G. australes* strains (two from the Atlantic and one from the Pacific Ocean) were placed in the seventh-place, near the bottom of the scale.

Despite several unsuccessful attempts to confirm toxicity in *Gambierdiscus* spp. [14], CTXs have not been confirmed for most *Gambierdiscus* spp., except for *G. australes* [114], *G. pacificus* [115], *G. polynesiensis* [53] and *G. excentricus* [116]. A putative CTX (2,3-dihydroxy P-CTX-3C) was detected by liquid chromatography-mass spectrometry (LC-MS/MS) in only one *G. australes* strain (CCMP 1653) from Hawaii (Pacific Ocean), at exponential phase [114]. In fact, *Gambierdiscus* species are common producers of MTXs and *G. australes* is one of the top producers [23]. MTX1 and MTX3 were detected by LC-MS/MS in *G. australes* by Munday et al. (2017) [23]. Moreover, in Rhodes et al. (2017) [17], LC-MS/MS confirmed the presence of MTX1 in all tested *G. australes* strains. This is in accordance with the results for the present study in which almost all strains of *G. australes* (12 out of 15) presented MTX-like activity. Nonetheless, a recent study including *G. australes* cultures from the present work, (IRTA-SMM-17-162, IRTA-SMM-17-164, IRTA-SMM-17-189, IRTA-SMM-17-244, IRTA-SMM-17-253, IRTA-SMM-17-271) were analysed by liquid chromatography coupled to low and high resolution mass spectrometry (LC-MS/MS) and (LC-HRMS), and MTX1, desulfo-MTX1 and didehydro-34 desulfo-MTX1 were not detected. By contrast, 44-methylgambierone (MTX3) was present in all of these strains [117].

The MTX family, previously included only molecules with a molecular weight of more than 3000 Da and having no activity on VGSCs [118]. Recently, 44-methylgambierone has been found in *G. belizeanus* and *G. australes* [119,120]. This molecule was previously defined as MTX3 [121]. Nonetheless, it is a molecule of 1060 Da which presents structural differences as compared to the previous MTXs and shows CTX-like activity more than MTX-activity. In human cortical neurons, 44-methylgambierone showed no signals of cell mortality at 0.01 to 20 nM for five days, whereas at 0.1 nM of MTX1 significant cell death was observed in 2h [119]. Furthermore, in human neuroblastoma cells, after 24h of exposure of cells to MTX3 at 10 to 50 nM cells did not show signs of toxicity, while with MTX1 at 0.1 nM, complete cell death was observed [119]. Hence, including 44-methylgambierone in the MTX group may lead to confusion on the role of the rest of MTXs in CP. Given that only one strain of *G. australes* strain produced a CTX analogue [114], and the

effects of 44-methylgambierone in neuro-2a cells could be similar to effects of CTXs, the CTX-like toxicity of the *G. australes* extracts of the present study could be potentially attributed to the effect of 44-methylgambierone. Even so, 44-methylgambierone exhibited very low toxicity by MBA, hence it is unlikely it contributes to CP [122].

Concerning *F. paulensis,* the CTX-like toxicities of the current study ranged from 8 to 16 fg CTX1B equiv. $cell^{-1}$. These toxicities were low in comparison to the *G. australes* strains. Previously, one *F. paulensis* strain (Dn35EHU) from the Balearic Islands presented low CTX-like toxicity by MBA [15]. In the same study, for the same strain, traces of 54-deoxyCTX1B and gambieric acid A (GA A) were detected by LC-HRMS. Recently, Estevez et. al (2020) [117] detected 44-methygambierone for the *F. paulensis* strain (IRTA-SMM-17-209), which is the same strain used in the current study. This is in accordance with the results of Rhodes et al. (2014) [86] and Larsson et al. (2019) [14], which detected 44-methylgambierone in *F. paulensis* from the Pacific Ocean. Therefore, like *G. australes*, the CTX-like toxicity of *F. paulensis* in neuro-2a CBA could be explained by the presence of CTX analogues and the 44-methylgambierone.

F. paulensis presents low toxicity in comparison to other *Fukuyoa* species. Litaker et al. (2017) [55] detected CTX-like toxicity in three *F. ruetzleri* strains by neuro-2a CBA with an average of 24.50 and 6.50 fg CTX3 equiv. $cell^{-1}$. Like *G. australes,* it is suggested that some strains of *F. paulensis* are non-CTX-producers because no signal of CTX-like toxicity and no CTX-analogues were found at early stationary phase for a cultured strain (VGO1185) from Brazil (Atlantic Ocean) by neuro-2a CBA [2]. Moreover, *F. paulensis* (previously *G. yasumotoi*) CAWD210 from New Zealand did not exhibit CTX-like toxicity by sea urchin embryo assay (SUEA) [86]. That is in concordance with the results of the present study and the increase in viability observed when neuro-2a cells were exposed to extracts from *F. paulensis*. Laza-Martínez et al. (2016) [15] detected MTX-like activity by MBA. However, in the present work, the MTX-like activity for *F. paulensis* was not detectable (n=2). Furthermore, there is no confirmation of MTX1, desulfo-MTX1 and didehydro-34 desulfo-MTX1 in *F. paulensis* strain (IRTA-SMM-17-209) by analytical methods [117].

Toxicities of several strains of *Gambierdiscus* from the Pacific have been largely studied, but information about the strains from the Mediterranean Sea is scarce despite the increasing identification of species in recent decades. To the best of our knowledge, the presence of CTX-like toxicity in *Gambierdiscus* strains has only been evaluated from the eastern Mediterranean region using three strains of *G. carolinianus, G. silvae* and *Gambierdiscus* sp.; all of them analysed by neuro-2a CBA. The *G. carolinianus* strain showed CTX-like activity in low quantities (< 4 fg

CTX3C equiv. cell^{-1}) [54], the *G. silvae* showed high CTX-like toxicity and the putative new species *Gambierdiscus* sp. exhibited low CTX-like activity [16]. The demonstration of CTX-like toxicity in strains in the Balearic Islands, and the fact that no evidence of ciguateric fish or CP has occurred in this area, could suggest that these populations are relatively new residents or that the densities of the populations are probably low.

5. Conclusions

Fukuyoa and *Gambierdiscus* cells found in samples from the Balearic Islands from 2016 to 2019 have been identified as *F. paulensis* and *G. australes*. These two species seem to be well-established in the area. Considering the other studies, CTX-toxicity exhibited by most of the *G. australes* and *F. paulensis* strains was low. However, one strain of *G. australes* (IRTA-SMM-17–168) was classified as a very high producer in comparison to previous studies. In addition, it is not possible to discard that some cells from the Balearic Islands could be a high CTX-producers. Even though CP cases have not yet been confirmed in the Mediterranean, the CTX-like toxicity present in the strains of *G. australes* and *F. paulensis* from the Balearic Islands may indicate that potential future cases of CP should not be dismissed. There is a clear need for continued studies and monitoring of benthic dinoflagellates in the region.

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Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.hal.2020.101913.

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iii. *Use of Mass Spectrometry to determine the Diversity of Toxins Produced by Gambierdiscus and Fukuyoa species from the Balearic Islands and Crete (Mediterranean Sea) and the Canary Islands (Northeast Atlantic)*

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ABSTRACT

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 ciguatera poisoning (CP) remains to be clarified. Ciguatoxins and maitotoxins are very toxic compounds produced by these dinoflagellates and were 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.

Keywords: maitotoxins; ciguatoxins; *Gambierdiscus*; *Fukuyoa*; LC-MS/MS; HRMS; QToF; ciguatera poisoning

Key Contribution: Metabolites were identified in several *Gambierdiscus* strains from the Balearic Islands, Greece and the Canaries. High resolution mass spectrometry confirmed the presence of 44-methyl gambierone (MTX3) and did not detect maitotoxin-1 (MTX1), desulfo-MTX1 and didehydro desulfo-MTX1 above the detection limit in Mediterranean strains of *G. australes*.

1. Introduction

Ciguatera Poisoning (CP) is described as a food intoxication endemic in tropical and subtropical areas of the world. The poisoning is caused by the consumption of fish or shellfish that accumulat toxic compounds produced by benthic dinoflagellates of the genus *Gambierdiscus* and *Fukuyoa* [1].

CTXs are considered the main toxins responsible for CP as their lipophilic character allows for intestinal absorption and accumulation. They are cyclic polyether compounds of around 1100 Da being classified as Pacific (P-CTXs), Caribbean (C-CTXs) and Indian (I-CTXs) ciguatoxins. Different CTX analogues from these groups have been detected in fish tissue associated to a CP case, while only a few P-CTXs were detected in *Gambierdiscus* extracts from the Pacific Ocean [3–7]. MTXs are water soluble cyclic polyethers containing one or two sulfate ester groups; these groups are responsible for the intermediate polarity of MTXs, and their low intestinal absorption casts doubts as to their involvement in CP [8]. Six MTX analogues have currently been identified: maitotoxin-1 (MTX1), maitotoxin-2

(MTX2), maitotoxin-3 (MTX3), maitotoxin-4 (MTX4), desulfo-MTX1 and didehydrodemethyl-desulfo-MTX1, all of which were isolated from different strains of *Gambierdiscus* [9–12]. MTX1 is the most toxic marine compound and the largest natural non-biopolymer toxin consisting of a ladder-shaped cyclic polyether compound containing two sulfate groups [9]. MTX2 was isolated from an Australian *Gambierdiscus* strain from Queensland [10]. Its structure has not yet been elucidated and it showed a lower potency than MTX1 by intraperitoneal injection in mice. MTX3 (44-methylgambierone) was first characterized by [10], but it was not until recently that its structure had been elucidated by its isolation from *G. belizeanus* and *G. australes*, being identified as a gambierone homologue [13 - 15]. MTX3, which is about one third the molecular weight of MTX1, showed a biological activity similar to CTX3C but with much lower potency, indicating that despite being grouped in the MTX group, this compound exhibits CTX-like activity rather than MTX-like activity [13]. MTX4 was recently isolated from *G. excentricus* extracts from the Canary Islands (Spain) [11]. Its structure is not yet elucidated but it has ion clusters and molecular mass in a similar range as MTX1, as well as sulfate esters; it was reported to exhibit a similar toxic effect of MTX1 in neuroblastoma cells detecting a high cytotoxicity and Ca^{2+} influx [11]. Other large but mono-sulfated MTXs were recently elucidated by their isolation from *Gambierdiscus* spp. from the Caribbean Sea, i.e., desulfo-MTX1 from *G. belizeanus* and didehydro-demethyl-desulfo-MTX1 from G. ribotype-2, showing the wide variety of MTXs that seem to be produced by these dinoflagellates [12] (Table 1). Furthermore, *Gambierdiscus* and *Fukuyoa* have shown to produce non-structurally related cyclic polyether compounds such as gambierol, gambieric acids, gambieroxide and gambierone [15-18].

Most of these compounds can be classified depending on their mechanism of action into two groups: (i) MTX-like compounds, associated with a massive Ca^{2+} influx causing a rapid cell death, including MTX1, MTX2 and MTX4; and (ii) CTX-like compounds, which create a disequilibrium in the voltage-gated sodium channels (VGSCs), including all CTXs, and with much lower potency, MTX3 and gambierone [13].

A wide variety of *Gambierdiscus* species are present in regions considered endemic for CP (the Pacific Ocean or the Caribbean Sea) [19]. These dinoflagellates have also been detected in CP-emerging regions such as the Canary Islands (Spain) and Madeira archipelago (Portugal), as well as in the Mediterranean Sea, from which no CP cases have been reported until now [20]. *Gambierdiscus excentricus* was the first *Gambierdiscus* species detected in the Canary Islands [21], but its detection led to further research that concluded with the detection of a large number of *Gambierdiscus* species (*G. australes*, *G. caribaeus*, *G. carolinianus*, *G. excentricus*, *G. silvae* and *Gambierdiscus* ribotype*3*) suggesting that the Canary Islands would be a "hot spot" for these dinoflagellates [22]. On the other hand, the only available data from the Selvagem Islands (Madeira, Portugal) is the detection of numerous strains of *G. australes*, which test positive in the Neuro2a cell-based toxicity assay (Neuro-2a CBA), suggesting they contain CTX- and MTX-like compounds [23]. In the Mediterranean Sea, the presence of *Gambierdiscus* was first reported in 2003 (*Gambierdiscus* sp.) in Crete (Greece), while to our knowledge only one study in 2016 attempted to characterize a strain of *F. paulensis* from Formentera (Balearic Islands, Spain) by LC-MS, however, the trace levels of toxic compounds present did not allow for conclusive results about the compounds produced [24,25].

Table 1. List of the Maitotoxins isolated from dinoflagellates of the genus *Gambierdiscus* and *Fukuyoa*.

MTX congener	Chemical formula	Monoisotopic mass (Da)	Reference
MTX1	$C_{164}H_{258}O_{68}S_2$	3379.6171 for the free acid form	[9]
MTX2	Unknown	3298 for the mono-sodium salt	[10]
MTX3	$C_{52}H_{78}O_{19}S$	1038.4858 for the free acid form	[13,14]
MTX4	Unknown	3292.486 for the free acid form	[11]
desulfo-MTX1	$C_{164}H_{258}O_{65}S$	3299.6603 for the free acid form	[12]
didehydro-demethyl-desulfo-MTX1	$C_{163}H_{254}O_{65}S$	3283.6290 for the free acid form	[12]

*This table include MTX3 which is 44-methylgambierone but previously it was classified as MTX.

As part of the ongoing EuroCigua project [26], *G. australes* and *F. paulensis* were isolated from Menorca and Mallorca (Balearic Islands, Spain), *Gambierdiscus* sp.2 from Crete (Greece), as well as *G. excentricus* from La Gomera (Canary Islands, Spain), showing CTX and MTX-activity. The aim of the present study was to describe the diversity of toxins produced by a selection of these strains of *Gambierdiscus* and *Fukuyoa* species using liquid chromatography coupled to low- and high-resolution mass spectrometry (LC-MS/MS and LC-HRMS). While LC-MS/MS was used for a rapid screening of the toxic compounds potentially present, allowing for the estimation of their concentrations, the purpose of LC-HRMS was the confirmation and structural characterization of the toxic compounds previously identified by LC-MS/MS.

2. Materials and Methods

2 .1. Reference Toxins and Chemicals

Maitotoxin-1 (MTX1) standard used for the LC-MS analysis was obtained from Wako Chemicals USA, Inc. (Richmond, VA, USA). MTX1 standard was dissolved in MeOH:H2O (1:1, v/v) being the stock solution 10 µg mL^{-1}. MTX4 qualitative laboratory reference material partially purified from *Gambierdiscus excentricus* was available from a previous study at the Phycotoxins Laboratory [11]. MTX3 and gambierone were identified as both compounds have identical retention times in our chromatography in both *G. australes* and *G. belizeanus*, the two species from which they have been originally isolated. MTX3 and gambierone qualitative laboratory reference material from *G. australes* and *G. belizeanus* was available at the Phycotoxins Laboratory [11]. HPLC-grade methanol and dichloromethane for extraction were purchased from Sigma Aldrich (Saint Quentin Fallavier, France). Milli-Q water was supplied by a Milli-Q integral 3 system (Millipore, Saint-Quentin-Yvelines, France). Water, acetonitrile, formic acid and ammonium formate used to prepare mobile phases were of LC-MS grade. All these chemicals were purchased from Sigma Aldrich (Saint Quentin Fallavier, France).

In order to carry out the toxicity evaluation by Neuro-2a CBA, CTX1B was provided by Dr. Lewis, University of Queensland and was stored in absolute methanol at -20 °C. Neuroblastoma murine cells (neuro-2a) were purchased in ATCC LGC standards (USA). Ouabain, veratridine, sodium pyruvate, thiazolyl blue tetrazolium bromide (MTT) were purchased from Merck KGaA (Darmstadt, Germany). Dimethyl sulfoxide (DMSO) and absolute methanol were purchased from Honeywell (Fürth, Germany) and from Chemlab (Zedelgem, Belgium), respectively. The incubator was purchased from Binder, Germany. The microplate reader KC4 was purchased from BIO-TEK Instruments, Inc.

2.2. *Gambierdiscus* and *Fukuyoa* Strains

All nine dinoflagellate extracts analyzed in this work were obtained from the collection of strains obtained in the EuroCigua project. All strains were cultivated at the IRTA laboratory (Tarragona, Spain) and the detailed information about these strains is shown in Table 2.

Table 2. Detailed information about the dinoflagellate extracts analyzed with the neuroblastoma cell-based assay.

Species	Strain Code	Location	Number of Cells Extracted	Volume of Culture (L)	CTX-like (fg CTX1B Equiv./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 (NQ)
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 [33].

2.2.1 Culturing, harvesting, toxin extraction and cell-based assay analysis

All strains were inoculated in 5L of medium ES (Provasoli 1968, modified by Jorge Diogène) with the salinity was adjusted to 36 in a 8L flat-bottom round glass-flasks. Cultures were maintained in filtered air and lightly turbulence (gentle bubbling) was supplied by air-pump system. The initial concentration of dinoflagellates was between 25 and 50 cells mL^{-1}. Strains were incubated at 24 ± 0.5 °C. The illumination was provided by fluorescent tubes and with photon irradiance of 100 µmol m^{-2} s^{-1} under 12:12 h L:D photoperiod.

When cultures arrived at late-exponential phase (after 20 ± 3 days), cultures were vigorously shaken, and 15 mL aliquot was taken and fixed by lugol's iodine solution (3 %) to estimate the cell concentration (cell mL^{-1}). Subsequently, the remaining volume of each strain was filtered and collected by a 10 µm plankton net (Holmbladsvej, Denmark) in sterile 50 mL Falcon tubes and centrifuged at 4300 g for 20 min (Alegra X-15R, Beckman Coulter). Supernatants were discarded and microalgal pellets of each strain were pooled in one 50 mL Falcon tube. Centrifugation was repeated and supernatant were discarded. Pellets were subsequently kept at -20 °C with absolute methanol (10 mL for 10^6 cells) until toxin extraction.

To extract the toxin from microalgal pellets, each pellet with methanol was sonicated using ultrasonic cell disrupter (Watt ultrasonic processor VCX750, USA). The tip amplitude was set at 37 %, 3 sec on/2 sec off for 15 minutes. The sample was then centrifuged at 600 g for 5 min at 4 °C. Supernatant was transferred to a glass vial. Then methanol was added to microalgal pellets, and the procedure of toxin extraction was repeated twice. After that, the methanol extract was evaporated to dryness with a rotary evaporator (Büchi Syncore, Switzerland) or dried under N_2 gas (Turbovap, Caliper, Hopkinton, USA) at 40 °C, then extracts were filtered with PTFE filters (0.2 µm) and stored at -20 °C. The neuro-2a CBA was performed according to Reverté et al. (2018) [66].

2.2.2. Sample pretreatment for LC-MS analysis

The methanol extract from the cell pellet extraction was evaporated to dryness under N_2 stream at 50 °C and CTX- and MTX-like compounds were partitioned as previously described [67]. Briefly, the residue from extraction was reconstituted in dichloromethane (50 mL by 1 million cells) and partitioned twice with MeOH:H_2O (3:2, v/v) (25 mL by 1 million cells). Both organic and aqueous layers were evaporated to dryness under N_2 stream at 50 °C and kept at -20 °C prior to the analysis. MTX-like compounds are supposed to partition into the MeOH:H_2O (3:2, v/v) whereas CTX-like compounds are supposed to partition into the dichloromethane layer. Dried residue from the aqueous methanol fraction was reconstituted in 0.5 mL of MeOH:H_2O (1:1, v/v) whereas the solid residue from the dichloromethane layer was reconstituted in 0.5 mL MeOH being both filtrated through 0.2 µm prior to the LC- MS analysis.

2.3. LC-MS analysis

2.3.1. LC-LRMS/MS (API 4000 QTrap)

LC-MS/MS analysis to monitor specific MTX congeners and gambierone was performed using a LC system (UFLC XR Nexera, Shimadzu, Japan) coupled to a hybrid triple quadrupole/ion-trap mass spectrometer API 4000 QTrap (SCIEX, Redwood City, CA, USA) equipped with a turboV® ESI source. Maitotoxins and gambierone were separated using a reversed-phase C_{18} Kinetex column (100 Å, 2.6 µm, 50 × 2.1 mm, Phenomenex, Le Pecq, France) with water (A) and 95% acetonitrile/water (B) both containing 2 mM of ammonium formate and 50 mM of formic acid. The column oven and the sample tray temperatures were set at 40 °C and 4 °C, respectively. The flow rate was set at 0.4 mL min^{-1}, the injection volume was set to 5 µL. Separation was achieved using the following mobile phase gradient: from 10 to 95% B in 10 min, keep at 95 % B for 2 min, return to 10 % B in 0.1 min and

equilibration for 3.9 min prior the next injection. The instrument control, data processing and analysis were conducted using Analyst software 1.6.3 (Sciex, Redwood city, CA, USA). LC-MS/MS analysis analyses were carried out in negative ion acquisition mode, monitoring the transitions showed in Table S5 in Multiple Reaction Monitoring (MRM) mode with a dwell time of 80 ms. Source conditions were curtain gas 25 psi, ionspray −4.5 kV, turbogas temperature of 500 °C, gas 1 and 2 set at 50 psi, an entrance and declustering potential of −10 V and −210 V, respectively. Positive ion acquisition mode was also used in the analysis of MTX3 and gambierone. Source conditions were curtain gas 25 psi, ionspray 4.5 kV, turbogas temperature of 500 °C, gas 1 and 2 set at 50 psi, an entrance and declustering potential of 10 V and 100 V, respectively.

The fragment ion monitored in negative ionization mode for all the MRM transition of the MTX-group of toxins was the hydrogenated sulfate anion *m/z* 96.9 $[HOSO_3]^-$ which was used as confirmatory transition. Quantitation of MTX1, MTX4, desulfo-MTX1 and didehydro-demethyl-desulfo-MTX1 was operated using the MRM transition $[M-2H]^{2-}/[M-2H]^{2-}$ whereas for MTX3 and gambierone $[M-H]^-$/ $[M-H]^-$ (Table S8). Due to the lack of the appropriate standards for the quantitation of each compound, MTX3, MTX4 and gambierone were quantified against the MTX1 calibration curve, assuming equal molar response and applying the same limit of detection (LOD) and limit of quantitation (LOQ) calculated for MTX1.

The MTX1 standard calibration range for the LC-MS/MS analysis consisted of seven concentrations ranging from 0.2 to 10 µg mL^{-1} in MeOH:H_2O (1:1, v/v). LOD and LOQ were determined with the ordinary least-squares regression data method [68,69]. The LOD was calculated as three times the standard deviation of the y-intercepts over the slope of the calibration curve; the LOQ was calculated as ten times the standard deviation of the y-intercepts over the slope of the calibration curve [68,69]. Therefore, LOD and LOQ for the MTX1 MRM transition $[M-2H]^{2-}/[M-2H]^{2-}$ were 0.32 and 0.97 µg mL^{-1}, respectively.

2.3.2. LC-HRMS and HRMS/MS (Q-Tof 6550 iFunnel)

LC-HRMS analyses were carried out using a UHPLC system 1290 Infinity II (Agilent Technologies, Santa Clara, CA, USA) coupled to a HRMS time of flight mass spectrometer Q-Tof 6550 iFunnel (Agilent Technologies, Santa Clara, CA, USA). Chromatographic separation was performed using a Kinetex C_{18} column (100 Å, 1.7 µm, 100 x 2.1 mm, Phenomenex, Le Pecq, France) at 40 °C with water (A) and 95 % acetonitrile (B) both containing 2 mM ammonium formate and 50 mM formic acid. The flow rate was 0.4 mL min^{-1} and the injection volume 5 µL. Gradient of mobile phase was carried out as follows: 5 % B was kept for 1 min, then increased

to 100 % B over 11 min, kept at 100 % B for 2 min and returning to the initial conditions in 0.5 min and then equilibrated the column for 4.5 min prior to the next injection.

Source conditions were set as follows: gas temperature, 160 °C; gas flow, 45 psi, 11 L min^{-1}; nebulizer,; sheath gas temperature, 250 °C; sheath gas flow, 11 L min^{-1}; capillary voltage, 4500 V and nozzle voltage, 500 V. The instrument was calibrated, using the Agilent tuning mix, in negative and positive ionization mode before each analysis.

LC-HRMS analyses were carried out in full scan and targeted MS/MS mode in positive and negative ionization mode in separate runs. Full scan analysis operated at a mass resolution of 40,000 Full width at Half Maximum (FWHM) over a mass-to-charge ratio (*m/z*) ranging from 100 to 3200 with a scan rate of 1 spectra/s. Targeted MS/MS was performed in a Collision Induced Dissociation (CID) cell at 45,000 FWHM over the scan rage from m/z 50 to 1700 with a scan rate of 10 spectra/s and a scan rate of 3 spectra/s applying three different collision energies in order to have a good fragmentation pathway. Two reference masses *m/z* 121.0509 (purine) and *m/z* 922.0099 (hexakisphosphazine) were continuously monitored during the entire run. Data acquisition was controlled by MassHunter software (Agilent technologies, CA, USA). Raw data were processed with Agilent MassHunter Qualitative Analysis software (version B.07.00, service pack 1) using the Find by Formula (FbF) algorithm screening with a Personal Compound Database and Library (PDCL) created by Phycotoxins laboratory (IFREMER, France).

3. Results

3.1. LC-MS/MS Analysis

In an attempt to separate MTXs and gambierones from CTXs, liquid/liquid partitioning of the crude extracts was carried out between MeOH:H2O (3:2) and dichloromethane. The methanol-soluble fraction (MSF) and the dichloromethane-soluble fraction (DSF) of each crude extract of dinoflagellate cell pellets were analyzed by LC-MS/MS monitoring MTXs, as well as gambierone (Table 2). A Multiple Reaction Monitoring (MRM) method in negative ionization mode screened for six MTXs (MTX1, MTX2, MTX3, MTX4, desulfo-MTX1 and didehydro-demethyl-desulfo-MTX1), as well as gambierone. The ESI^- MRM method was based on monitoring a qualitative transition from the (pseudo-)molecular ion to the hydrogenated sulfate anion m/z 96.9 $[HOSO3]^-$ at high collision energy, which is typicalof these compounds, and for quantitation based on monitoring the pseudo-

molecular ion itself $[M-2H]^{2-}$/$[M-2H]^{2-}$ for MTX1, MTX4, desulfo-MTX1 and didehydro-demethyl-desulfo-MTX1. This was due to the single-charged ion of these compounds being outside the mass range of the mass spectrometer (50–2800 Da), whereas $[M-H]^-$/ $[M-H]^-$ was selected for MTX3 and gambierone. Due to the absence of MTX2 MS/MS fragmentation data, the double-charged $[M-2H]^{2-}$/$[HOSO3]^-$ and triple-charged $[M-3H]^{3-}$/$[HOSO3]^-$ molecular anions to the sulfate anion m/z 96.9 $[HOSO3]^-$ were selected as MRM transitions for this compound, assuming similar MS/MS fragmentation behavior of MTX1 and MTX2 [27].

The identification of MTX1, MTX3, gambierone and MTX4 was carried out by comparing retention times as well as ion ratios with the reference materials available (Table S8). On the other hand, the lack of structural information and reference material of MTX2 limited its identification. Therefore, the absence of this compound was reported as not detected above the limit of detection of any ion transition monitored. The lack of reference material of desulfo-MTX1 and didehydro-demethyl-desulfo-MTX1 also limited its identification, with the approach followed being the same as that described for MTX2. However, the accurate mass of these compounds was available, only hampering its identification in LC-HRMS by the uncertainty of the limit of detection of our method for these compounds.

44-methyl-gambierone (MTX3) was identified in all samples of *G. australes* from Menorca and Mallorca (Balearic Islands, Spain), detecting both quantitative m/z 1037.6 $[M-H]^-$ / m/z 1037.6 $[M-H]^-$ and qualitative m/z 1037.6 $[M-H]^-$ / m/z 96.9 $[HOSO3]^-$ MRM ion transitions in negative ionization mode. MTX3 concentrations in *G. australes* strains ranged from 344 to 1661 pg MTX1 equivalent $cell^{-1}$ (eq. $cell^{-1}$) taking into account the total amount detected in the sum of MSF + DSF. *Gambierdiscus* sp.2 from Crete (Greece) contained significantly less MTX3 (4.3 pg MTX1 eq. $cell^{-1}$). The recovery of MTX3 in the MSF ranged between 66.7 % and 84.1 % for *G. australes* for the Balearic and Crete strains (Table 3). MTX3 was also present in one *F. paulensis* from Menorca (Balearic Islands, Spain) at a concentration of 10.5 pg MTX1 eq. $cell^{-1}$. In this case, however, the recovery of MTX3 in the MSF was only 17.7 % (Table 3). This difference compared to the recovery observed in the MTX3 detected in *G. australes* must be explored; at this stage, we can only presume a matrix effect during the partitioning step. Gambierone was only identified in the one *Gambierdiscus* strain from Crete, albeit at a significant concentration of 776 pg MTX1 eq./cell for the sum of MSF + DSF. The recovery of gambierone in the MSF was 92.2 % (Table 3).

A putative gambierone analogue, with an earlier retention time than gambierone on the C_{18} column, was detected in all *G. australes* from Menorca and Mallorca

(Balearic Islands, Spain) at concentrations ranging from 148.6 to 523.4 pg MTX1 eq. $cell^{-1}$ and an average recovery of the putative gambierone analogue in the MSF of 99.7 % (Table 3). Negative ionization mode detected both quantitative m/z 1023.5 $[M-H]^-$/ m/z 1023.5 $[M-H]^-$ and qualitative ion transitions m/z 1023.5 $[M-H]^-$ /m/z 96.9 $[HOSO3]^-$ with the same ion ratios as gambierone. This compound was not present in the other three strains of *Gambierdiscus*.

Further confirmation of MTX3, gambierone and the putative gambierone analogue was carried out in positive ionization mode and not only in MRM, but also in full scan and enhanced product ion modes, confirming the presence of MTX3 and gambierone due to the detection of their common fragment m/z 803 as well as their specific fragments m/z 233 and m/z 219, respectively. This was already reported by Boente-Juncal et al. [13], and a common fragment with m/z 109 was assigned to the fragmentation of the side chain in the last ring (Figures S1 and S2). The putative gambierone analogue did not show any common or specific gambierone fragments but showed a fragmentation pattern similar to these compounds, including water losses and sulfate loss followed by water losses (Figures S1 and S2). MTX4 was confirmed in a strain of *G. excentricus* from La Gomera (Canary Islands, Spain). Retention time as well as MRM ion ratios transitions m/z 1646.2 $[M-2H]^{2-}$/m/z 1646.2 $[M-2H]^{2-}$ and m/z 1646.2 $[M-2H]^{2-}$/m/z 96.9 $[HOSO3]^-$ were consistent with those obtained in the MTX4 reference material. The recovery of MTX4 from the DSF to the MSF was 99.3 % (See Table 3). The MTX1, MTX2, desulfo-MTX1 and didehydro-demethyl-desulfo-MTX1 were not detected in any *Gambierdiscus* or Fukuyoa samples from the Mediterranean Sea and the *G. excentricus* from Canary Islands (Spain), neither in the MSF nor in the DSF.

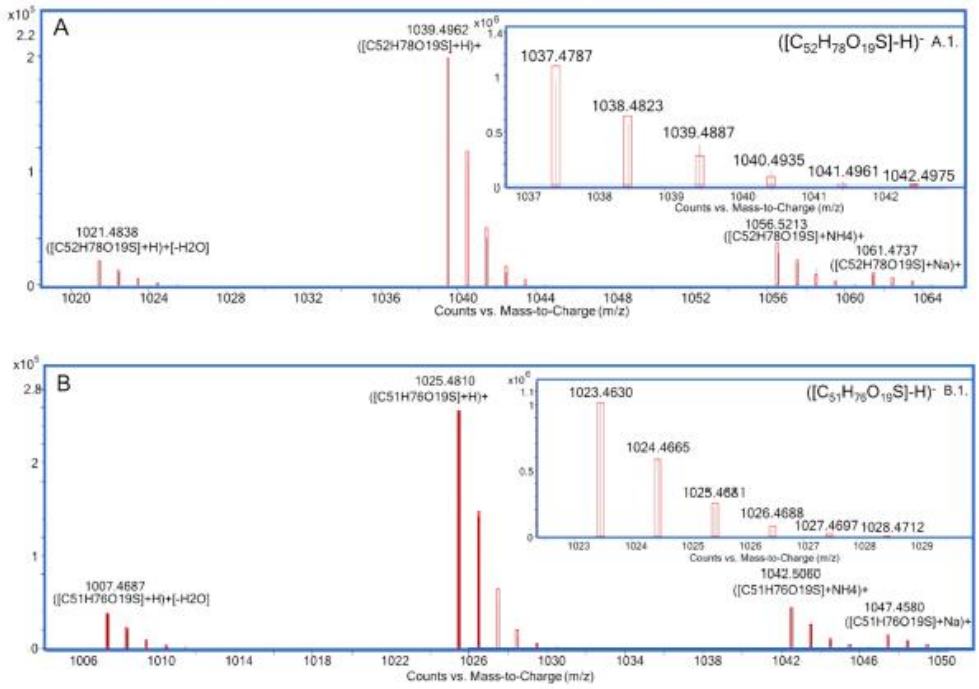

Figure 1. LC-HRMS analysis in MS full scan mode of MTX3 detecte in *G. australes* (A) ESI^+ mode, (A1) ESI^- mode; putative gambierone detected in *Gambierdiscus* sp.2 (B) ESI^+ mode (B1) ESI^- mode.

Table 3. Results obtained after the liquid chromatography mass spectrometry (LC-MS/MS) analysis of the dinoflagellate extracts using a triple-stage quadrupole instrument. Results are expressed in pg MTX1 eq. cell^{-1}; MSF: Methanol Soluble Fraction; DSF: Dichloromethane Soluble Fraction; n.d.: not detected.

Species	Strain Code	Location	pg MTX1 eq./Cell (% of TOTAL)															
			MTX1	MTX2	MTX3 MSF (%)	MTX3 DSF (%)	Σ	MTX4 MSF (%)	MTX4 DSF (%)	Σ	desulfo-MTX1	didehydro-demethyl-desulfo-MTX1	Gambierone MSF (%)	Gambierone DSF (%)	Σ	P-Gambierone Analogue MSF (%)	P-Gambierone Analogue DSF (%)	Σ
G. australes	IRTA-SMM-17-189	Torret, Menorca, Balearic Islands, Spain	n.d.	n.d.	241 (70.2)	102 (29.8)	344.0		n.d.		n.d.	n.d.		n.d.		148 (99.7)	0.4 (0.3)	149
G. australes	IRTA-SMM-17-162	St. Adeodat, Menorca, Balearic Islands, Spain	n.d.	n.d.	575 (79.9)	150 (20.1)	720		n.d.		n.d.	n.d.		n.d.		275 (99.6)	1.0 (0.4)	276
G. australes	IRTA-SMM-17-164	St. Adeodat, Menorca, Balearic Islands, Spain	n.d.	n.d.	1108 (66.7)	553 (33.3)	1661		n.d.		n.d.	n.d.		n.d.		522 (99.8)	1.2 (0.2)	523
G. australes	IRTA-SMM-17-271	Macarella, Menorca, Balearic Islands, Spain	n.d.	n.d.	1107 (83.7)	215 (16.3)	1322		n.d.		n.d.	n.d.		n.d.		408 (99.9)	0.4 (0.1)	409
F. paulensis	IRTA-SMM-17-209	Sacaleta, Menorca, Balearic Islands, Spain	n.d.	n.d.	1.8 (17.7)	8.6 (82.3)	10.5		n.d.		n.d.	n.d.		n.d.			n.d.	
G. australes	IRTA-SMM-17-253	Anguila, Mallorca, Balearic Islands, Spain	n.d.	n.d.	781 (72.3)	300 (27.7)	1081		n.d.		n.d.	n.d.		n.d.		229 (99.8)	0.5 (0.2)	229
G. australes	IRTA-SMM-17-244	Camp de Mar, Mallorca, Balearic Islands, Spain	n.d.	n.d.	403 (84.1)	75.9 (15.9)	479		n.d.		n.d.	n.d.		n.d.		173 (99.6)	0.7 (0.4)	174
Gambierdiscus sp.2	0010G-CR-CCAUTH	Kolimpari, Crete, Greece	n.d.	n.d.	3.0 (70.1)	1.3 (29.9)	4.3		n.d.		n.d.	n.d.	716 (92.2)	60.3 (7.8)	775.9		n.d.	
G. excentricus	IRTA-SMM-17-407	Playa de vueltas, La Gomera, Canary Islands, Spain	n.d.	n.d.		n.d.		36.8 (99.3)	0.3 (0.7)	37.1	n.d.	n.d.		n.d.			n.d.	

3.2. LC-HRMS/MS Analysis

The presence of the compounds previously identified and quantified by LC-MS/MS was confirmed by LC-HRMS. Mass spectral detection was performed in full scan and targeted MS/MS mode in negative (ESI^-) and positive (ESI^+) ionization mode. Both DSF and MSF of dinoflagellate extracts were analyzed in ESI^- and ESI^+ full scan mode, and screening with an in-house database allowed for identification of several compounds based on their characteristics reported in the literature.

MTX3 was confirmed in all *G. australes* in both ESI^- and ESI^+ full scan mode at 7.6 min. Negative ionization mode showed the detection of a single ion corresponding to the deprotonated molecule $[M-H]^-$ with Δppm < 1 ppm in all samples of this species (Figure 1A1, Table S1). At the same retention time, positive ionization mode showed a prominent ion corresponding to the molecular ion $[M+H]^+$ and also pseudo-molecular ions $[M+H-H2O]^{+,}$ $[M+NH4]^+$, $[M+Na]^+$ with Δppm < 3.7 ppm in all ions of these samples (Figure 1A) (See Table S1).

Gambierone was identified at 7.4 min in the *Gambierdiscus* sp. from Crete (Greece) showing a similar ion pattern as the one detected for MTX3 in both ESI^- and ESI^+ full scan mode. Negative ionization mode showed the detection of a single ion matching the deprotonated molecule $[M-H]^-$ *m/z* 1023.4630 (Δppm= +0.1 ppm) (Figure 1 B.1, Table S2). Positive ionization mode showed, at the same retention time, a prominent ion corresponding to the molecular ion $[M+H]^+$ *m/z* 1025.4810 (Δppm= +3.5 ppm) and also the pseudo-molecular ions $[M+H-H_2O]^+$ *m/z* 1007.4687 (Δppm= +1.8), $[M+NH_4]^+$ *m/z* 1042.5060 (Δppm= +1.9) and $[M+Na]^+$ *m/z* 1047.4580 (Δppm= -1.3) (Figure 1 B, Table S2).

MTX3 and gambierone were further confirmed in targeted MS/MS in both ESI^- and ESI^+ mode. ESI^- targeted MS/MS selected the deprotonated molecule $[M-H]^-$ applying 30 eV, 50 eV and 70 eV detecting the hydrogen-sulfate fragment $[HOSO_3]^-$ in both compounds (Figure 2 A & B). An ion representing a water loss $[M-H-H_2O]^-$ was also detected in low abundance, as well as the fragments specific for MTX3, *m/z* 977.4535 (Δppm= -4.0 ppm) and *m/z* 959.4428 (Δppm= -4.2 ppm), and those specific for gambierone, *m/z* 963.4392 (Δppm=-2.6 ppm) and *m/z* 945.4302 (Δppm= -1.0 ppm). Fragmentation pathways are also proposed on the basis of the observed fragments, including the two common fragments with theoretical *m/z* 899.3741 and *m/z* 839.3529, as well as water losses common to both MTX3 and gambierone (Figure 2 A.1 & B.1) (Table 4).

Table 4. Accurate masses (measured and theoretical) of informative ions of MTX3 and gambierone ESI⁻ and ESI⁺ mode.

Ion	MTX3 Molecular Formula	MTX3 m/z Measured	MTX3 m/z Theoretical	MTX3 Δ ppm	Gambierone Molecular Formula	Gambierone m/z Measured	Gambierone m/z Theoretical	Gambierone Δ ppm
ESI⁻								
[M-H]⁻	**$C_{52}H_{77}O_{19}S^-$**	**1037.4774**	1037.4785	−1.1	$C_{51}H_{75}O_{19}S^-$	1023.4612	1023.4629	−1.6
$[M-H-H_2O]^-$	$C_{52}H_{75}O_{18}S^-$	1019.4625	1019.4680	−5.3	$C_{51}H_{73}O_{18}S^-$	1005.4463	1005.4523	−6.0
	$C_{50}H_{73}O_{17}S^-$	977.4535	977.4574	−4.0	$C_{49}H_{71}O_{17}S^-$	963.4392	963.4417	−2.6
H_2O	$C_{50}H_{71}O_{16}S^-$	959.4428	959.4468	−4.2	$C_{49}H_{69}O_{16}S^-$	945.4302	945.4312	−1.0
	$C_{43}H_{63}O_{18}S^-$	899.3707	899.3741	−3.7	$C_{43}H_{63}O_{18}S^-$	899.3707	899.3741	−3.7
$-H_2O$	$C_{43}H_{61}O_{17}S^-$	881.3613	881.3635	−2.5	$C_{43}H_{61}O_{17}S^-$	881.3618	881.3635	−1.9
	$C_{41}H_{59}O_{16}S^-$	839.3501	839.3529	−3.4	$C_{41}H_{59}O_{16}S^-$	839.3517	839.3529	−1.5
$-H_2O$	$C_{41}H_{57}O_{15}S^-$	821.3362	821.3424	−7.5	$C_{41}H_{57}O_{15}S^-$	821.3306	821.3424	−14.3
$[HOSO_3]^-$	$HOSO_3^-$	96.9611	96.9601	10.3	$HOSO_3^-$	96.9615	96.9601	14.4
ESI⁺								
$[M+H]^+$	$C_{52}H_{79}O_{19}S^+$	1039.4911	1039.4931	−1.9	$C_{51}H_{77}O_{19}S^+$	1025.4749	1025.4774	−2.4
$[M+H-H_2O]^+$	$C_{52}H_{77}O_{18}S^+$	1021.4795	1021.4825	−2.9	$C_{51}H_{75}O_{18}S^+$	1007.4636	1007.4668	−3.2
$[M+H-2H_2O]^+$	$C_{52}H_{75}O_{17}S^+$	1003.4692	1003.4720	−2.8	$C_{51}H_{73}O_{17}S^+$	989.4528	989.4563	−3.5
$[M-SO_3+H]^+$	$C_{52}H_{79}O_{16}^+$	959.5333	959.5363	−3.1	$C_{51}H_{77}O_{16}^+$	945.5171	945.5206	−3.7
$[M-SO_3-H_2O+H]^+$	$C_{52}H_{77}O_{15}^+$	941.5232	941.5257	−2.7	$C_{51}H_{75}O_{15}^+$	927.5070	927.5100	−3.2
$[M-SO_3-2H_2O+H]^+$	$C_{52}H_{75}O_{14}^+$	923.5125	923.5152	−2.9	$C_{51}H_{73}O_{14}^+$	909.4968	909.4995	−3.0
$[M-SO_3-3H_2O+H]^+$	$C_{52}H_{73}O_{13}^+$	905.5022	905.5046	−2.7	$C_{51}H_{71}O_{13}^+$	891.4849	891.4889	−4.5
$[M-SO_3-4H_2O+H]^+$	$C_{52}H_{71}O_{12}^+$	887.4911	887.4940	−3.3	$C_{51}H_{69}O_{12}^+$	873.4743	873.4783	−4.6
$[M-SO_3-5H_2O+H]^+$	$C_{52}H_{69}O_{11}^+$	869.4812	869.4835	−2.6	$C_{51}H_{67}O_{11}^+$	855.4656	855.4678	−2.6
	$C_{43}H_{65}O_{18}S^+$	901.3854	901.3886	−3.6	$C_{43}H_{65}O_{18}S^+$	901.3884	901.3886	−0.2
$-H_2O$	$C_{43}H_{63}O_{17}S^+$	883.3756	883.3780	−2.7	$C_{43}H_{63}O_{17}S^+$	883.3744	883.3780	−4.1
$-OSO_3H$	$C_{43}H_{63}O_{14}^+$	803.4182	803.4212	−3.7	$C_{43}H_{63}O_{14}^+$	803.4173	803.4212	−4.9
	$C_{15}H_{21}O_2^+$	233.1533	233.1536	−1.3	$C_{14}H_{19}O_2^+$	219.1375	219.1380	−2.1
	$C_{15}H_{19}O^+$	215.1429	215.1430	−0.5	$C_7H_9O^+$	109.0644	109.0648	−3.6
	$C_8H_{11}O^+$	123.0800	123.0804	−3.2	$C_6H_9^+$	81.0697	81.0699	−2.2
	$C_7H_9O^+$	109.0645	109.0648	−2.8				
	$C_7H_{11}^+$	95.0852	95.0855	−3.2				
	$C_6H_9^+$	81.0696	81.0699	−3.7				

Positive mode ESI⁺ targeted MS/MS of MTX3 and gambierone molecular ion $[M+H]^+$, *m/z* 1039.4931 and *m/z* 1025.4774 respectively, at an average CE of 20, 40 and 60 eV allowed for the unambiguously confirmation of both compounds. MTX3-targeted MS/MS spectra showed a series of: 1) water losses and sulfite loss plus water losses (Δppm<4ppm), 2) the ion with theoretical *m/z* 803.4212 also common to gambierone, and the ions corresponding to those already reported in LC-MS/MS at *m/z* 233.1553 (Δppm= -1.3 ppm) and *m/z* 109.0645 (Δppm= -2.8 ppm), assigned to the fragmentation of MTX3 I-ring (Figure 3A) (Table 4).

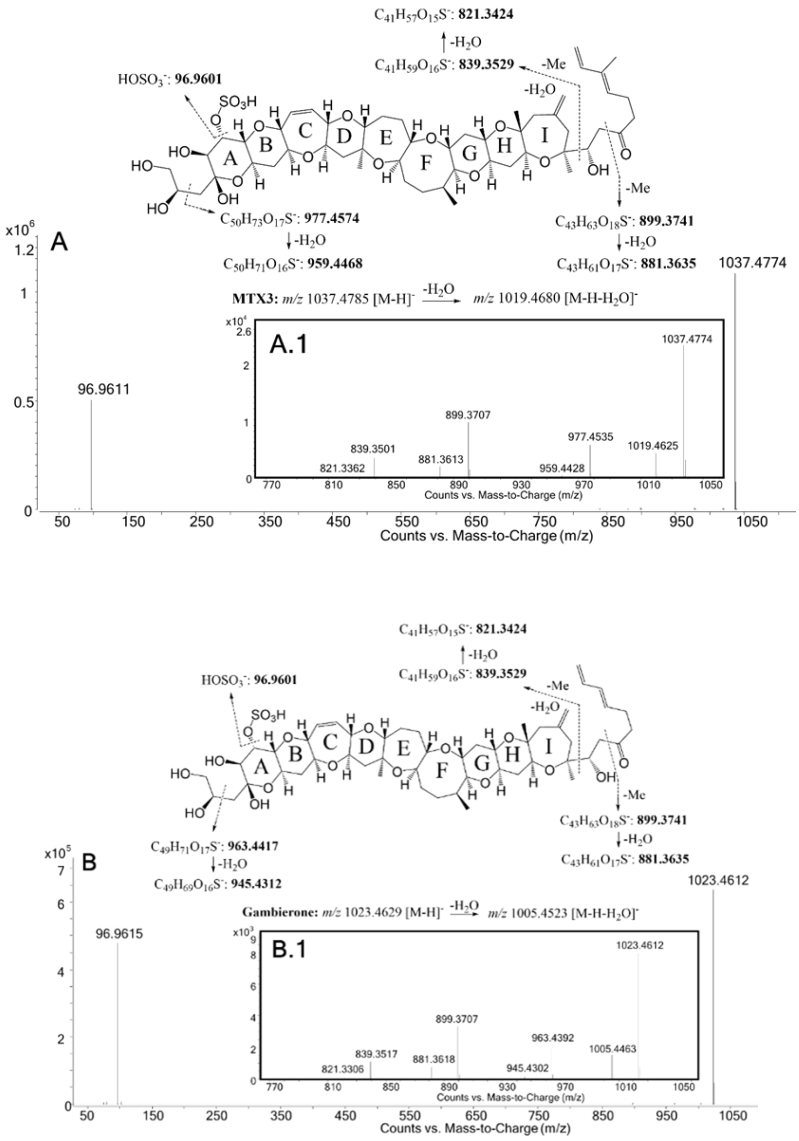

Figure 2. ESI- Targeted HRMS/MS spectra of: MTX3 in *G. australes*, **A**) Average CE of 30 eV, 50 and 70 eV, **A.1**) Zoom from *m/z* 770 to *m/z* 1050 at 70 eV; gambierone in *G. spp.*, **B**) Average CE of 30 eV, 50 and 70 eV, **B.1**) Zoom from *m/z* 770 to *m/z* 1050 at 70 eV.

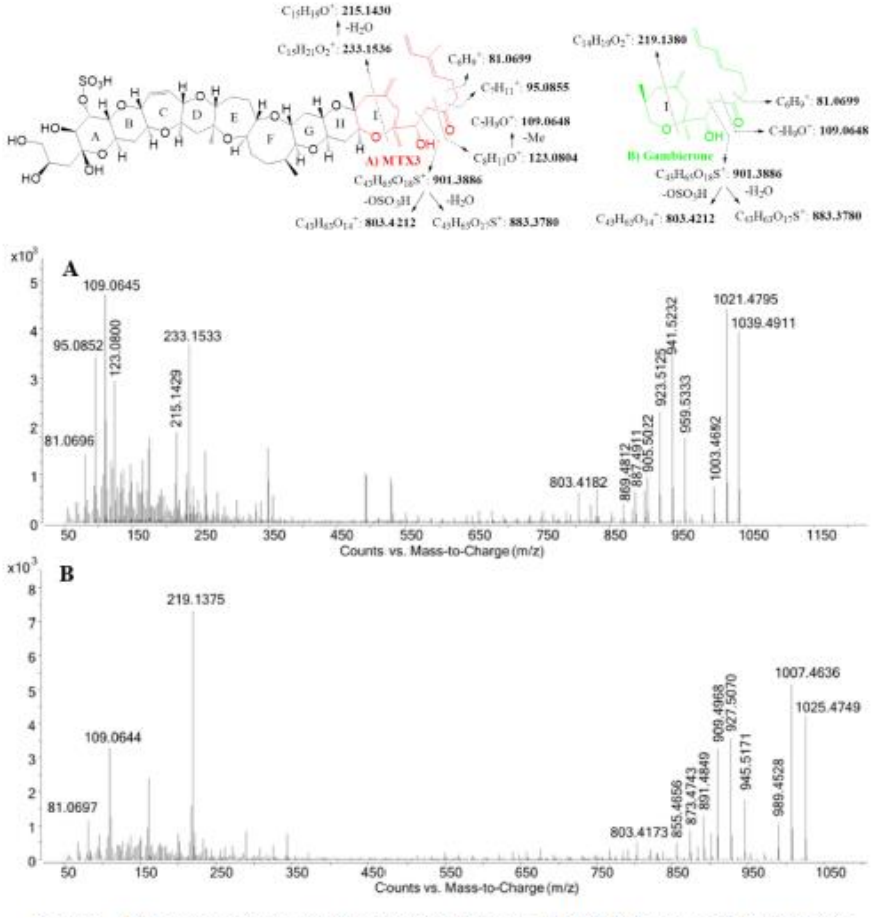

Figure 3. +ESI averaged 20 eV, 40eV and 60eV and targeted HRMS/MS spectra of: (A) MTX3 in *G. australes*,(B) putative gambierone in *Gambierdiscus* sp.2

The same fragmentation pattern was observed for gambierone with the detection of water losses and sulfite loss plus water losses (Dppm < 5 ppm), the ion with m/z 803.4173 (Δppm = -4.9 ppm), and the gambierone specific ions m/z 219.1375 (Δppm = -2.1 ppm) and m/z 109.0644 (Δppm = -3.6 ppm) (Figure 3B) (Table 4).

Due to the lack of the molecular formula of MTX4, its confirmation in the *G. excentricus* from the Canary Islands (Spain) could not be carried out by screening with the database. However, the availability of MTX4 reference material allowed to confirm this compound by comparing retention time (7.5 min), as well as further ESI^- targeted MS/MS selecting the $[M-2H]^{2-}$ and detection of the two fragment ions $[HOSO_3]^-$ and $[SO_3]^-$ (Figure 4).

The putative gambierone analogue detected by LC-MS/MS was identified by LC-HRMS ESI^+ full scan analysis using the algorithm Find-By-Molecular-Feature (FBF) detecting, at a retention time of 6.0 min, a prominent ion with *m/z* 1042.4911 assigned to $[M+NH_4]^+$ as well as $[M+H]^+$ *m/z* 1025.4632, $[M+Na]^+$ *m/z* 1047.4427 and $[M+K]^+$ *m/z* 1063.4108 (Figure S3). The mass differences (Δppm) for these ions were higher than 13, indicating that this compound is probably not a gambierone isomer (Table S3, Figure S3). It was also observed that under the LC-HRMS conditions the ion pattern of this compound is different to both gambierone and the putative gambierone analogue in low- and high-resolution MS, where the prominent ion is the protonated molecule $[M+H]^+$. ESI^- targeted MS/MS of the $[M-H]^-$ of the putative gambierone revealed a hydrogenated sulfate anion loss typical of MTX-like compounds, whereas no specific or common fragments to gambierone or MTX3 were detected between *m/z* 770 and *m/z* 1010 (Figure S4). Positive ionization mode targeted MS/MS of the $[M+H]^+$ also revealed a similar fragmentation pattern as gambierone and MTX3 with sulfate loss plus water losses, however, large mass differences (Δppm > 20) were observed, compared to gambierone and no fragments common to gambierone were detected (Table S4 & Figure S5).

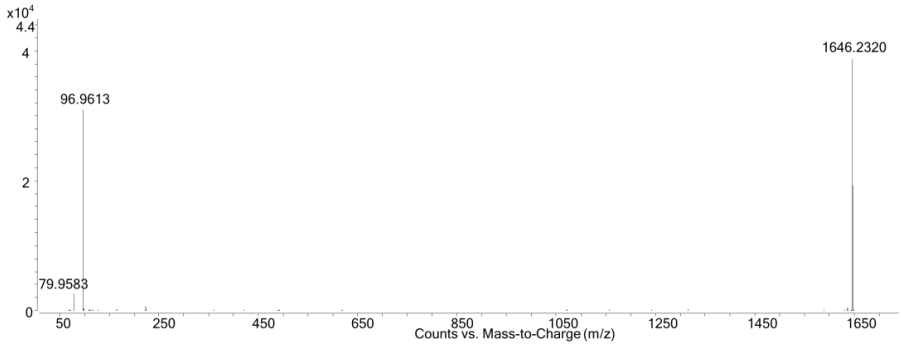

Figure 4. ESI^- Targeted HRMS/MS spectra at 110 eV of MTX4 from *G. excentricus* extract.

The screening of raw data with the database of the ESI^- and ESI^+ full scan data also detected a putative gambieroxide at a retention time of 5.3 min in the MSF of all *G. australes* strains (Table S5). Negative ESI full scan mode allowed for detection of the deprotonated molecule $[M-H]^-$ of the putative gambieroxide with Δppm < 3 ppm (Figure S6). Positive ESI full scan acquisition showed a prominent ion at *m/z* 1212.6019 corresponding to $[M+NH_4]^+$ with Δppm = +3.0 ppm, and $[M+Na]^+$ was also detected at *m/z* 1217.5553 but with lower intensity and a Δppm = +1.2 ppm (Figure S7). Negative ESI-targeted MS/MS of $[M-H]^-$ *m/z* 1193.5572 of the putative gambieroxide only revealed a fragment at *m/z* 453.1964 which was not identified in the molecule [70] whereas ESI^+-targeted MS/MS of $[M+NH_4]^+$ *m/z* 1212.5983 showed a prominent fragment at *m/z* 1159.5493 corresponding to $[M+H-2H_2O]^+$ (-1.1 ppm) and followed by six water losses molecules. The lack of authentic gambieroxide MS/MS data or standard limited the confirmation of this compound (Figure S8 & S9).

As above mentioned, the DSF was also analyzed and compared with the corresponding compounds reported in the literature. Two compounds matching gambieric acid C and D were identified in both the ESI^+ and ESI^- MS full scan in *G. australes*. These compounds partially coeluted on the C_{18}-column with retention times of 8.895 min for the compound tentatively identified as gambieric acid C and 8.928 min for the compound tentatively identified as gambieric acid D.

For both putative gambieric acid C and D, the deprotonated molecular cluster $[M-H]^-$ was detected with Δppm < 2.5 ppm in all samples of *G. australes* (Figure 5 A.1 & B.1) (See Table S6 and S7). Positive ESI full scan mode showed at the same retention time three prominent ions corresponding to the molecular ion $[M+H]^+$ and also pseudo-molecular ions $[M+NH_4]^+$, $[M+Na]^+$ with Δppm <5 ppm in all ions of these samples (Figure 5A & 5B, Tables S6 & S7).

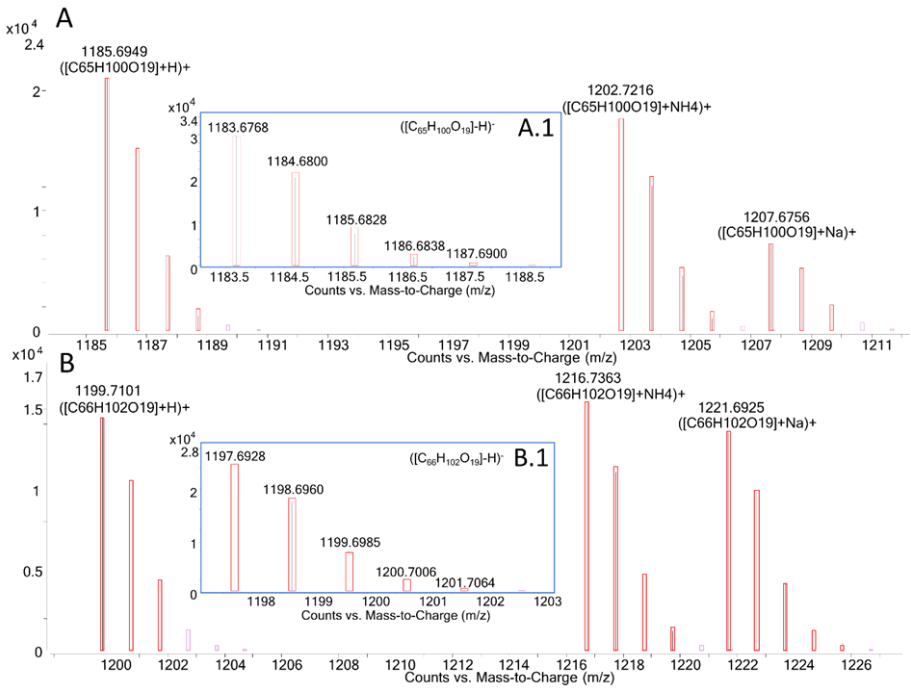

Figure 5. LC-HRMS full scan analysis of: gambieric acid C, A) ESI^+, A.1) ESI^-; gambieric acid D, B) ESI^+, B.1) ESI^- in *G. australes* extract.

Positive ESI targeted MS/MS selecting gambieric acid C and D $[M+H]^+$ *m/z* 1185.6932 and *m/z* 1199.7088 respectively, showing a common fragment with *m/z* 135.1174, assigned to the fragmentation of the side chain containing an ester group (Figure 6). Putative gambieric acid C spectra showed two prominent ions *m/z* 1039.6331 (Δppm= -2.0 ppm) and *m/z* 943.5394 (Δppm= -2.0 ppm), both followed by four water losses (Figure 6A). Putative gambieric acid D spectra showed a similar fragmentation pattern with the detection of *m/z* 1053.6477 (Δppm= -3.0 ppm) and *m/z* 957.5542 (Δppm= -2.9 ppm) both followed by water losses, with the most intense being the first water loss of both ions (Figure 6B).

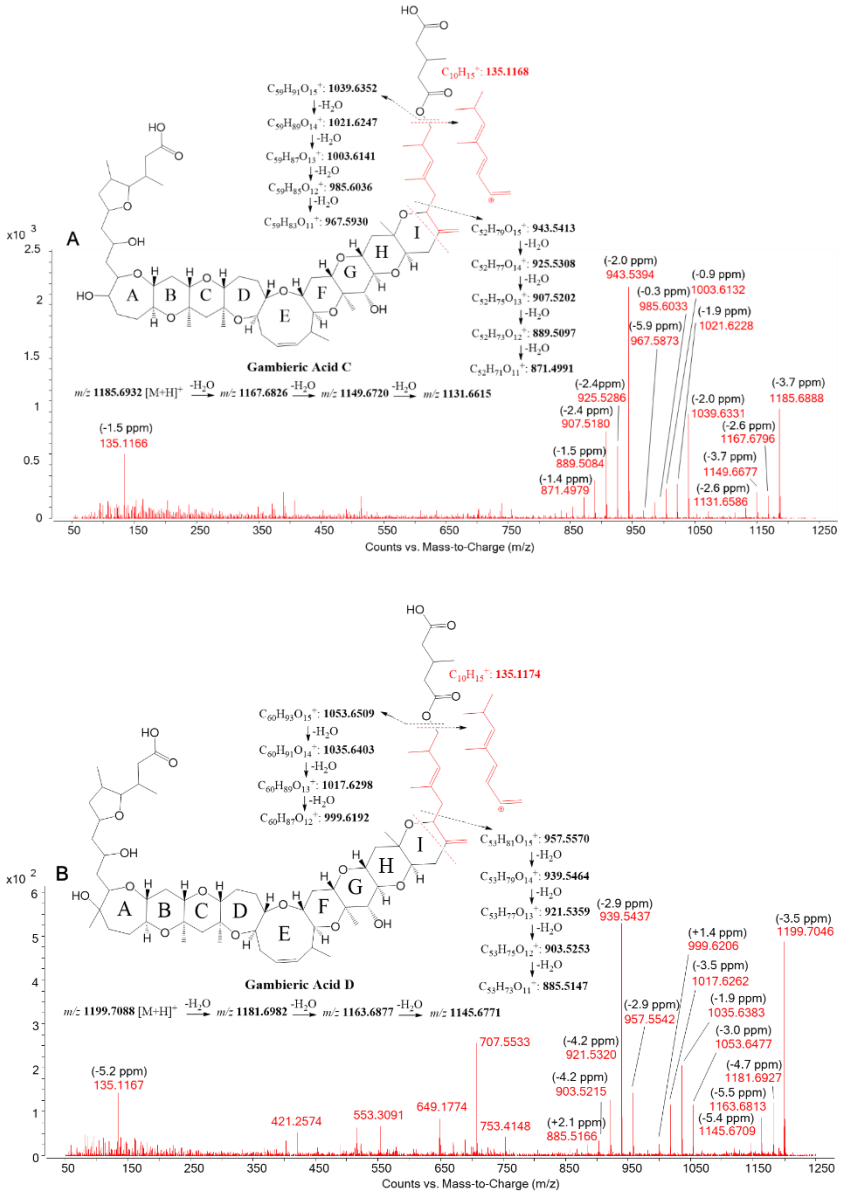

Figure 6. ESI⁺ targeted HRMS/MS spectra at an average of 20, 40 and 60 eV of (**A**) putative gambieric acid C and (**B**) putative gambieric acid D; both detected in *G. australes.*

ESI$^-$ targeted MS/MS was also carried out selecting the deprotonated molecular ions of putative gambieric acid C and D [M-H]$^-$, *m/z* 1183.6786 and *m/z* 1197.6943 respectively. Despite the difficulty in fragmentation of the molecules in this ionization mode and the low abundance of the fragments, both compounds showed a similar fragmentation pattern detecting a single water loss molecule [M-H-H_2O]$^-$ *m/z* 1165.6633 (Δppm= -4.0 ppm) and *m/z* 1179.6758 (Δppm= -6.7 ppm) respectively, a fragment corresponding to cleavage on the alfa carbonyl group of the ester *m/z* 1055.6275 (Δppm= -3.6 ppm) and *m/z* 1069.6389 (Δppm= -7.5 ppm) and a common fragment with *m/z* 112.9852 (Figure S10).

4. Discussion

The present study identified and quantified by LC-LRMS/MS and further confirmed via LC-HRMS the presence of maitotoxins as well as gambierone and other cyclic polyether compounds in dinoflagellates of the genus *Gambierdiscus* and *Fukuyoa* from the Mediterranean Sea and one *G. excentricus* from the Eastern Atlantic (Canary Islands, Spain).

LC-MS/MS negative ionization in MRM mode allowed for the sensitive quantitation of various MTXs. The two mono-sulfated MTXs recently isolated from *Gambierdiscus* of the Caribbean Sea, desulfo-MTX1 and didehydro-demethyl-desulfo-MTX1, were also monitored, assuming that due to their similarities to MTX1 they would have a similar fragmentation pattern monitoring therefore $[M-2H]^{2-}/[M-2H]^{2-}$ as a quantitative transition and $[M-2H]^{2-}/ [HOSO_3]^-$ as qualitative transition. Quantitation of the different MTXs and gambierone was carried out using MTX1, the only commercially available maitotoxin. This quantitation may be adequate for MTX4 which is a compound chemically similar to MTX1, not only in its molecular weight, but also in its fragmentation pattern. MTX3 (=44-methyl-gambierone) and gambierone, which are one-third the molecular weight of MTX1 and MTX4, were also quantified using this approach as they also contain a sulfate group and with results comparable to a commercially available analogue. This approach is traceable but results in somewhat increased uncertainty on any estimated concentration of these compounds.

Among the dinoflagellate species analyzed in this study, *G. australes* appears to be the species that produces the widest variety of hitherto reported *Gambierdiscus* metabolites: MTX3, gambieric acids C and D, a putative gambierone analogue and a putative gambieroxide. All *G. australes* strains analyzed were from the same geographical region, Mallorca and Menorca which are located in the western

Mediterranean (Balearic Islands, Spain). The detection of MTX3 in this species is in agreement with the strains of *G. australes* recently isolated from Raoul Island (New Zealand) [4]. No MTX1 was detected in any strain of *G. australes* from Balearic Islands while, in contrast, this compound was detected by LC-MS/MS with MTX3 in strains of the same species from Kochi (Japan) as well as Macaulay Island (New Zealand) [11,28].

The recovery of MTX3 from the DSF was 75 % which indicates that the liquid/liquid partition using aqueous MeOH and dichloromethane must be further optimized to quantitatively separate MTX3 from CTXs. This partition behavior also shows that MTX3 has a somewhat lipophilic character which may result in MTX3 being absorbed and accumulated as CTXs in fish and the human digestive system. A recent study showed the accumulation of MTX1 in carnivorous fish tissues (liver and muscle of snapper) after the exposure to *G. australes* [29]. While MTX3 clearly has much lower CTX-like activity compared to CTX3C [13], MTX3 was detected in much higher concentrations, up to 1662 pg MTX1 eq. $cell^{-1}$, suggesting that MTX3 should be monitored in fish muscle in order to explore its possible role in CP.

The absence of MTX1 and the detection of a putative gambierone analogue in all *G. australes* pointed out another possible difference in the toxin profiles of *G. australes* from the Mediterranean Sea and the Pacific Ocean. The use of LC-HRMS showed the ability to conclude that this compound, which initially seemed to be a gambierone isomer by full scan and MRM mode in LC-MS/MS, is not a gambierone isomer due to its high Δppm compared to authentic gambierone. Toxicity of purified fractions containing this compound should be evaluated to check its possible interest for the overall assessment of the toxicity of *G. australes.*

Putative gambieric acids C and D were detected in the DSF of all strains of *G. australes*, but not in any other species. Again, this suggests a strong lipophilic character and the potential to accumulate in fish flesh and potentially pass through the human intestinal barrier. Despite the lack of reference material for gambieric acids C and D to compare retention time, the HRMS ESI^- and ESI^+ full scan and targeted MS/MS analysis showed confident data to conclude that the compounds detected should match these potent antifungal compounds, initially isolated from *G. toxicus* from French Polynesia in 1992 [17,30], i.e., prior to this species being separated into several other species. Gambieric acid D was recently detected in the tissue of a shark involved in a CP in the Indian Ocean, suggesting the stability of some of these compounds through the food web [4]. The toxicity of gambieric acids C and D and their role in CP is not clear, the only toxicological data is in mouse lymphoma cells L5178Y showing a moderately low toxic effect [17,30].

Despite the detection of a putative gambieroxide in the MSF of all strains of *G. australes* by LC-HRMS, its confirmation is not conclusive due to the lack of authentic gambieroxide to compare retention time as well as fragmentation in targeted MS/MS. Gambieroxide was first isolated from *G. toxicus* GTP2 strain collected at Papeete in French Polynesia and has a structure similar to yessotoxin [18].

The only *F. paulensis* analyzed in this work was also from Menorca (Balearic Islands, Spain) and showed a low amount of MTX3, 10.5 pg MTX eq. $cell^{-1}$, which may explain the poor recovery of 18 % from the MSF reinforcing the inadequacy of this liquid/liquid partition for this particular compound. MTX3 was also detected by LC-MS/MS in *F. paulensis* from Australia and New Zealand [31,32]. In the Western Mediterranean Sea (Formentera, Balearic Islands, Spain), a positive response was observed in the mouse bioassay (MBA) for both MSF and DSF of a *F. paulensis* and the inconclusive detection by LC-HRMS of gambieric acid A and 54-deoxy-CTX1B in trace levels [25].

*Gambierdiscus sp.*2 from Crete (Greece) showed a profile with low MTX3 concentration (4.2 pg MTX1 eq. $cell^{-1}$) and gambierone at much higher concentration (776 pg MTX1 eq. $cell^{-1}$). The biological activity of gambierone is similar to CTX3C but of lower potency (similar to MTX3) and since approximately 8 % partitioned into the DSF, it should also be monitored in fish tissue from CP cases [15]. The presence of *Gambierdiscus* in Crete Island was first reported in 2003 by [24] while it was not until 2017 when a putative MTX3 was detected by LC-MS/MS in a *G. carolinianus* strain from this region [24].

The availability of a strain of *G. excentricus* from La Gomera (Canary Islands, Spain) allowed to confirm that MTX4 is the main MTX present in this species as previously reported [11]. The liquid/liquid partitioning step appeared adequate for MTX4 as 99.3 % of MTX4 were recovered in the MSF.

Supplementary Materials: The following are available online at http://www.mdpi.com/2072-6651/12/5/305/s1, Figure S1. LC-MS/MS Full scan analysis of: A) MTX3 from *G. australes*; B) gambierone from *Gambierdiscus* sp; C) putative gambierone analogue from *G. australes*, Figure S2. LC-MS/MS spectra resulting from enhanced product ion scan at an average CE of 20, 40 and 60 eV of: A) MTX3 from *G. australes*; B) gambierone from *Gambierdiscus* sp; C) putative gambierone analogue from *G. australes*, Table S1. Accurate mass measurements using LC-HRMS full scan analysis in ESI^- and ESI^+ mode for MTX3, Table S2. Accurate mass measurements using LC-HRMS full scan analysis in ESI^- and ESI^+ mode for gambierone, Figure S3. Putative gambierone analogue detected in LC-HRMS using

the Find by Molecular Feature (FMF) algorithm in *G. australes* at 6.08 min, Table S3. m/z measured values for the putative gambierone analogue and Δ ppm calculated in base of gambierone theoretical values, Figure S4. ESI-targeted MS/MS analysis of the putative gambierone analogue selecting $[M-H]^-$ ion in *G. australes* at 6.08 min. m/z measured values for the putative gambierone analogue and Δ ppm calculated in base of gambierone theoretical values, Figure S5. ESI^+targeted MS/MS analysis of the putative gambierone analogue selecting $[M+H]^+$ ion in *G. australes* at 6.08 min, Table S4. Accurate mass measurements for the putative gambierone analogue and Δ ppm calculated in base of gambierone theoretical values, Table S5. Accurate mass measurements using LC-HRMS full scan analysis in ESI^- and ESI^+ mode for putative gambieroxide, Figure S6. ES^+ LC-HRMS full scan analysis of putative gambieroxide detected in *G. australes* at 5.32 min, Figure S7. ESI^+ LC-HRMS full scan analysis of putative gambieroxide detected in *G. australes* at 5.32 min, Figure S8. ESI^--targeted HRMS/MS spectrum of putative gambieroxide selecting $[M-H]^-$ ion in *G. australes* at a collision energy of 50 eV, Figure S9. ESI^+-targeted HRMS/MS spectrum of putative gambieroxide selecting $[M+H]^+$ ion in *G. australes* at a collision energy of 20 eV, Table S6. Accurate mass measurements using LC-HRMS full scan analysis in ESI- and ESI^+ mode for putative gambieric acid C, Table S7. Accurate mass measurements using LC HRMS full scan analysis in ESI^- and ESI^+ mode for putative gambieric acid D, Figure S10. ESI-targeted MS/MS spectra at an average of 20, 40 and 60 eV of: A) gambieric acid C; B) gambieric acid D detected in *G. australes*, Table S8. MRM transitions monitored using the LC-MS API 4000 QTrap

5. Conclusions

This study allowed for the characterization of the toxins produced by different species of dinoflagellates from the Mediterranean Sea. LC-MS/MS was used for a first and rapid screening, allowing a quantitative estimation of the toxins involved, while LC-HRMS allowed for their confirmation and characterization. Dinoflagellates from the Mediterranean Sea showed toxin profiles similar to those detected in CP endemic regions. Strains of *G. australes* from Mallorca and Menorca (Spain, Mediterranean Sea) produced MTX3, putative gambieric acid C and D, a putative gambierone analogue and putative gambieroxide, while MTX1 was absent in contrast to the same species from temperate waters of the Pacific Ocean. F. paulensis from the same region only produced MTX3, and the strain of *Gambierdiscus* sp.2 from Crete (Greece) produced MTX3 and gambierone. Further research needs to be carried out in order to evaluate the possible presence of CTXs in fish from the Mediterranean Sea, considering that the presence of these

dinoflagellates is associated with the production and accumulation of CTXs through the marine food web.

Author Contributions: Conceptualization, P.E., M.S., A.G.-M., J.D. and P.H.; methodology, P.E., M.S., A.T., M.R.-A.; investigation, P.E., A.T., M.R.-A., K.A. and M.S.; resources, A.G.-M., J.D. and P.H.; data curation, P.E., M.S.; writing—original draft preparation, P.E., MS. and P.H.; writing—review and editing, A.G.-M., J.M.L.-M., M.R.-A., J.D. and P.H.; supervision, J.M.L.-M., A.G.-M. and P.H.; project administration, A.G.-M. and P.H.; funding acquisition, A.G.-M., J.D. and P.H. All authors have read and agreed to the published version of the manuscript.

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iii. Evaluation of CTX-like compounds in fish from waters in the Balearic Islands (western Mediterranean Sea)

(unpublished results)

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ABSTRACT

Ciguatera Poisoning (CP) occurs when seafood contaminated with ciguatoxins (CTXs) is consumed. CP was typically from tropical and subtropical regions. At present, CP is an emergent disease in temperate areas. Although no CP cases have been confirmed in the Mediterranean Sea, this area is currently of interest because of the recent findings of the CTX-producing species. In the present work, the CP risk was evaluated in the Balearic Islands (western Mediterranean) by examining the CTX-like toxicity using neuroblastoma cell-based assay. Flesh and liver extracts of thirty fish from various species included carnivorous, herbivorous, and omnivorous. None of these fish exhibited CTX-like activity, which agrees with the nonexistent CP reported cases. At present, CP risk seems negligible. The present study provides the first information on the potential presence of CTXs in several fish from this area. Nonetheless, since the number of analyzed samples is restricted, additional research is required.

1. Introduction

Ciguatera Poisoning (CP) is an illness foodborne that affects between 50,000 to 500,000 people per year mainly in the tropical areas [1]. CP occurs from the consumption of fish that contain ciguatoxins (CTXs) [2]. CTXs are produced by two genera of benthic dinoflagellates, *Gambierdiscus* and *Fukuyoa* [2–4]. These genera are mainly epiphytes and when herbivores eat the macroalgae or filter feeders ingest them, these toxins enter the food web [2]. CTXs were accumulated until to end of the food web. Large predators such as barracuda (*Sphyraenidae*), amberjack (*Seriola* spp.), moray eels (*Muraenidae*), groupers (*Serranidae*), snappers

(*Lutjanidae*) are typically high accumulators of CTXs [5–7]. However, herbivorous or omnivorous such as the parrotfish (*Scaridae*) and more recently, invertebrates have also been involved in it [8,9].

CTXs are large polycyclic-ether rings that are divided into two large groups: CTX1B and CTX3. Currently, more than 38 analogs have been described [10]. It is suggested that CTXs in fish are the oxidized forms of microalgal CTXs after the oxidation reactions in the seafood tissues and these reactions may increase the toxicity of molecules [11,12]. CTXs bind to the voltage-gated sodium channels (VGSCs). Consequently, sodium permeability increases, causing repetitions of potential firings [13]. Besides, CTXs block the delayed-rectifier and transient 'A-type' potassium channels that also increase neuronal excitability [14]. The most common symptoms of CP are gastrointestinal (diarrhoea, abdominal pain), neurological (paresthesia, unusual temperature perception, and taste alteration) and cardiovascular (bradycardia and hypotension) [15]. Severe cases can produce death, but they are rare [16].

Protecting consumers from CP is difficult since CTXs have no odour and colour; they are heat and acid-basic stable, consequently, the cooking processes cannot eliminate them. The United States Food and Drug Administration (US FDA) and EFSA (European Food Safety Authority) stated that the safety level for human in flesh is 0.01 ng of CTX1B kg^{-1}. Products with CTXs are banned in the market of the European Union (EU). Nonetheless, no regulatory limits have been established in the EU and there is no validated method for analysis. The detection and quantitation of CTXs are challenging since standards are scarce and toxins are found in very low levels in seafood.

Among approaches for CTX detection, there is the neuroblastoma cell-based assay (Neuro-2a CBA), which are based on the toxicological effect of toxins in the neuro-2a cell line [17]. As mentioned before, CTXs bind to the VGSCs and the potassium channels, which increments the sodium levels in the cell. CTXs at nanomolar levels do not affect the viability of neuro-2a cells. But cytotoxicity is observed in neuro-2a cells at very low concentrations of CTXs (picomolar) in the presence of two drugs that enhances the CTX-effect: ouabain and veratridine [17]. Ouabain blocks the sodium-potassium ion pump and veratridine blocks the sodium channels [18,19]. The viability of neuro-2a can be easily measured using a colorimetric assay [20]. Even though evaluating the CTX-effect is not a confirmatory method, it can be useful to discriminate between toxic (CTX-like toxicity) and non-toxic fish which can estimate the CP risk of a region.

In the Mediterranean, CP has not ever been confirmed and no CTXs are currently confirmed to be present in Mediterranean seafood. Though cases of CP have been reported in the Mediterranean Sea, all in the coast of Israel (eastern Mediterranean), they have been questioned. In 1973 a possible case of CP for a rabbitfish (*Siganus luridus*) was described [21]. After in 1989, other case was reported by ingestion of salema (*Sarpa salpa*) [22]. Later in 2002, a CP case for a rabbitfish was again described by Raikhlin- Eisenkraft (2002) [23]. In the two latter cases, symptoms were electrical charges associated with dysphasia, dysarthria, palpitations, hallucinations, and nightmares. Haro et al. [24], after analyzing the CP cases in the Mediterranean, concluded that they probably had been misdiagnosed and they were actually cases of ichthyoallyeinotoxism.

Gambierdiscus and *Fukuyoa* species have been reported in the eastern and western Mediterranean Sea [25–27]. Our previous studies suggested that the levels of CTX production for *G. australes* from the Balearic Islands are similar to those for *G. australes* in the Canary Islands where CP is present.

In the present study, we aimed to examine the potential presence of CTXs in fish from the Balearic Islands at levels below the recommended regulatory thresholds. The study constitutes a first screening tool before instrumental subsequent analysis. This information contributes to knowing more about the risk of CP in the Balearic Islands. The toxicity of CTX-like toxicity of 30 fish samples was evaluated by neuroblastoma cell-based assay (neuro-2a CBA). The assessment was performed by assessing the CTX-like toxicity using muscle and liver tissues.

2. Materials and Methods

2.1 Fish sampling and sample preparation

Fish were provided by fishermen. Samples were collected from Majorca (November 2018, October 2019) and Minorca (September 2019). Species were morphologically identified, and weight (kg) was recorded. Once in the lab, all fish were dissected and flesh and liver of thirty fish of eleven species were obtained (10 ± 0.1 g for tissue). Fish tissues were kept at -20 °C until tissue extraction for toxin recovery. The extraction protocol was implemented following based on Yogi et al. (2011) [6] and modified by Dr. Jean Turquet [16]. The sample was heated at 70 °C for 10 min in a water bath. Then, an acetone homogenization (2 mL g^{-1} wet weight of tissue) was performed with an Ultraturrax blender (IKA, T25 Basic, Germany) for 3 min. The homogenate was centrifuged at 3,000 g for 15 min at room temperature, and the supernatant was recovered and filtered using a 0.22 µm PTFE filter (Whatman,

Sigma-Aldrich). The homogenization process was repeated twice. The supernatants were pooled and evaporated in a Syncore® Polyvap evaporator (Büchi R-200, Flawil, Switzerland) at 40 ± 5 °C. After drying the acetonic phase, the aqueous residue was raised with Milli-Q water to 4 mL and diethyl ether was added, mixed by vortex, to initiate a water:diethyl ether partition (1:4, v:v) kept at room temperature for 24h . Afterward, the diethyl ether phase was recovered and dried under N_2 gas (Turbovap, Zymark corp, Hopkinton, USA) at 40 °C. The dried extract was dissolved in methanol: water (2:8, v:v) and the extract was partitioned twice with n-hexane (1:2, v:v). The hexane layers were discarded, and the methanolic phase was dried with the evaporator at 60 ± 5 °C. Finally, the resulting residues were re-dissolved in 4 mL of HPLC-grade methanol and preserved at -20 °C until analysed with the neuro-2a CBA.

2.2 Reagents

In order to semiquantify the toxicological effect, the CTX1B provided by Prof. Richard J. Lewis (The Queensland University, Australia) and it was used as standard. Neuroblastoma murine cells (neuro-2a) were purchased from ATCC LGC standards (USA). Poly-L-lysine, foetal bovine serum (FBS), L-glutamine solution, ouabain, veratridine, phosphate buffered saline (PBS), penicillin, streptomycin, RPMI-1640 medium, sodium pyruvate, thiazolyl blue tetrazolium bromide (MTT), N-(3-dimethylaminopropyl)-N'-ethylcarbodiimide hydrochloride (EDC), N-Hydroxysuccinimide (NHS), Tween 20, bovine serum album (BSA), poly horseradish peroxidase-streptavidin (polyHRP-streptavidin) and 3,3',5,5'-tetramethylbenzidine (TMB) liquid substrate were supplied by Merck KGaA (Germany). Dimethyl sulfoxide (DMSO) and absolute methanol were purchased from Honeywell (Spain) and Chemlab (Spain), respectively.

2.3 Evaluation of CTX-like toxicity by neuro-2a CBA

The evaluation of CTX-like toxicity was performed using the neuro-2a CBA following the procedure described in Caillaud et al., (2012) [17]. Briefly, neuro-2a cells (cell line CCL-131, ATCC LGC standards, USA) were seeded in 96 well-plates at 40,000 cells $well^{-1}$. After 24 h, ouabain and veratridine (O/V) were added to a final concentration at 140 µM and 14 µM respectively, then, 10 µL of each sample (serial dilutions of extract or standard) was added to each well in triplicate. CTX1B was used as a standard. The extract or standard was evaporated and resuspended in 5% FBS RPMI medium. Then, neuro-2a cells with ouabain and with or without veratridine (O/V+ or O/V-) were exposed to serial dilutions of the extract or standard. Previously an evaluation of the maximum concentration of the extract without matrix effect in flesh and liver was conducted. Tested concentrations

ranged between 0.2 to 12 pg mL^{-1} for CTX1B, 25 to 150 ng equivalents (equiv.) mL^{-1} of flesh and 6.25 to 50 ng equiv. mL^{-1} of liver. Controls consisted on Neuro-2a cells exposed to 5 % FBS and not to extracts or standard in both conditions (O/V- and O/V+). All conditions were tested in triplicate. After 24h of incubation, the neuro-2a cell viability was measured following the MTT colorimetric assay [20]. The CTX-like activity of the extracts was estimated according to the standard dose-response curve following Caillaud et al. (2012) and was expressed as µg equivalents (equiv.) of CTX1B per Kg of tissue. The limit of quantitation (LOQ) of the method was defined as the amount of CTX1B that causes 20 % of cell viability inhibition (IC_{20}) in O/V+ and that can be expressed according to the maximum concentration of tested tissue [16].

3. Results

The maximum exposure concentration of tissues was 200 ng equiv of tissue mL^{-1} for flesh and 60 ng equiv of tissue mL^{-1} for liver and the LOQs were 0.00334 ± 0.0023 µg CTX1B equiv. Kg^{-1} for flesh (n=30) and 0.03906 ± 0.0467 µg CTX1B equiv. Kg^{-1} for liver (n=28). Figure 1 shows an example of the response of neuro-2a cells by exposition of flesh (A) and liver (B) extracts. In figure 1(A), at concentrations lower than 75 mg mL^{-1} of flesh extract, a nontoxic effect was observed in both treatments (O/V+ and OV/-). Whereas at a concentration of 150 mg mL^{-1}, unspecific toxicity was observed in both treatments. In figure 1 (B), at concentrations lower than 25 mg mL^{-1} for liver extract, nontoxic effect was observed in both treatments (O/V+ and O/V-). While at concentrations of 50 mg mL^{-1}, unspecific toxicity was observed in both treatments. Therefore, though extract of fish of flesh and liver exhibited toxicity, it cannot be attributed to CTX-like toxicity.

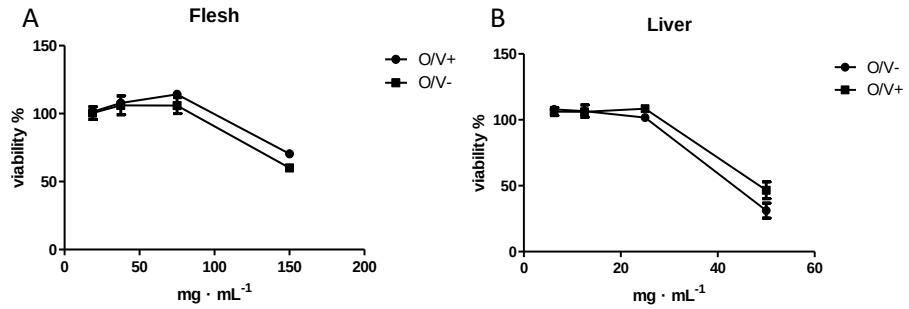

Fig. 1. Dose response curve for neuro-2a cells after the exposition of flesh (A) and liver (B) extracts of white seabream (*Diplodus sargus*) under treatments O/V- (without ouabain and veratridine), O/V+ (with ouabain and veratridine).

Table 1. Quantitations of CTX-like toxicity using the neuro-2a CBA of samples (flesh and livers). Quantitations and LOQ are expressed as µg CTX1B equiv. Kg^{-1} ± SD.

Code	Common name	*Species*	Location	Weight (kg)	LOQ flesh	LOQ liver
1	Drusky grouper	*Ephinelus marginatus*	Majorca	2.3	0.0029	0.0112
2	Red porgy	*Pagrus pagrus*	Majorca	0.6	0.00565	0.0896
3	Red porgy	*Pagrus pagrus*	Majorca	0.6	0.0029	0.0896
4	Mediterranean moray	*Muraena helena*	Majorca	0.4	0.0029	0.0264
5	Mediterranean moray	*Muraena helena*	Majorca	0.3	0.0029	0.0528
6	Mediterranean moray	*Muraena helena*	Majorca	0.6	0.0029	0.0264
7	Mediterranean moray	*Muraena helena*	Majorca	0.6	0.0029	0.0132
8	Mediterranean moray	*Muraena helena*	Majorca	0.7	0.0027	0.0264
9	Yellowmouth barracuda	*Sphyraena viridensis*	Majorca	0.7	0.0027	0.0224
10	Yellowmouth barracuda	*Sphyraena viridensis*	Majorca	0.7	0.0027	0.0448
11	White seabream	*Diplodus sargus*	Majorca	0.4	0.0054	0.0224
12	White seabream	*Diplodus sargus*	Majorca	0.3	0.0054	0.0896
13	Greater amberjack	*Seriola dumerili*	Majorca	0.8	0.0054	0.0448
14	Greater amberjack	*Seriola dumerili*	Majorca	0.6	0.0054	0.1808
15	Common dolphinfish	*Coriphaena hippurus*	Majorca	1.1	0.0054	0.1295
16	Common dolphinfish	*Coriphaena hippurus*	Majorca	1.1	0.0108	0.1295
17	Greater amberjack	*Seriola dumerilii*	Minorca	1.9	0.0005	-
18	Mediterranean barracuda	*Shyraena shyraena*	Minorca	1.2	0.0010	0.0081
19	European conger	*Conger conger*	Minorca	0.9	0.0020	0.0081
20	Yellowmouth barracuda	*Sphyraena viridensis*	Minorca	1.1	0.0010	0.0081
21	Red porgy	*Pagrus pagrus*	Minorca	0.4	0.0005	-

22	European conger	*Conger conger*	Minorca	0.6	0.0005	0.0042
23	White seabream	*Diplodus sargus*	Minorca	0.3	0.0010	0.0042
24	Mediterranean moray	*Muraena helena*	Minorca	3	0.0010	0.0042
25	European conger	*Conger conger*	Minorca	1.8	0.0010	0.0042
26	Greater amberjack	*Seriola durmerili*	Minorca	0.4	0.0042	0.0042
27	Mediterranean moray	*Muraena helena*	Minorca	0.8	0.0021	0.0131
28	White seabream	*Diplodus sargus*	Minorca	0.2	0.0033	0.0042
29	Yellowmouth barracuda	*Sphyraena viridensis*	Majorca	0.4	0.0066	0.0053
30	Common dolphinfish	*Coryphaena hippurus*	Majorca	0.9	0.0066	0.0263

4. Discussion

Working with CBA assays has advantages for the consumer due to their sensitivity. In addition, the global toxicity of the sample allows us to detect CTX compounds that cannot be detected some analytical methods as liquid chromatography mass spectrometry (LC-MS/MS) due to the lack of standards. These characteristics make cell-based assay suitable to be a method used as a CP prevention tool. In fact, in the Canary Islands, neuro-2a CBA was implemented in 2011 to detect potential CTXs [28, 29]. Moreover, in previous studies, neuro-2a assay has been used as a screening tool for CTX-like compounds and after CTXs have been confirmed and characterized by analytical methods successfully [28, 30]. However, it should be noted that we cannot forget the interferences of other compounds from the matrices. Matrices can cause nonspecific toxicity, making it impossible to discern if the sample has compounds present that increase sodium levels in cells. In our study at tested concentrations any CTX-like toxicity was detected. Thus, currently, implementing controls guidance for fishery products in the Balearic Islands seems not an urgent issue. However, the findings of CTX-producing species evidence the necessity to explore the toxin profile of the *Gambierdiscus* and *Fukuyoa* species and the seafood from the Balearic Islands.

5. Conclusions

Even though, Gambierdiscus and Fukuyoa species are present in the Balearic Islands, given the lack of CTX-like toxicity in fish the present study estimates that the CP risk in the Balearic Islands is low.

Author statement

Àngels Tudó: Investigation, Formal analysis, Writing - review & editing; Maria Rambla-Alegre: Methodology, Investigation, Formal analysis, Writing - review & editing; Núria Sagristà: Investigation, Formal analysis, Writing - review & editing; Esther Dàmaso: Investigation, Formal analysis, Writing; Mònica Campàs: Conceptualisation, Methodology, Formal analysis, Supervision; Jorge Diogène: Conceptualisation, Methodology, Formal analysis, Writing - Writing - review & editing, Supervision, Project administration, Funding acquisition.

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3.2. CP HAZARD IDENTIFICATION IN THE CANARY ISLANDS

i. Further advance of *Gambierdiscus* Species in the Canary Islands, with the First Report of *Gambierdiscus belizeanus*

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ABSTRACT

Ciguatera Poisoning (CP) is a human food-borne poisoning, known from ancient times mainly in tropical and subtropical areas, which occurs when fish or very rarely invertebrates contaminated with ciguatoxins (CTXs) are consumed. CTXs are produced by the genus of marine benthic dinoflagellates: *Gambierdiscus*. The presence of *Gambierdiscus* species in a region is one indicator of CP risk. The Canary Islands (North Eastern Atlantic Ocean) is an area where CP cases have been reported since 2004. In the present study, samplings for *Gambierdiscus* cells were conducted in this area during 2016 and 2017. *Gambierdiscus* cells were isolated and identified as *G. australes*, *G. excentricus*, *G. caribaeus* and *G. belizeanus* by molecular analysis. *G. belizeanus* was reported for the first time in the Canary Islands in El Hierro. *Gambierdiscus* isolates were cultured and the CTX-like toxicity of forty-one strains was evaluated with the neuroblastoma cell-based assay (neuro-2a CBA). *G. excentricus* exhibited the highest CTX-like toxicity (9.5-2566.7 fg CTX1B equiv. $cell^{-1}$) followed by *G. australes* (1.7-452.6.2 fg CTX1B equiv. $cell^{-1}$). By contrast, the toxicity of *G. belizeanus* was low (5.6 fg CTX1B equiv. $cell^{-1}$) and *G. caribaeus* did not exhibit CTX-like toxicity. In addition, for the *G. belizeanus* strain, the production of CTXs was evaluated with a colorimetric immunoassay and an electrochemical immunosensor. As a result, *G. belizeanus* produces two types of CTX congeners (CTX1B and CTX3C series congeners) and can contribute to CP in the Canary Islands.

Keywords: Ciguatera; ciguatoxins (CTXs), *Gambierdiscus*; neuroblastoma cell-based assay; immunoassay; immunosensor

Key Contribution: *G. belizeanus* was reported for the first time in the Canary Islands (El Hierro). *G. belizeanus* produced CTX1B and CTX3C series of congeners.

1. Introduction

Gambierdiscus [1] species are marine benthic dinoflagellates that produce secondary metabolites such as ciguatoxins (CTXs) and maitotoxins (MTXs). CTXs are lipid-soluble polyethers [2], which are introduced in food webs when filter feeders and herbivorous organisms eat free-swimming microalgal cells, macroalgae, or substrates that are colonized by benthic dinoflagellates [3]. Then, CTXs are transferred, transformed, and bioaccumulated through the food webs. Humans can get poisoned after the consumption of CTX-contaminated fish or very rarely some invertebrates (crustaceans, gastropods, echinoderms and bivalves) and suffer a disease known as Ciguatera Poisoning (CP) [4].

CTXs activate voltage-gated sodium channels (VGSCs) of cells, resulting in intracellular sodium increase and causing the repetitive firing of action potentials [5,6]. As a consequence, a few hours after the consumption of CTXs, gastrointestinal symptoms appear, typically followed by cardiac and neurological disorders. The neurological symptoms can last weeks, months, and even years [7]. The number of people who suffer from the disease is unknown, mainly due to the variability of symptoms, which leads to misdiagnoses and under-reporting. Annually, it is estimated that about 10,000–500,000 people suffer from the illness [8,9]. Even though CP is one of the most relevant poisonings worldwide, so far, there is no specific treatment [8]. CP was typical from tropical and subtropical regions, but during recent decades, CP cases have increased [10,11] and they have appeared in temperate zones through the importation of tropical ciguateric fish [12] or by the consumption of local ciguateric fish [13,14]. Climate change could change the geographical distribution of the dinoflagellates and the migration patterns of ciguateric fish contributing to the geographical expansion of CP or increasing population densities of CTX-producing species in temperate areas [15,16]. In Europe, outside the boundaries of endemic areas in intertropical climates, new CP cases appeared in the North Eastern Atlantic Ocean after the consumption of fish from the Selvagens Islands (Portugal) and the Canary Islands (Spain) [17,18]. In the Canary Islands, CP is an illness of concern. In one decade (2008–2018), more than one hundred people have suffered from CP [19]. To prevent CP cases, the local

authorities of this area have implemented the neuroblastoma cell-based assay (neuro-2a CBA) [20] to evaluate the possible presence of CTXs in the flesh of certain species of fish through the assessment of CTX-like toxicity [21].

It should be noted that only a few *Gambierdiscus* species have been confirmed to be CTXs producers [22,23], the toxin production is often very low, and not all the species produce the same quantities of toxins [22,24,25]. Therefore, the composition of species in the local areas could be an indicator of the level of risk to catch a ciguateric fish. One of the main factors to explain the latitudinal presence of Gambierdiscus species is the temperature [26], but other factors could be involved.

The Canary Islands are a transition zone between the oligotrophic waters associated with the Canary Current (CC), which is the subtropical gyre of the North Atlantic Ocean, and the eutrophic waters produced by the upwellings of deep cold waters with high nutrients along the African coast [27]. The east part of the Archipelago is semiarid; it is influenced by aeolian dust from the African continent and by the cold waters from the African upwelling system [28]. In contrast, the west is more humid, with more oceanic conditions and a minor influence of the African continent and the upwellings [28]. These conditions cause a longitudinal oceanographic east–west gradient of productivity (≈100 g of carbon m^{-2} yr^{-1}) and the sea surface temperature (SST) (1–2 °C), which could explain the geographical distribution of the *Gambierdiscus* species.

Regarding the presence of *Gambierdiscus* species, in the Canary Islands, *Gambierdiscus* sp. was reported in 2004 [29]. Afterwards, in 2011, *G. excentricus* was described as a new species [30]. After that, the new species *G. silvae* [31] and *G. excentricus* were considered endemic from the Canary Islands. During the last decades, several samplings in the islands showed the high biodiversity and the wide geographical distribution of the *Gambierdiscus* genus [32,33]. At present, six species have been recorded in the Canary Islands, *G. australes* [31], *G. belizeanus* (in the current study), *G. caribaeus* [33], *G. carolinianus* [33], *G. excentricus*, and *G. silvae* [31], and none of them is limited to the Canary Islands.

The present study reports for the first time *G. belizeanus* in the Canary Islands. Previously, *G. belizeanus* was reported in Belize in the West Atlantic Sea [34], in Cuba [35], Cancun, St. Barthelemy, St. Marteen, and St. Thomas [36,37] in the Caribbean Sea. Additionally, it was detected in the Saudi Arabia in the Red Sea [38] and in Australia [39], Malaysia [40], and Kiribati Island [41] in the Pacific Ocean. *G. belizeanus* is considered a low toxin producer [24,38]. Among *Gambierdiscus* species from the Canary Islands, the evaluation of CTX-like toxicity has revealed that *G. excentricus* is one of the highest CTX-producing species within the genus

Gambierdiscus and the most likely contributor to CP in the Atlantic Ocean [24,25,30].

Globally, it is not understood what triggers CP cases [4]. To fully understand the process of CP, to elucidate factors that may trigger CP, and to prevent the cases, it is necessary to identify and monitor the ciguateric fish but also to identify the CTX-producing species, their distribution, their physiology, and their toxicity.

The current study aimed to characterize the biodiversity and the geographical distribution of the *Gambierdiscus* genus in the seven most important islands of the Canary Islands Archipelago and to evaluate the potential CTX production of *Gambierdiscus* species to complement previous studies. For that purpose, samplings in the seven big islands of the Canary Archipelago were performed between October 2016 and October 2017. Isolates of *Gambierdiscus* cells were brought into culture. The molecular identification and morphological characterization of cultures contributed to the new report of *G. belizeanus* in the Archipelago. In addition, CTX-like toxicity was evaluated for forty-one strains of four species (*G. australes*, *G. belizeanus*, *G. caribaeus*, and *G. excentricus*) with the neuro-2a CBA, and the production of CTXs by the *G. belizeanus* strain was analyzed by a colorimetric immunoassay and an electrochemical immunosensor. New strategies within the microalgal field for future research of CP in the Canary Islands are further discussed.

2. Methods

2.1. Reagents and equipment

CTX1B standard solution was obtained from Prof. Richard J. Lewis (The Queensland University, Australia) [5] and calibrated (correction factor of 90%) in relation to the NMR-quantified CTX1B standard solution from Prof. Takeshi Yasumoto (Japan Food Research Laboratories, Japan). 51-OH-CTX3C standard solution was kindly provided by Prof. Takeshi Yasumoto (Japan Food Research Laboratories, Japan) and was used as a model for the series of CTX3C congeners. Neuroblastoma murine cells (neuro-2a) were purchased from ATCC LGC standards (USA). Poly-L-lysine, foetal bovine serum (FBS), L-glutamine solution, ouabain, veratridine, phosphate buffered saline (PBS), penicillin, streptomycin, RPMI-1640 medium, sodium pyruvate, thiazolyl blue tetrazolium bromide (MTT), N-(3-dimethylaminopropyl)-N'-ethylcarbodiimide hydrochloride (EDC), N-Hydroxysuccinimide (NHS), Tween 20, bovine serum album (BSA), poly horseradish peroxidase-streptavidin (polyHRP-streptavidin) and 3,3',5,5'-tetramethylbenzidine (TMB) liquid substrate were supplied by Merck KGaA

(Germany). Dimethyl sulfoxide (DMSO) and absolute methanol were purchased from Honeywell (Spain) and Chemlab (Spain), respectively. Taq Polymerase and Dynabeads M-270 Carboxylic Acid (2×10^9 beads mL^{-1}) were purchased from Invitrogen (Spain). QIAquick PCR Purification Kit was obtained from Qiagen (Germany). For the DNA amplification, a Mastercycler nexus gradient thermal cycler purchased from Eppendorf (Spain) was used. The 3G8, 10C9 and 8H4 monoclonal antibodies (mAbs) had been prepared by immunizing mice with keyhole limpet hemocyanin (KLH) conjugates of rationally designed synthetic haptens [80–86]. A microplate Reader KC4 from BIO-TEK Instruments, Inc. (Vermont, USA) was used to perform colorimetric measurements and Gen5 software was used to collect and process the data. Arrays of eight screen-printed carbon electrodes (DRP-8 × 110), a boxed connector (DRP-CAST8X) and a magnetic support (DRP-MAGNET8X) were purchased from Dropsens S.L. (Spain). A PalmSens potentiostat (Houte, Netherlands) connected to an 8-channel multiplexer (MUX8) was used to perform amperometric measurements. Data from potentiostat were collected and evaluated with PalmSens PC software (Houte, Netherlands).

2.2. Sampling area and the strategy

The Canary Islands are located in the north-east Atlantic Ocean (28.36715°, 17.61396°) between 100 km and 600 km west of the north-west African coast. Samplings were performed in seven big islands during 2016 and 2017. Gran Canaria, Fuerteventura, Lanzarote were sampled in October 2016 and La Palma, Tenerife, El Hierro and La Gomera in October 2017. Additionally, samplings were carried out in Gran Canaria in April 2017. Sampling locations are shown on a map on Figure 1 and Table 1 shows the details. In total, 53 sampling stations were studied. In each station, the temperature, salinity, pH, oxygen saturation and oxygen concentration were recorded with a multiparametric probe (YSI 556 MPS). The coordinates of the stations, date and the environmental data are shown in Table S1. In each sampling station, two samples were obtained: epilithic (surface rasping of rocky substrates) and epiphytic (sampling of macrophytes). Samples were collected using a plastic bottle (Nalgene, HDPE, 1 L). For the first sample, the substrates were scratched using the bottleneck, and for the second macroalgae were gently removed from their substrate and introduced into the bottle under the water. Then, the bottles were manually shaken, and each sample was filtered through a 300 µm nylon mesh to remove the detritus and the larger grazers. The filtered water was stored in a plastic bottle (Nalgene, HDPE, 125 mL).

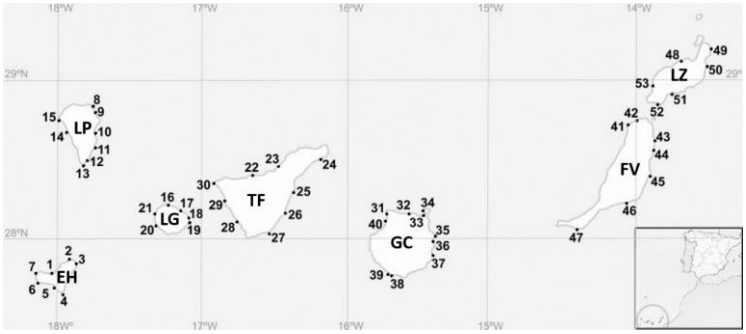

Figure 1. Sampling stations in the Canary Islands for the current study. Numbers correspond to locations described in Table 1. EH (El Hierro), FV (Fuerteventura), GC (Gran Canaria), LG (La Gomera), LP (La Palma), LZ (Lanzarote) and TF (Tenerife).

Table 1. Description of the sampling stations of the present study in the Canary Islands. Ref.: correspond to the locations in Fig. 1. Details of the date, coordinates and environmental data are compiled in Table S1.

Ref.	Island	Location	Ref.	Island	Location
1	El Hierro	Charco Azul	27	Tenerife	La Tejita
2	El Hierro	Charco Manso	28	Tenerife	La Caleta
3	El Hierro	Tamaduste	29	Tenerife	El Pto. de Santiago
4	El Hierro	La Restinga	30	Tenerife	Punta de Teno
5	El Hierro	Tacoron	31	Gran Canaria	Punta Sardina
6	El Hierro	Orchilla	32	Gran Canaria	El Puertillo, Bañaderos
7	El Hierro	Verodal	33	Gran Canaria	Las Canteras
8	La Palma	La Fajana	34	Gran Canaria	El Confital
9	La Palma	El Puerto Espíndola	35	Gran Canaria	Melenara
10	La Palma	Los Cancajos	36	Gran Canaria	Playa Tufia
11	La Palma	Salemera	37	Gran Canaria	Agüimes, Playa El Cabrón
12	La Palma	El Puerto de Trigo	38	Gran Canaria	Arguineguín El Pajar
13	La Palma	El Faro Fuencaliente	39	Gran Canaria	Arguineguín Sta. Águeda
14	La Palma	Tazacorte	40	Gran Canaria	Las Charcas de Agaete
15	La Palma	Puntagorda	41	Fuerteventura	Caleta del Río, El Cotillo
16	La Gomera	Vallehermoso	42	Fuerteventura	Majanicho
17	La Gomera	La Caleta	43	Fuerteventura	Playa Jabalito
18	La Gomera	Playa de Ávalos	44	Fuerteventura	Puerto Lajas
19	La Gomera	Playa de la Cueva	45	Fuerteventura	Puerto Caleta del Fuste
20	La Gomera	Playa de Vueltas	46	Fuerteventura	Gran Tarajal
21	La Gomera	Alojera	47	Fuerteventura	Morro Jable
22	Tenerife	Charca del Viento	48	Lanzarote	Caleta Caballo
23	Tenerife	Puerto del Sauzal	49	Lanzarote	Las Cocinitas
24	Tenerife	Playa las Teresitas	50	Lanzarote	Charco del Palo
25	Tenerife	El Puertito	51	Lanzarote	Puerto Calero
26	Tenerife	Punta de Abona	52	Lanzarote	Playa Mujeres
			53	Lanzarote	El Golfo

2.3. Isolation and culturing

Samples were observed under a light microscope (Leica Microsystems GmbH, Germany) to isolate *Gambierdiscus* and *Fukuyoa* cells by the capillary method [87]. Each dinoflagellate cell was placed individually in one well of an untreated Nunc 24 well plate (Thermo Fisher Scientific) with 1mL of modified ES medium [88]. The culture medium was constituted with seawater from L'Ametlla de Mar (Spain), Mediterranean Sea (40.8465 °; 0.772432 °), which was aged for two months in the dark and was filtered through an activated carbon filter of PTFE (Thermo Fisher Scientific) and after through a 0.22 µm cellulose acetate filter (Merck KGaA, Germany). The salinity was adjusted to 36 with Milli-Q water. After 2-3 weeks, fifty-two cultures achieved at least 20 cells mL^{-1} and cells were transferred to fresh medium for maintenance in 28 mL round bottom glass tubes (Thermo Fisher Scientific). Cells were cultured at 24 ± 1 °C, with photon irradiance of 100 µmol photons m^{-2} s^{-1} and 12:12 light:dark cycle. Light was provided by fluorescent tubes with white light. Irradiance was measured by an irradiometer (QSL-2100 Radiometer, Biospherical Instruments, San Diego, USA).

2.4. Molecular identification

To identify the fifty-two cultures at the species level, the D8-D10 region of the LSU rDNA was used [23,89]. The cultures were transferred to 100 mL Erlenmeyer flasks at a final concentration of 50 cells mL^{-1}. Afterwards, 50 mL of culture were harvested at the exponential phase by centrifugation (4300 g, 20 min). The resulting cell pellet was processed for DNA extraction using the phenol/chloroform/isoamylalcohol extraction (PCI) protocol according to Toldrà et al. [90]. Genomic DNA was quantified and checked for its purity using a NanoDrop 2000 spectrophotometer (Thermo Fisher Scientific). The D8-D10 of the LSU region was amplified by PCR using the primers FD8 and RB [89]. Amplifications were carried out in a Mastercycler nexus gradient thermal cycler (Eppendorf, Spain) as it was described in Reverté et al. [66]. The similarity of sequences was checked by BLAST (National Centre of Biotechnology Information, NCBI) and deposited in GenBank. Sequences of >562 were aligned using MAFFT v.7 [91] with the G-INS-1 progressive method. The final alignment consisted of 42 seqs from the current study with 562 positions. The origin of the sequences and the date of collection are shown in Table S2. The phylogenetic relationships were inferred by Maximum Likelihood (ML) using RaxML v.8 [92] and by Bayesian inference (BI) using Mr. Bayes v.3.2.2 [93]. The model of evolutionary reconstruction for ML analysis was estimated using JModelTest 2.1.10 [94]. In the BI approach, two analyses were run in parallel, 10^6 generations, and four chains in each run. The parameters used for analysis were nst

= mixed and rates = gamma. By default, 25% of the trees were discarded. The stability of the chains was checked using Tracer v.1.7.1 [95].

2.5. Morphological characterization

Monoclonal cultures were cultivated and acclimated for a minimum of one year before experimentation to reduce the variability of the response that stress can produce [96]. The morphological characterization was conducted in 4 strains: IRTA-SMM-16-286 (*G. australes*) from Lanzarote, IRTA-SMM-17-407 (*G. excentricus*) from La Gomera, IRTA-SMM-17-03 (*G. caribaeus*), these three strains were reported in Gaiani et al. [97], and IRTA-SMM-17-421 (*G. belizeanus*) from El Hierro. A 5 mL aliquot of each culture at the late exponential phase was fixed with 3% Lugol's iodine solution. Then, the aliquots were stained using Calcofluor White M2R (Sigma Aldrich, Spain), according to Fritz and Triemer [98]. The thecae were described following the nomenclature proposed by Fraga et al. [26]. The depth (dorso-ventral axis) and the width (transdiameter lateral extremes of cingulum) of 50 individuals of each strain were measured using an epifluorescence microscope (LEICA DMLB and NIKON eclipse 80i) equipped with an Olympus camera (Olympus DP70). The software used for measurements was Olympus DP controller (Olympus Corporation). Cell dimensions were expressed as mean ± standard deviation (SD). In addition, cells of *G. belizeanus* (IRTA-SMM-17-421) were observed by scanning electron microscopy (SEM). For that, 10 mL of cultures at the initial exponential growth phase were fixed with glutaraldehyde at a final concentration of 4 % for 2 h at room temperature. After that, 3 mL of culture were collected with a syringe by applying a low pressure on a 5 µm Nuclepore Track-Etch Membrane (Thermo Fisher Scientific). Previously, the membrane had been coated with poly-L-lysine and held in a plastic filter mould of 13 mm diameter (PALL, life Science). The membrane with the cells was rinsed twice; once with seawater (autoclaved and filtered with active carbon 0.22 µm) and a second time with seawater/MilliQ water (50:50, v:v). Afterwards, dehydration was performed in a graded EtOH series of 30, 50, 70, 80, 90 and twice with 96 %. Filters were transferred to vessels with absolute EtOH and sent to SEM facilities of the Institut de Ciències del Mar (ICM-CSIC). Then, filters were submitted to critical-point drying with liquid carbon dioxide in a BAL-TEC CPD030 unit (Leica Microsystems, Austria). Dried filters were mounted on stubs with colloidal silver, then sputter-coated with gold in a Q150R S (Quorum Technologies Ltd). Cells were observed with a Hitachi S3500N scanning electron microscope (Hitachi High Technologies Co., Ltd, Japan) at an accelerating voltage of 5 kV.

2.6. Production of microalgal extracts for toxin evaluation

Forty-one cultures of *Gambierdiscus* were inoculated in 500 mL Fernbach at an initial concentration of 50 cells mL^{-1}. When cultures arrived at the late exponential-early stationary phase, aliquots of microalgal cultures were collected, fixed with 3 % Lugol's iodine solution for cell counting under the light microscope. Then, cultures were harvested by centrifugation (4300 g, 20 min), obtaining between 1×10^5 - 1.6 x 10^6 cells of each strain. Cell pellets were kept with absolute methanol (1 mL of methanol for 1 x 10^6 cells) at -20 °C. For toxin extraction, each microalgal pellet was sonicated using an ultrasonic cell disrupter (Watt ultrasonic processor VCX750, USA) at 3 sec on and 2 sec off, 34 % amplitude for 15 min. After that, the sample was centrifuged (600 g, 5 min) and the supernatant was removed and kept in a glass vial. Then, new absolute methanol was added to the cell pellet and the whole process of toxin extraction was repeated twice. Supernatants were pooled and evaporated under N_2 (Turbovap, Caliper, Hopkinton, USA) at 40 °C. Dried extract was dissolved with methanol and kept at -20 °C until toxin evaluation.

2.7. Evaluation of CTX-like toxicity with the neuroblastoma cell-based assay

The CTX-like toxicity of forty-one cultures of *Gambierdiscus*, consisting of *G. australes* (n=29), *G. excentricus* (n=10), *G. caribaeus* (n=1) and *G. belizeanus* (n=1), was evaluated using the neuro-2a CBA. This assay is used to detect compounds that target voltage-gated sodium channels (VGSCs). The assay uses ouabain, which blocks the Na^+/K^+-ATPase ion pump and inhibits the efflux of Na^+ [99] and veratridine, which activates the VGSC, enhancing the influx of Na^+ [100]. The neuro-2a CBA is based on the reduction of viability of the neuro-2a cells when the extract presents CTXs or molecules that activate VGSCs after the ouabain and veratridine treatment [101].

The neuro-2a CBA and the data analysis were conducted following the protocol described in Caillaud et al. [102]. CTX1B was the molecule of reference (standard) to quantify the CTX-like toxicity of the extracts, and a dose-response curve was obtained each day of experimentation. To be able to discriminate CTX-like toxicity from other types of toxicity, neuro-2a cells were exposed to extracts or standards with and without ouabain and veratridine. It was considered that samples contained CTX-compounds or other compounds that target VGSCs when toxicity was not observed in the O/V- conditions but was observed in the O/V+ conditions. In the cases that toxicity was observed in both conditions (O/V- and O/V+), it indicates the presence of a non-specific toxic compounds other that target the VGSCs.

Concentrations of CTX1B ranged between 0.2 and 25 pg mL^{-1}, and concentrations of microalgal extracts ranged between 0.3 and 3000 cells equiv. mL^{-1}. The concentrations of microalgal extracts were chosen based on the toxic effect observed in the neuro-2a cells from previous screening experiments. After 24 h of exposure, the viability of the neuro-2a cells was assessed by the quantitative colorimetric MTT assay [101]. Data analysis was performed using SigmaPlot software 12.0 (Systat Software Inc., USA). Matrix effect was considered when significant toxicity appeared in the neuro-2a cells without O/V-. Significant toxicity was described as the inhibition of more than 20 % of the cell viability. The normality of the CTX-like toxicities was checked using the Shapiro-Wilk test. Then, a one-way ANOVA was used to test if significant differences in CTX-like toxicities occurred among *G. excentricus* and *G. australes*, and if significant differences occurred between the islands within these species. The statistical test and graphs were performed with R studio [103].

2.8. Evaluation of the presence of two series of CTX congeners (CTX1B and CTX3C) in *G. belizeanus* with a colorimetric immunoassay and an electrochemical immunosensor.

The use of 3G8 and 10C9 mAbs in the screening of the *Gambierdiscus* extracts allowed the detection of two series of CTXs congeners (CTX1B and CTX3C) thanks to the high affinities of these capture antibodies for their CTX targets. In particular, 3G8 mAb binds to the left wing of CTX1B and 54-deoxyCTX1B [84] and 10C9 mAb binds to the left wing of CTX3C and 51-hydroxyCTX3C [82]. The 8H4 mAb, used as a reporter antibody, binds to the right wing of all the four congeners [104]. For this reason, quantitations are expressed in fg $cell^{-1}$ of CTX1B equiv. when only the 3G8 mAb was incubated with the microalgal extract, and in fg $cell^{-1}$ of 51-hydroxyCTX3C equiv. in presence of only the 10C9 mAb. The use of separated mAbs allows the discrimination between the two series of CTX congeners. Therefore, when both antibodies are incubated with the extract, a global response is obtained, and thus quantitations can be provided either in fg $cell^{-1}$ of CTX1B equiv., or 51-hydroxyCTX3C equiv. In this work, the obtained quantitations when the two antibodies are incubated together are provided only in CTX1B equiv. for comparison with neuro-2a CBA results. Analyses of *G. belizeanus* extracts were performed as described in Gaiani et al. [97]. Briefly, magnetic beads (MBs) were activated with an EDC and NHS solution and incubated with 3G8 or 10C9 mAbs. In particular, 3G8 mAb binds against the left wing of CTX1B and 54-deoxyCTX1B. Instead, 10C9 mAb binds specifically to the left wing of CTX3C and 51-hydroxyCTX3C [84,86]. Figure 2 shows the structure of the four CTX congeners (CTX1B and CTX3C congeners) that the antibodies recognise. After the incubation, the mAb-MB conjugates were washed,

placed into new tubes in a separate or mixed way, exposed to microalgal extract (previously evaporated and suspended in PBS-Tween) or CTX standard (CTX1B or 51-hydroxyCTX3C) for calibration purposes. Afterwards, a blocking step was performed with PBS-Tween-BSA. Then, the conjugates were incubated with biotin-8H4 mAb [104]. The 8H4 mAb binds to the right wing of CTX1B and 54-deoxyCTX1B and has cross-reactivity with the right wing of CTX3C and 51-hydroxyCTX3C. Finally, immunocomplexes were incubated with polyHRP-streptavidin, washed, and re-suspended in PBS-Tween. The colorimetric immunoassay was performed incubating the immunocomplexes with TMB (HRP enzyme substrate) and reading the absorbance at 620 nm using an automated plate spectrophotometer. Measurements were performed in triplicate. The electrochemical immunosensor was performed placing the immunocomplexes on the working electrodes of an 8-electrode array, incubating with TMB, and measuring the reduction current using amperometry (−0.2 V (vs. Ag) for 5 s). Measurements were performed in quadruplicate.

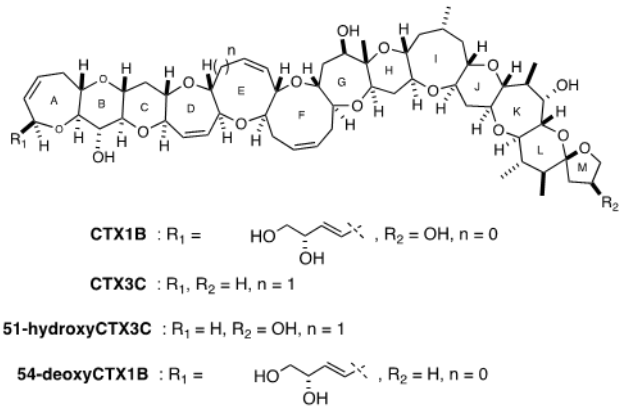

Figure 2. Structure of CTX1B and CTX3C congeners recognized by the antibodies used in this work.

3. Results and discussion

3.1. Molecular identification

Fifty-two strains including four species [*G. australes* (n = 32), *G. excentricus* (n = 18), *G. caribaeus* (n = 1) and *G. belizeanus* (n = 1)] were identified using sequences of the LSU D8-D10 rDNA region. The results of the BLAST analysis were well supported by the trees obtained using the Maximum Likelihood (ML) and the Bayesian Inference (BI) methods. Figure 3 shows the topology of the ML phylogenetic tree with

bootstrap support values (bt) and the posterior probability (pp) of BI analysis displayed at branch nodes. Topography with the two phylogenetic trees was very similar. In both trees, the strains of this study are well defined within their respective clades for *G. australes*, *G. excentricus*, *G. caribaeus* or *G. belizeanus* with bt/pp values of 96/1.00, 100/1.00, 98/0.92, 96/1.00, respectively. Further, *G. pacificus* species were split into two clades. One sequence is grouped in the ML tree with *G. lewisii* with a high bootstrap value (>70). In contrast, in the BI tree, the clade of *G. lewisii* with *G. pacificus* appear but less well supported (0.83 pp). The sequence of IRTA-SMM-17-421 was in the *G. belizeanus* cluster. This sequence exhibited 99% of similarity by BLAST with the isolate RS2-B6 of *G. belizeanus* (KY782638) from the Red Sea [38]. The genetic distance, pairwise distance (p-distance), between those two sequences was 0.011 substitutions per site. The strain IRTA-SMM-17-421 jointly with RS2-B6 has a deletion of 121 bp as described previously in Catania et al. [38], and this may indicate that it could be a ribosomal pseudogene. After excluding the deletion, the p-distance between IRTA-SMM-17-421 and *G. belizeanus* sequences ranged between 0.002 and 0.019 substitutions per site.

3.2 Morphological characterization

The depth and width were measured in 50 cells for each species: *G. australes*, *G. excentricus*, *G. caribaeus* and *G. belizeanus* using the Calcofluor White stain method under light microscopy. Measurements for each species are shown in Table 2.

Table 2. Morphological sizes (average depth and width (± SD) of *Gambierdiscus* species of this study measured by light microscopy. The ranges of values are shown in parentheses.

Species	Depth (µm)	Width (µm)
G. australes	71.13 ± 7.06 (60.6 – 98.4)	65.40 ± 6.54 (53.4 – 82.1)
G. belizeanus	64.12 ± 5.28 (52.8 – 76.2)	59.64 ± 5.95 (46.5 – 76.0)
G. caribaeus	87.20 ± 11.19 (61.2 – 116.5)	86.45 ± 11.44 (63.17 – 119.7)
G. excentricus	90.25 ± 8.90 (72.2 – 109.7)	82.97 ± 9.06 (67.8 – 106.7)

Cell morphology can vary depending on the culture conditions, growth phase, and different genotypes [32,42]. The morphological characterization showed that some cells of *G. australes* in the present work were smaller than the original description (76.0 - 93.0 µm of depth, 65.0 - 84.0 µm of width) in Chinain et al. [43]. But overall, values are according to measurements presented in Bravo et al. [32], Litaker et al. [44], Rhodes et al. [23]. For *G. belizeanus,* the minimum measurements of depth in the present study are in accordance with the original description of 53-67 µm in Faust [34]; but, the minimum value for width (described in Faust as length), is lower than the first description (54-63 µm of width). Moreover, the maximum value for depth and width are higher than the description of Faust [34]. *G. caribaeus* cells from the current study were bigger than the original description in Litaker et al. [44] and cells of Bravo et al. [32]. In reference to *G. excentricus*, all measurements are in accordance with the original description Fraga et al. [26] and the values were similar to Bravo et al. [32] and Hoppenrath et al. [45].

3.2.1. Morphological characterization of *G. belizeanus*

Cells were anterior-posteriorly compressed. The plate formula of *G. belizeanus* was Po, 4', 6'', ?, 5''', 2'''' based on Fraga et al. [26]. The cells were heavily aerolated (Fig. 4A, 4B, and 4C). Limits of the thecae are well defined by intercalary bands. These two latter characteristics are typical of *G. belizeanus* [23,105,109]. The 2' plate is rectangular (Fig. 4A) and 2'''' plate is pentagonal (Fig. 4B). Figure 4D shows the apical pore plate (Po).

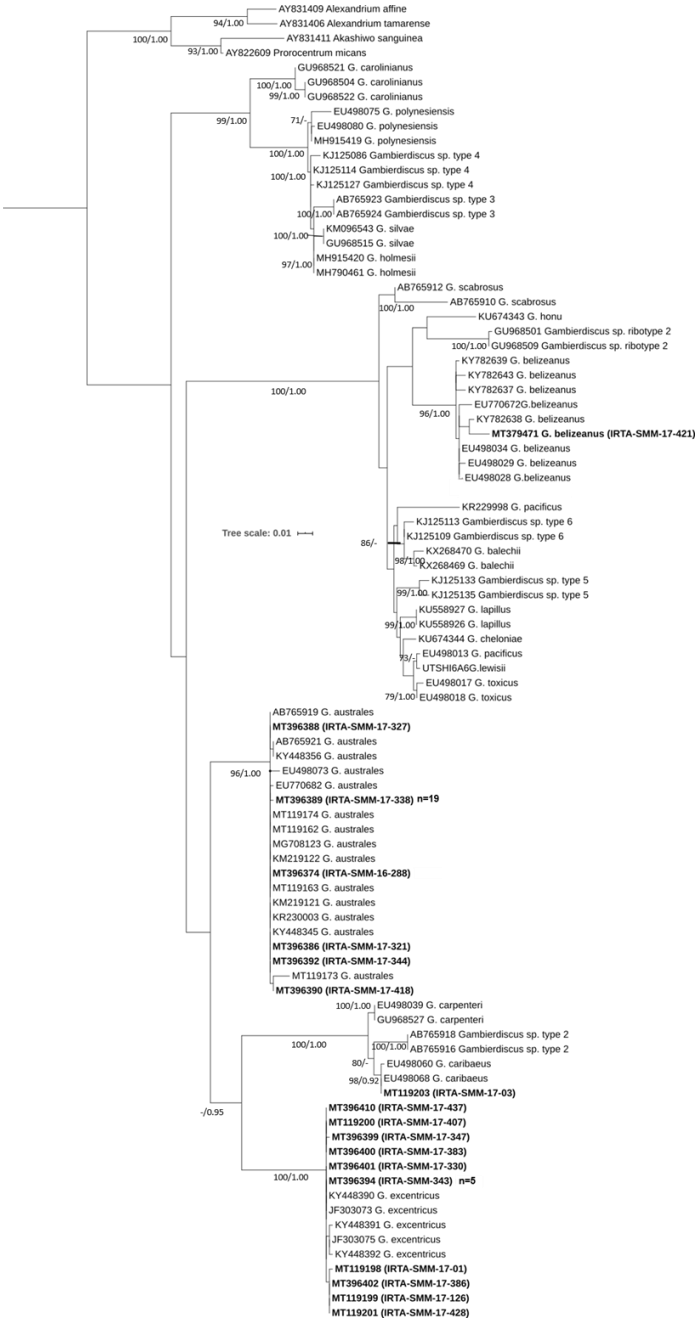

Figure 3. Phylogenetic tree of the LSU D8-D10 region (rDNA) using Maximum likelihood analysis. Sequences in bold represent the strains of this study. The number of clones (n) with the same haplotypes is shown in parentheses. Values at nodes represent bootstrap values (≥70) and the Bayesian posterior probability (≥0.95) (bt/pp).

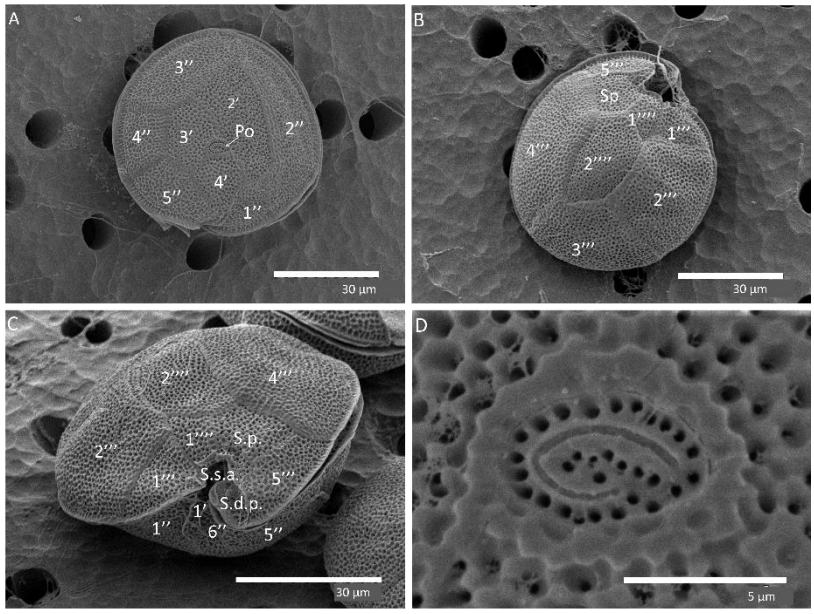

Figure 4. SEM images of *G. belizeanus* (IRTA-SMM-17-421): apical (A), antapical (B), ventral (C) views, detail of Po plate and pores (D).

3.3. Distribution of the *Gambierdiscus* species in the Canary Islands

The observation of the samples under light microscopy showed that cells of *Gambierdiscus* spp. were found at 21 stations of the 53 stations sampled in the seven islands in 2016 and 2017. *Gambierdiscus* cells co-occurred with cells of the genera *Coolia*, *Ostreopsis*, *Prorocentrum*, *Amphidinium*, *Karenia* and *Trichodesmium*, among others. Details of the islands and the number of identifications for each station are shown in Figure 5. Overall, *G. australes* was the most abundant and it was present in all the islands. *G. excentricus* was the second most abundant. It was present in four islands Gran Canaria, Tenerife, La Gomera, and La Palma, excluding the eastern islands (Lanzarote and Fuerteventura) and the western island (El Hierro). *G. caribaeus* and *G. belizeanus* were identified in El Hierro.

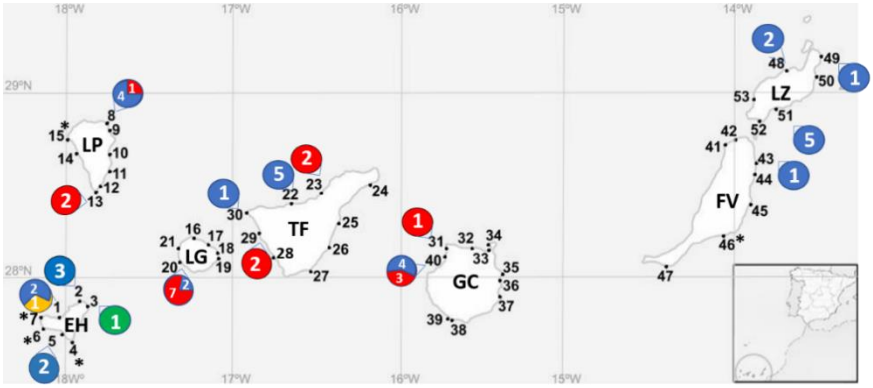

Figure 5. Distribution of each species in the stations of the Canary Islands during 2016-2017. Station numbers are represented in bold. The presence of *Gambierdiscus* species determined with molecular analysis is presented with a circle and includes the number strains identified for each species. The asterisk represents the presence of *Gambierdiscus* sp. Colours 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).

Canary Islands were considered outside of the geographical distribution of the *Gambierdiscus* genus, and the first reports of the genus were considered the result of a recent arrival in the islands [30]. The first reports of the *Gambierdiscus* genus in the Canary Islands were occasional, in one or two islands, and they were the result of the collection of very few samples [30,31]. The finding of the new species *G. excentricus* and *G. silvae* in the Canary Islands, and the rapid increase in the number of species in consecutive samplings combined with the occurrence of CP cases in the islands [13] elicited the alarm to conduct urgent systematic and wide samplings in the area in order to understand the distribution and origin of the dinoflagellates and their potential contribution to CP cases. Thus, Rodríguez et al. [33] collected samples in numerous stations in La Graciosa (Chinijo Archipelago) in March 2015 and in the big islands excluding La Palma and La Gomera in September-October 2015. Rodríguez et al. [33], numerous isolates were identified, and *Gambierdiscus* cell abundances were determined in the sampling stations. The authors found a high richness of the *Gambierdiscus* genus in the Canary Islands, reporting five species: *G. excentricus*, *G. silvae*, *G. australes*, *G. caribaeus* and *G. carolinianus*. This unexpected richness revealed a more likely ancient origin of the genus, contrary to what had been previously considered as a recent introduction [46]. In October 2017, Bravo and collaborators [32], who performed systematic samplings in La Gomera and La Palma, complemented the geographical distribution of the *Gambierdiscus* species, among other issues. To date, samplings in the Canary

Islands have been performed during very specific temporal scales, mainly in periods from October to September. In the current study, systematic samplings were performed in a higher number of stations, including new ones in the seven big islands. It is important to remark that a general bias in the identification process for the presence of species in a given area occurs since identification is a result of success growth under laboratory conditions. In Rodriguez et al. [33], this factor was partially avoided due to the identification of single cells in addition to identification of cultured strains. In the present study, single-cell identification was not appropriate since isolates were required to establish monoclonal cultures to enable evaluation of their toxicity. Despite these limitations, the current study has contributed to a better understanding of the biodiversity of the genus in the Canary Islands, while providing a review and update of the geographical distribution of the species. A new contribution of the current work is the first report of *G. belizeanus* in the Canary Islands Archipelago (El Hierro) and *G. australes* in La Palma.

Gambierdiscus species have distinct lower and upper thermal limits and optimal temperatures for their growth [47]. Additionally, they are considered to have a high intraspecific variable response in growth depending on the temperature, salinity and irradiance [47,48], and the response could be influenced by the geographical origin of the isolate [48]. Even so, biodiversity in the islands could follow a geographical pattern depending on the maximum and minimum temperatures on each island. For instance, as mentioned before, SST of the western part of the Archipelago is higher. Therefore, a priori, the west could be more suitable for the warmer tropical species. This phenomenon has already been observed in fish [49]. Overall, the range of SST during 1972-2012 in the Archipelago was 15.9 °C in March-April and 25.5 °C in August-October [50]. The optimal temperatures for *Gambierdiscus* species are between 20 and 28 °C, and the maximal growth temperatures usually are >25 °C . Hence, it is expected that high abundances and more diversity of *Gambierdiscus* species would be found during August-October.

The distribution of *G. australes* and *G. excentricus* observed in the current study was similar to that reported by Rodriguez et al. [33] and Bravo et al. [32]. Table 3 compiles the records of *Gambierdiscus* species in the Canary Archipelago from the literature together with the results of the current study. *G. australes* is present in all seven big islands. This data are consistent since *G. australes* is the *Gambierdiscus* species with the widest optimal temperature range, between 19-28 °C [26,51]. In the current study, its presence is not dominant in all the islands, being slightly less abundant in La Gomera. This observation is in accordance with the previous results of Bravo et al. [32] reported for this island, where *G. excentricus* was the dominant taxa followed by *G. silvae*, *G. caribaeus* and *G. australes*. The second most dominant

species in the islands is *G. excentricus*, although it is not reported in El Hierro. Although physiological data have not been reported for *G. excentricus*, its distribution in the Canary Islands is coherent considering that El Hierro has the most tropical conditions and that *G. excentricus* is more commonly present in temperate areas [26,45,52]. *G. silvae* was recorded in the central islands: Tenerife, La Gomera and Gran Canaria. Overall, longitudinal distribution of *G. silvae* is quite broad, including the Caribbean Sea and the Atlantic Sea [33,52]. However, compared to other species, one *G. silvae* strain, originating from Caribbean Sea, showed a narrow range of tolerance to temperature and the maximal temperature for growth was low (24.8 °C) [48]. This result should be contrasted with maximal temperature of other *G. silvae* strains, but these data are presently lacking. *G. carolinianus* is present only in Tenerife. In Kibler et al. [47], the responses to environmental factors among strains were highly variable, but globally, *G. carolinianus* was well adapted to low temperatures (15 °C). Additionally, optimal temperatures for growth were also low (21.8-27.9 °C) [48]. Finally, *G. caribaeus* was reported in La Gomera and El Hierro, while *G. belizeanus* was present only in El Hierro. Experimentally, strains of *G. belizeanus* and *G. caribaeus* exhibited a wide range of temperature [48], but their range of temperature for maximal growth is considered high, since their temperature ranges are 26.1-29.1 °C [48] and 25-31 °C [48,53], respectively. Thus, the geographical distribution of both species in the Canary Islands is in accordance with the high-temperature adaptation in the laboratory.

In reference to the level of biodiversity, Tenerife and La Gomera have the highest richness of *Gambierdiscus* species. Tenerife is the biggest island and, consequently, more different habitats could be available. Additionally, its location in the middle of the Archipelago provides intermediate conditions. However, there are some inconsistences when trying to explain the biodiversity in the islands according to temperatures. For instance, experimentally, *G. belizeanus* has an optimal temperature range similar to *G. caribaeus* reported in El Hierro and in La Gomera. In contrast, *G. belizeanus* has not been reported in La Gomera. This may indicate that the presence of species is not yet well recorded or that more important factors for *Gambierdiscus* species are still unidentified. For instance, the exposure of the station to wave action was an important factor influencing the variability of macroalgae in the Canary Islands [54]. Additionally, it is important to highlight that there is high intraspecific variation in the physiologic response under laboratory conditions. In other words, the strains from the Canary Islands may differ from other strains tested in previous studies.

Table 3. Literature review of the distribution of the *Gambierdiscus* species in the Canary Islands. (El Hierro), FV (Fuerteventura), GC (Gran Canaria), LG (La Gomera), LP (La Palma), LZ (Lanzarote) and TF (Tenerife).

Species	Islands	Date of sampling	Reference
G. australes	LZ, FV, GC	Sept.- Oct. 2015	Rodríguez et al., 2017
		Oct. 2016	This study
	EH	Sept.- Oct. 2015	Rodríguez et al., 2017
		Oct. 2016	This study
	TF	2013	Fraga and Rodríguez, 2014
		Sept.- Oct. 2015	Rodríguez et al., 2017
		Oct. 2017	This study
	LG	Oct. 2017	Bravo et al., 2019; this study
		March 2004	Fraga et al., 2011
	LP	Oct. 2017	This study
G. belizeanus	EH	Oct. 2017	This study
G. caribaeus	EH	Sept.- Oct. 2015	Rodríguez et al., 2017
		Oct. 2016	Soler-Onis et al., 2016
	LG	Oct. 2017	This study
		Oct. 2017	Bravo et al., 2019
G. carolinianus	TF	Sept.- Oct. 2015	Rodríguez et al., 2017
G. excentricus	TF	March 2004	Fraga et al., 2011
		2013	Fraga and Rodríguez, 2014
		Sept.- Oct. 2015	Rodríguez et al., 2017
		Oct. 2017	This study
	LZ	Sept.- Oct. 2015	Rodríguez et al., 2017
		Oct. 2017	This study
	FV	Sept.- Oct. 2015	Rodríguez et al., 2017
		Oct. 2016	This study
	GC	March 2004	Fraga et al., 2011
		Oct. 2017	This study
	LP	March 2004	Fraga et al., 2011
	LG	March 2004	Fraga et al., 2011
		Oct. 2017	Bravo et al., 2019; this study
G. silvae	TF	2013	Fraga and Rodríguez, 2014
		Sept.- Oct. 2015	Rodríguez et al., 2017
	GC	Winter 2010	Fraga and Rodríguez, 2014
	LG	Oct. 2017	Bravo et al., 2019

The prevalence of the *Gambierdiscus* isolates in the Canary Islands since 2004 shows a good adaptation of the genus to the conditions found in the Archipelago. During recent decades, oceans have suffered a warming trend and the SST in the Canary Islands is projected to increase 0.25 °C decade^{-1} [56]. The SST between 1985-2018 in the Canary Islands did not surpass 26 °C [50], which is far from the lethal temperatures for *Gambierdiscus* species (~31 °C) [47,57]. Globally, the abundances of *Gambierdiscus* in the Canary Islands could be higher during the next years influenced by rising temperatures. Nonetheless, in the easternmost islands, the trend of the upwelling is not clear [56] and how upwelling could affect to *Gambierdiscus* cell densities under the influence of the SST in the eastern islands is still to be studied.

3.4. Evaluation of CTX-like toxicity with the neuro-2a CBA.

The evaluation of the CTX-like toxicity was conducted for 41 extracts from cultures at late exponential-early stationary phase of the four *Gambierdiscus* species: *G. australes*, *G. excentricus*, *G. caribaeus* and *G. belizeanus*, using the neuro-2a CBA. Obtaining CTX purified standards from microalgae is a very difficult task since the CTX production in microalgae is often very low. For this reason, the toxicological evaluation often is carried out with CTX standards purified from fish. It has been found that CTX4A, CTX4B, and CTX3C are produced by the alga, and they are oxidized to the analogs CTX1B, 52-epi-54-deoxyCTX1B, 54-deoxyCTX1B, 2-hydroxyCTX3C, and 2,3-dihydroxyCTX3C [58]. In the present study, the reference molecule was CTX1B [59], it's a typically CTX found in large carnivorous fish in the Pacific Ocean [59,60] and which has never been found in microalgae.

As expected, in each experiment, the standard CTX1B displayed a non-significant reduction of viability with O/V+ treatment, whereas a typical sigmoid curve was exhibited in the O/V- treatment. The average of the maximum exposed concentration of cells without toxicity in the O/V- treatment for *G. australes*, *G. excentricus, G. belizeanus* and *G. caribaeus* ranged between 2-201, 0.2-50, 160 and 6800 cells mL^{-1}, respectively. The one-way ANOVA showed that differences of CTX-like toxicities among the *G. excentricus* and *G. australes* were significant (p-value<0.01). Moreover, the one-way ANOVA test for the CTX-like toxicity for the islands between species was significant; differences were for Fuerteventura for *G. australes* and in la Palma for *G. excentricus*. These islands only have one tested strain. If these islands were not considered in the analysis, then the one-way ANOVA was not significant. Table 4 shows the results of the CTX-like toxicity expressed as fg CTX1B equiv. cell^{-1}. The most toxic species was *G. excentricus* with a range of 9.5-2566.7 fg CTX1B equiv. cell^{-1}. The most toxic strain was IRTA-SMM-17-330 from La Palma. The second most toxic species was *G. australes* with a range of 1.7-452.6 fg

cell^{-1}, followed by *G. belizeanus* which presented 5.6 ± 0.1 fg CTX1B equiv. cell^{-1}. *G. caribaeus* did not show toxicity at 6800 cells mL^{-1} with an LOD of 0.42 fg CTX1B equiv. cell^{-1}. Table S3 shows the neuro-2a cell viability and the CTX-like estimations obtained by the exposure to *Gambierdiscus* spp. extracts in O/V^{+} and O/V^{-} conditions. The differences of CTX-like toxicity among strains of *G. excentricus* (n = 10) and *G. australes* (n = 29) from different islands are shown in a boxplot in Figure 6.

Table 4. CTX-like toxicity of Gambierdiscus spp. using the neuro-2a CBA. CTX like toxicity and the limit of detection (LOD) are expressed as fg CTX1B equiv. cell−1. Ref: number of the station according to Fig. 1, EH (El Hierro), FV (Fuerteventura), GC (Gran Canaria), LG (La Gomera), LP (La Palma), LZ (Lanzarote) and TF (Tenerife).

Strain Code	CTX-like toxicity	Island	Station	Strain Code	CTX-like toxicity	Island	Station
G. australes				*G. australes*			
IRTA-SMM-17-004	205.1 ± 34.5	LZ	48	IRTA-SMM-17-316	51.5 ± 6.9	TF	22
IRTA-SMM-17-006	127.7 ± 85.0	LZ	51	IRTA-SMM-17-307	37.3 ± 12.6	TF	30
IRTA-SMM-16-288	106.1 ± 75.3	LZ	51	IRTA-SMM-17-436	98.6 ± 25.4	LG	20
IRTA-SMM-16-290	46.1 ± 22.2	LZ	51	IRTA-SMM-17-393	44.6 ± 11.5	LG	20
IRTA-SMM-16-292	39.7 ± 10.5	LZ	49	IRTA-SMM-17-344	41.2 ± 0.1	LP	8
IRTA-SMM-16-286	33.6 ± 6.5	LZ	51	IRTA-SMM-17-335	29.1 ± 8.6	LP	8
IRTA-SMM-16-293	32.7 ± 10.0	LZ	51	IRTA-SMM-17-287	11.3 ± 2.3	LP	8
IRTA-SMM-17-007	15.8 ± 1.7	LZ	48	IRTA-SMM-17-288	5.7 ± 3.8	LP	8
IRTA-SMM-17-002	452.6 ± 23.2	FV	43	IRTA-SMM-17-389	226.3 ± 24.5	EH	1
IRTA-SMM-17-103	118.7 ± 30.3	GC	40	IRTA-SMM-17-324	160.4 ± 17.2	EH	5
IRTA-SMM-17-107	12.2 ± 2.1	GC	40	IRTA-SMM-17-418	68.3 ± 9.5	EH	2
IRTA-SMM-17-112	1.9 ± 0.6	GC	40	IRTA-SMM-17-321	31.9 ± 15.0	EH	5
IRTA-SMM-17-106	1.7 ± 0.1	GC	40	IRTA-SMM-17-425	27.8 ± 3.3	EH	2
IRTA-SMM-17-358	138.9 ± 17.7	TF	22	IRTA-SMM-17-327	7.2 ± 0.3	EH	2
IRTA-SMM-17-291	82.8 ± 22.2	TF	22				
G. belizeanus				*G. caribaeus*			
IRTA-SMM-17-421	5.6 ± 0.1	EH	1	IRTA-SMM-17-003	Neg. LOD <0.42	EH	3
G. excentricus				*G. excentricus*			
IRTA-SMM-17-001	1149.3 ± 212.3	EH	1	IRTA-SMM-17-386	12.8 ± 2.8	TF	23
IRTA-SMM-17-126	226.7 ± 22.1	GC	40	IRTA-SMM-17-429	1525.9 ± 634.1	LG	20
IRTA-SMM-17-128	9.5 ± 2.6	GC	40	IRTA-SMM-17-432	962.1 ± 154.7	LG	20
IRTA-SMM-17-404	1257.64.8 ± 319.3	TF	29	IRTA-SMM-17-413	18.1 ± 5.7	LG	20
IRTA-SMM-17-405	1153.4 ± 238.8	TF	29	IRTA-SMM-17-330	2566.7 ± 333.3	LP	13

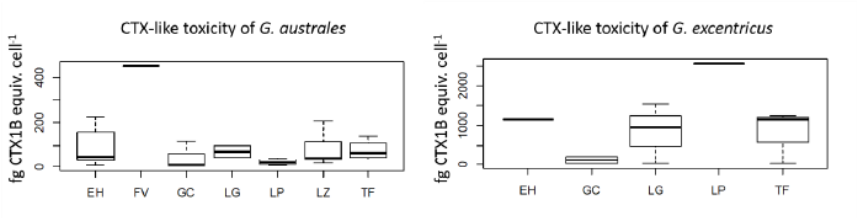

Figure 6. Distribution of CTX-like toxicity of G. australes and G. excentricus according to island of origin. EH (El Hierro), FV (Fuerteventura), GC (Gran Canaria), LG (La Gomera), LP (La Palma), LZ (Lanzarote) and TF (Tenerife).

In parallel to the identification attempts of the *Gambierdiscus* species in the Canary Islands and the determination of their geographical distribution, efforts to confirm if the local populations of *Gambierdiscus* produce CTXs and contribute to the CP of the Canary Islands have been made. Currently, although the CTX-like toxicity has been described for almost all species from the Canary Islands [24,25,61], the confirmation of the CTXs production in isolates from the Canary Islands has only been determined for *G. excentricus* by Paz et al. [62]. The production of CTXs seems to be very low and infrequent compared to the production of MTXs [22]. For instance, maitotoxin-4 (MTX4) was detected in strains of *G. excentricus* from Tenerife (VGO791 and VGO792) by Pisapia et al. [63] and from la Gomera (IRTA-SMM-17-407) by Estevez et al. [64]. In the evaluation of CTX-like toxicity by neuro-2a CBA of the crude extracts, other compounds can interfere, since the evaluated toxicity in neuro-2a CBA is a composite effect, and sometimes other compounds can cause unspecific mortality [25]. In Estevez et al. [64], the toxic effect on neuro-2a cells of the methanolic crude extract of the *G. excentricus* (IRTA-SMM-17-407) was very high, making the identification/quantitation of CTX-like toxicity impossible. This effect was likely due to the presence of MTX4, also was described in the same paper, since MTXs have high toxicity to neuro-2a cells [65]. In neuro-2a CBA, the use of controls with and without ouabain and veratridine allow the discrimination of CTX-like toxicity. Nevertheless, high amounts of MTXs, for example, and eventually other compounds, could also mask the presence of CTXs.

Fraga and collaborators [30] observed that *G. excentricus* from the Canary Islands produces compounds with high CTX-like toxicity. Afterwards, Pisapia et al. [24] reported that *G. silvae* was 100-fold less toxic and *G. australes* 1000-fold less toxic than *G. excentricus*. Thus, *G. excentricus* and *G. silvae* seemed to be the top producers of CTX-like toxicity in the Atlantic Ocean. Nonetheless, after Reverté et al. [66] and Rossignoli et al. [25], this statement is controversial. Both studies listed *G. australes* and *G. excentricus* as the most toxic species in the Canary Islands, and

not *G. silvae*. In particular, Rossignoli et al. [25] examined the CTX-like toxicity using the neuro-2a CBA for the isolates of the five species originating from the Canary Islands. In that study, the highest CTX-like activity was for *G. excentricus* (VGO1361) with 510 fg CTX1B equiv. cell^{-1} followed by *G. carolinianus* (VGO1197) with 101 fg CTX1B equiv. cell^{-1}, *G. australes* (VGO1252) with 107 fg CTX1B equiv. cell^{-1} , *G. caribaeus* (VGO1367) with 90 fg CTX1B equiv. cell^{-1} and G*. silvae* (VGO1378) with 77 fg CTX1B equiv. cell^{-1}. These results can be compared with our study because both studies used the same molecule of reference (CTX1B) and the same method for the toxicological evaluation.

Among the strains of the present work, *G. excentricus* exhibited the highest toxicity levels. These values are higher than those of Rossignoli et al. [25], being more similar to the levels measured by Fraga et al. [25] of 1100 fg CTX1B equiv. cell^{-1}. The second most toxic species was *G. australes*. The mean values of the current study were similar to the highest values of Rossignoli et al. [25] (31-107 fg CTX1B equiv. cell^{-1}) and globally lower than the values of Reverté et al. [61] (200 to 600 fg CTX1B equiv. cell^{-1}). The *G. caribaeus* strain (IRTA-SMM-17-03) did not exhibit CTX-like toxicity, this in accordance with Rossignoli et al. [25]. In fact, in previous studies, *G. caribaeus*, *G. belizeanus* and *G. carolinianus* were classified as low CTX producers [25,60]. *G. caribaeus* showed no CTX-like toxicity by CBA [66]. Referring to *G. belizeanu*s, the CTX-like toxicity values of the current study are higher than the average of the toxicity for the *G. belizeanus* strains from the Red Sea of 0.038 fg of CTX1B equiv. cell^{-1} [38]. In the Red Sea, there is no confirmation of any CP cases [127] and *G. belizeanus* being the only *Gambierdiscus* species reported in that area. Even though only one isolate has been evaluated, its low toxicity, together with its restricted geographical distribution to El Hierro, suggest that the contribution of *G. belizeanus* to CP of the Canary Islands may be negligible, although more strains should be evaluated.

Literature shows there is a high variation of CTX-like response between isolates within the same species [24,25,61]. This variation has been observed also in the current study. For *G. excentricus* and *G. australes*, the strains with the highest toxicity were 160 and 100-fold more toxic than the least toxic strain of the same species, respectively. This variability is higher than the obtained intraspecific values in Litaker et al. [37], Pisapia et al. [24], and Rossignoli et al. [25]. These differences may be related to the large number of strains that have been evaluated in the present study.

According to our results, the CTX-like toxicity levels have no clear pattern relating to the islands of origin. This is relevant because data of the fish from the official control program of Ciguatera from the Canary Islands showed that ciguateric fish

follow an east-west gradient. Toxic fish were more likely to be caught in Lanzarote (53 %), followed by Fuerteventura (21 %), Gran Canaria (18 %), El Hierro (15 %), Tenerife (14 %), La Palma (5 %) and La Gomera (2 %) [21]. Sanchez-Henao et al. [21] suggested that the percentage of Lanzarote results must be considered with caution, since, some samples were not accompanied by the total information and may not reflect the reality. Thus, the global tendency of ciguateric fish could be explained by the *Gambierdiscus* cell abundances from the samplings of Rodriguez et al. [33], which showed higher abundances in the east than in the west. However, for some fish species, the east-west gradient of CTX-like toxicity is not followed [21]. For instance, the major percentage of CTX positive groupers was found in El Hierro followed by Lanzarote and the other islands. As it has been mentioned before, the cell densities were lower in El Hierro than in Lanzarote and Fuerteventura. In addition, the presence of the most toxic species, *G. excentricus,* is not confirmed in El Hierro. Therefore, these results of ciguateric fish should be compared with cell abundances by seasonality and include temporal series for different years. Additional other factors could contribute to toxin production and bioaccumulation in fish, as well as the mobility of fish. After all, it is still early to establish a list of the riskiest areas for CP, as the relation between microalgae and fish are unknown in the Canary Islands, and further research should be undertaken.

3.5 Evaluation of the presence of two series of CTX congener equivalents (CTX1B and CTX3C) in *G. belizeanus* with a colorimetric immunoassay and an electrochemical immunosensor

The role of the antibodies in the immunosensing tool is not to confirm the presence of individual CTXs congeners as in instrumental analysis techniques, but to screen the presence of compounds with wings structurally similar to those of the four CTX targets (CTX1B, 54-deoxyCTX1B, CTX3C and 51-hydroxyCTX3C). Therefore, and because of the sandwich format of the assay, the analysis is indicating the presence of compounds structurally related to two series of CTXs congeners, although no evidence can be obtained about which specific CTXs congeners are present. It is important to mention that the strategy will detect only the CTXs recognised by the antibodies (the four major CTX congeners previously mentioned), but it is also important to highlight that the antibodies have been demonstrated to not cross-react with brevetoxin A, brevetoxin B, okadaic acid and maitotoxins.

Analyses with the immunoassay and the immunosensor revealed the presence of the two series of CTXs congeners in the *G. belizeanus* extract. Immunoassay results when antibodies were used separately showed a higher concentration of 51-hydroxyCTX3C equiv. (0.28 ± 0.02 fg cell^{-1}) than of CTX1B equiv. (0.15 ± 0.03 fg cell^{-1}). As expected, the use of both antibodies simultaneously resulted in higher

toxin content (0.40 ± 0.02 fg of CTX1B equiv. cell^{-1}). Similar results were obtained with the electrochemical immunosensor. The use of 10C9 antibody resulted in a higher concentration (0.17 ± 0.08 fg 51-hydroxyCTX3C equiv. cell^{-1}) than the one obtained with 3G8 (0.13 ± 0.03 fg of CTX1B equiv. cell^{-1}). Again, the use of both antibodies together provided a higher toxin content (0.35 ± 0.04 fg CTX1B equiv. cell^{-1}). Both immunochemical tools providing similar CTX quantitations of CTX congeners in *G. belizeanus* extract. The use of these techniques not only revealed the presence of CTXs, but also allowed the discrimination between two series of CTX congeners (CTX1B and CTX3C). When using the mAbs separately, results showed slightly higher contents for the CTX3C series than for the CTX1B series. In fact, 51-hydroxyCTX3C equiv. contents were similar to those obtained in a previous study for *G. caribaeus* IRTA-SMM-17-03 (0.13-0.21 fg cell^{-1}), *G. australes* IRTA-SMM-17-286 (0.16-0.37 fg cell^{-1}), and *G. excentricus* VGO791 (0.16-0.31 fg cell^{-1}), both originating from the Canary Islands [68]. Regarding CTX1B equiv. contents, toxin contents in *G. belizeanus* were similar to those obtained for *G. caribaeus* IRTA-SMM-17-03 (0.13-0.24 fg cell^{-1}) and surprisingly, *G. excentricus* strains IRTA-SMM-17-01, IRTA-SMM-17-407 and IRTA-SMM-17-432 (0.09-0.19 fg cell^{-1}). It is important to note that CTX contents obtained with the immunochemical tools do not fully agree with those obtained with CBA. The reason is the different principle of recognition of both systems: whereas CBA is based on the toxic effect of a compound on the cell viability, the immunochemical tools are based on a structural recognition and affinity interaction between antibodies and their target molecules. Nevertheless, the analysis of microalgal extracts with different techniques provides complementary information and contributes towards improved characterization of the different *Gambierdiscus* species.

3.6. Future research strategies to understand CP in the Canary Islands

Currently, no fishery management strategies have been implemented in the Archipelago to prevent CP cases. However, during recent years, the official control of CTX-like toxicity evaluation of the harvested fish has been used as the first step to prevent CP cases [21,69]. The official control obliges all analysed fish to be stored and frozen until CTX-like toxicity results are available, influencing its commercial value.

In order to prevent and to identify the future trends of CP in the Canary Islands, knowledge about the microalgal communities should be considered. The link between dinoflagellates and CP remains uncertain, and the key vectors of the transfer of CTXs into fish and eventually shellfish are still unclear. It is essential to identify the principal involved species. One of the first big steps in the microalgal field is the unequivocal identification of the species and the estimation of their

abundance in the environmental samples. It is not clear whether only the genus *Gambierdiscus* can contribute to CP [22]. For example, *Coolia tropicalis* produces 44-methylgambierone (previously reported as MTX3) [70], which in human neuroblastoma cells (SH-SY5Y cell line) induces current sodium as the CTX3C but with lower potency [71], additionally, neither of these toxins induce cell death in human cortical neurons when they were exposed at 20 nM concentration [72] Even so, a recent study considers unlikely that 44-methylgambierone contributes to CP due to its low toxicity by mouse bioassay (MBA) [73]. It is necessary to clarify the toxin profiles of microalgae by instrumental analysis and analyse the toxicity of each compound to evaluate which species are low or high producers of CTX analogues. At present, there is a lack of knowledge of which compounds microalgae can produce and how these compounds interact with the food webs. Therefore, we need to identify these compounds in the microalgal, fish and invertebrate matrixes and establish the interactions. For that reason, there continues to exist big gaps in the detection of fish and microalgal toxins [74,75].

Another issue to be solved is related to the identification of *Gambierdiscus* species, which should be conducted to clarify the taxonomy and improve the molecular diagnoses. During the last years, there have been several attempts to identify *Gambierdiscus* species in environmental samples, and they have achieved good results [52,76,77], but they are not implemented in monitoring programs extensively.

The sampling method still requires standardization. Given that *Gambierdiscus* cells are found in a heterogeneous distribution, it is necessary to perform exhaustive geographical and temporal samplings using a standardized method. Artificial substrates have been used to sample benthic dinoflagellates such as *Gambierdiscus* spp., showing a reduction in the variability of densities of several samples collected at the same station [78]. However, when using artificial substrates, some ecological data are dismissed. For example, samplings in macroalgae substrates could aid in recognizing the potential preference by *Gambierdiscus* for particular macroalgae species or species assemblages. A priori, macroalgae are more visible and easier to monitor. Hence, understanding the population dynamics of the preferred macroalgae for *Gambierdiscus* could be relevant to explain the trend of the dinoflagellates. Additionally, identifying the grazers of these macroalgae could help to understand the accumulation through the food web. Nevertheless, it has to be taken into account that free-living cells could contribute to CP [3].

Additionally, to understand the future trend of populations in local areas, it is essential to identify critical factors for such regional populations. To this end, experimental design in field, such as environmental, ecological, and anthropogenic

activities that potentially can modulate the *Gambierdiscus* populations, should be considered. This field data should be combined with experimental/laboratory data. Other factors should be examined to understand, for instance, which variables affect the toxin content of cells.

4. Conclusions

The present study provides more data of *Gambierdiscus* species distribution and toxicity in the Canary Islands. The new report of *G. australes* in La Palma and the new finding of *G. belizeanus* in the Canary Islands (El Hierro) shows that data in the geographical and temporal scale is still scarce. Like the previous studies, the evaluation of the CTX-like toxicity shows that *G. excentricus* and *G. australes* are the species presenting the highest CP risk. These species are widely distributed in the Canary Islands. Further investigations are needed on the CTX-producing species, sampling methods, toxin profiles in microalgae and fish, relations between macroalgae, microalgae and fish, and accumulation of CTXs throughout the food webs. Until these issues are solved, it will be challenging to implement the best decisions to prevent CP at the local level of the Archipelago.

Supplementary Materials:

All authors have revised and approved the final version of the article.

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ii. Toxicity Characterisation of *Gambierdiscus* Species from the Canary Islands

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ABSTRACT

In the last decade, several outbreaks of ciguatera poisoning (CP) have been reported in the Canary Islands (central northeast Atlantic Ocean), confirming ciguatera as an emerging alimentary risk in this region. Five *Gambierdiscus* species, *G. australes, G. silvae, G. excentricus, G. carolinianus* and *G. caribaeus*, have been detected in macrophytes from this area and are known to produce the ciguatoxins (CTXs) that cause CP. A characterization of the toxicity of these species is the first step in identifying locations in the Canary Islands at risk of CP. Therefore, in this study the toxicity of 63 strains of these five *Gambierdiscus* species were analysed using the erythrocyte lysis assay to evaluate their maitotoxin (MTX) content. In addition, 20 of the strains were also analysed in a neuroblastoma assay (neuro-2a CBA) to determine their CTX-like toxicity. The results allowed the different species to be grouped according to their ratios of CTX-like and MTX-like toxicity. MTX-like toxicity was especially high in *G. excentricus* and *G. australes* but much lower in the other species and lowest in *G. silvae*. CTX-like toxicity was highest in *G. excentricus*, which produced the toxin in amounts ranging between 128.2 ± 25.68 and 510.6 ± 134.2 fg CTX1B equivalents (eq) cell^{-1} (mean ± SD). In the other species, CTX concentrations were as follows: *G. carolinianus* (100.84 ± 18.05 fg CTX1B eq. cell^{-1}), *G. australes* (31.1 ± 0.56 to 107.16 ± 21.88 fg CTX1B eq. cell^{-1}), *G. silvae* (12.19 ± 0.62 to 76.79 ± 4.97 fg CTX1B eq. cell^{-1}) and *G. caribaeus* (<LOD to 90.37 ± 15.89 fg CTX1B eq. cell^{-1}). Unlike the similar CTX-like toxicity

of *G. australes* and *G. silvae* strains from different locations, *G. excentricus* and *G. caribaeus* differed considerably according to the origin of the strain. These differences emphasise the importance of species identification to assess the regional risk of CP.

1. Introduction

Gambierdiscus is a genus of marine benthic dinoflagellates that produces maitotoxins (MTXs) and ciguatoxins (CTXs) [1,2,3]. These cyclic polyether neurotoxins are among the five most potent natural toxins isolated to date [4]. Because they accumulate in coral reef fish, they can be transferred through the marine food web [5,6,7,8]. In fact, CTXs are the main toxins responsible for ciguatera fish poisoning (CP) [9], a clinical syndrome caused by eating CTX-contaminated fish. The risks to human health related to the consumption of these toxins in fish have been assessed by the EU Food Safety Authority (EFSA) Panel on Contaminants [10]. CP is widespread in tropical and sub-tropical marine areas, including the Caribbean Sea, Indian Ocean, Polynesia and other areas of the Pacific Ocean [11]. However, over the past decade, an increase in the incidence of CP in areas where ciguatera is endemic [12,13,14] and several outbreaks of ciguatera in more temperate regions, such as Madeira [15,16,17] and the Canary Islands (NE Atlantic Ocean) [18], suggest an expansion of this disease is occurring.

The first reported CP outbreak in the Canary Archipelago was described in 2004. Five people were affected [19]. Two additional episodes happened in 2008 causing the intoxication of 11 people [20]. Since then, several CP episodes affected 113 people (Canary Government, 2017), [21,22]. Because of recurrent outbreaks, CP has been designated as a notifiable disease in the Canary Islands since 2015 (Canary Government, 2017).

The main fish species involved in CP in this area are amberjack (*Seriola spp*.), dusky grouper (*Epinephelus marginatus*) and Wahoo (*Acantocybium solandri*). The weight limits above which risks might occur are 14 kg, 17 kg and 35 kg respectively. Although CP occurs when toxic fish is ingested, CTXs are produced by microalgae belonging to *Gambierdiscus* genus. In the Canary Islands, ciguatera outbreaks have been related to *Gambierdiscus* species, including *G. australes*, *G. excentricus*, *G. silvae*, *G. carolinianus* and *G. caribaeus* [23], all of which have been isolated from macrophytes. An expansion of the *Gambierdiscus* distribution toward higher latitudes has been attributed to the increase in the ocean temperature caused by climate change.

CTXs are selective activators of voltage-dependent Na^+ channels in cells [24,25,26,27] whereas MTXs are water-soluble and alter the ion transport systems, causing an increase in free intracellular Ca^{2+} [28,29,30,31,32]. Although highly toxic [33], MTXs do not induce CP, because of their low oral potency and inability to accumulate in the muscle tissue of fish [7]. The extensive literature survey by Munday [34] found no published records of the oral toxicity of MTX and the only evidence of MTX accumulation in fish liver and viscera recorded before 2014 came from two studies published in the 1970s [35,36]. Whether MTX can cause CP via other routes remains to be investigated.

Numerous methodologies based on different approaches (e.g., toxicological symptoms, antibody recognition, mass spectrometry, etc.,) have been developed for the detection of CTX and MTX, but their use is often problematic (due among other things to, antibody cross-reactivity for CTX from different origins). In addition to better sampling procedures to allow an efficient extraction and concentration of the toxins, improved clean-up procedures to remove impurities that negatively impact sample analyses are needed. From an analytical perspective, the complexity of the sample matrix, the very low levels of toxin detected, and the lack of reference toxin material have greatly hampered the development of reliable methods for CTX and MTX determinations. Moreover, there are currently no chemical methods with the required sensitivity and specificity to rapidly monitor either of these toxins [37], and detection by LC-MS/MS [38] may lead to misidentifications. Consequently, to date, CTXs have been identified only in a few *Gambierdiscus* strains, i.e., those of *G. toxicus* [39], *G. polynesiensis* [2], *G. ruetzeri* [40], *G. pacificus* [3], *G. silvae* and *G. excentricus* [41]. In the absence of a rapid, cost-effective and reliable screening test for CTXs, health authorities around the world have relied on guidelines aimed at preventing high-risk fish species and fish life stages from entering the commercial market to reduce the risk of CP [42]. This strategy takes into account that CTXs can bioaccumulate over time, such that older and larger fish are more likely to be contaminated with higher levels of CTXs. However, few studies have directly examined the relationship between fish size and the presence of CTX in individual fish species [43]. A further problem is that there are no accredited methods for the analysis of CTXs and MTXs in fish samples.

A previous study showed that toxicity is more closely related to genetics rather than to environmental (temperature, ph and salinity) factors [44]. Among *Gambierdiscus* species in the Canary Islands, both *G. excentricus* [45,46] and *G. australes* [47] are known to produce high levels of CTX. However, information on other regional members of this genus is scarce. Therefore, in this study we characterised the relative toxicity of 63 *Gambierdiscus* strains of the

five *Gambierdiscus* species described in the Canary Islands thus far (Figure 1). An erythrocyte lysis assay (ELA) based on the method of Holland et al. [48] was performed to determine and compare MTX production by the tested strains. In addition, CTX production by the 20 most representative strains was assessed using the neuroblastoma cell-based assay (Neuro-2a assay). Compared to instrument-based methods, the ELA and the neuro-2a assay are more sensitive and do not require precise standards, since both assess the effect of the toxins on cells. Consequently, the assays have been widely used to detect MTX-like and CTX toxicities, respectively. The particular advantages of the ELA as a screening assay is that it requires relatively little starting material and is fast, highly reproducible and cost-effective.

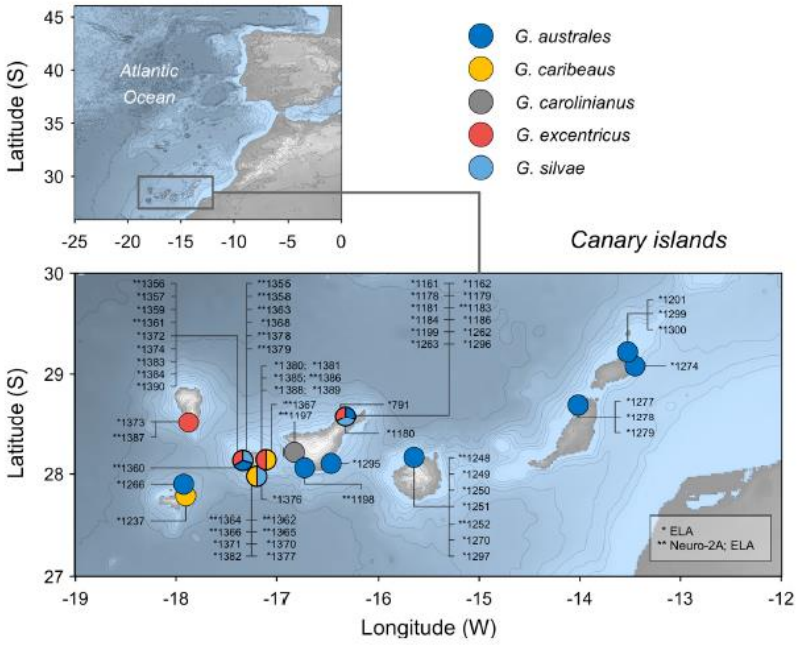

Figure 1. *Gambierdiscus* strains from Canary Islands examined in this study. * Analysed using the erythrocyte lysis assay (ELA); ** analysed using both the ELA and the neuro-2a cell assay.

2. Materials and Methods

2.1. Microalgal Cultures

Microalgal culture isolates of *Gambierdiscus* spp. (*n* = 67) used in the analysis belong to the Culture Collection of Harmful Microalgae of the Spanish Institute of Oceanography (CCVIEO). Sixty-three strains were sourced from seven different locations in the Canary Islands. However, since there was only one strain of *G. carolinianus* from the Canary Islands, four additional strains from Cuba were added to the analysis, VGO1397, CUB-9B5, CUB-9C6 and CUB-9C8, all of them were analysed by ELA and the VGO1397 strain was also analysed by neuro-2a.

The five species analysed were *G. australes* (*n* = 28), *G. excentricus* (*n* = 17), *G. caribaeus* (*n* = 10), *G. silvae* (*n* = 7) and *G. carolinianus* (*n* = 5). They were cultured in flasks containing different volumes of K/2 medium without silicates and prepared in seawater from the Ría de Vigo, with the salinity adjusted to 35. The cultures were incubated at 25 °C with a 12:12 L:D photoperiod.

2.2. Neuro-2a Cell-based assay

Gambierdiscus spp. cultures were cultivated until a biomass of at least 1.5×10^6 cells was reached. Toxins were extracted following the method proposed by Caillaud et al. [3], with modifications. Briefly, the cells were harvested by filtration onto 47-mm Whatmann filters and, under sonication (2 min by extraction, Footswitch 40), were extracted twice with equal volumes of methanol (5 mL for 1×10^6 cells) and twice with equal volumes of aqueous methanol (MeOH:H_2O 50:50) followed by centrifugation at 10,395 *g* for 10 min. The four supernatants were pooled and frozen at -20 °C until used. A volume of 1 mL of the pooled sample was filtered through 0.45 µm PTFE syringe filters and used for the neuro-2a assay.

CTX-like toxicity was evaluated following the neuroblastoma cell-based assay proposed by Caillaud et al. [49,58]. This assay measures cell viability of neuroblastoma cells (Neuro-2a) exposed to extracts when these extracts contain compounds that activate voltage gated sodium channels, that in the presence of ouabain and veratridine treatment cells die. Ouabain blocks the sodium and potassium ATPase pump inhibiting provoked efflux of sodium by compounds of the sample, and veratridine activates sodium compounds which increase and exacerbate the sodium levels.

Neuroblastoma murine cells (Neuro-2a) were obtained from the CIC cell bank of the University of Granada and maintained in RPMI-1640 medium (Gibco, Life Technologies, Carlsbad, CA, USA) supplemented with 5% foetal bovine serum

(Sigma-Aldrich, St. Louis, MO, USA), 1 mM sodium pyruvate, 2.5 mg L-glutamine mL^{-1}, 150 units penicillin mL^{-1} and 50 µg streptomycin mL^{-1} (Gibco Life Technologies, Carlsbad, CA), at 37 °C in a 5 % CO_2 humidified atmosphere, as previously described in Cañete and Diogène [59].

Prior to the experiments, neuro-2a cells were cultured in a 96-well microplate at 1.7 × 10^5 cells mL^{-1} and incubated for 24 h under the same conditions as described for cell maintenance. Thereafter, triplicate samples of the cells were exposed for 24 h to eight concentrations (½ serial dilutions) of CTX1B standard from the Pacific region (provided by R. Lewis [6]) or *Gambierdiscus* extracts (serial dilutions) with or without ouabain and veratridine (O/V: to a final concentration of 0.14 mM and 0.014 mM respectively). In order to calibrate the experiments, the mean and standard deviation of the IC_{50} were estimated. Assays were done in triplicate. CTX-like toxicity was determined by measuring the cell viability using the colorimetric [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium] MTT (Sigma-Aldrich) [60] assay described in Manger et al. (1993) [61]. Absorbance was read at 570 nm using an automated multi-well scanning spectrophotometer (Biotek, Synergy HT, Winooski, VT, USA) and the results were expressed as a percentage of viability compared to the corresponding control (with and without O/V). The cell viability data were analysed using the statistical and programming software R 2.1.12 (R Foundation for Statistical Computing, Vienna, Austria, 2013) [62]. A dose–response curve fit with a sigmoid regression curve (with variable slope) was established for each experiment to estimate the concentration of *Gambierdiscus* extracts or standards that inhibited cell viability by 20% (IC_{20}) and 80% (IC_{80}) for each experimental condition (with and without O/V). Viabilities close to 50% (IC_{50}) were further used as a toxicological parameter for qualitative and quantitative estimations of the content of CTX compounds produced, expressed as fg CTX1B equivalents (eq.) per cell. The limit of detection was defined as the concentration inhibiting viability by 20% (IC_{20}). Matrix effects of extracts were evaluated in wells lacking O/V. The CTX1B standard curve for this assay ranged from 0.2 to 25 pg mL^{-1}.

2.3. Erythrocyte Lysis Assay (ELA)

Gambierdiscus strains from the Canary Islands listed in Figure 1 and strains from Cuba detailed in 5.1 section were analysed by ELA. Aliquots of 3 mL and 10 mL were removed from each culture for cell counting and MTX extraction, respectively. The samples to be counted were fixed in Lugol solution and counted in a Sedgewick Rafter cell chamber. MTX was extracted by centrifuging the 10 mL aliquots at 2600 g for 10 min at 4 °C. The cell-free supernatants were carefully discarded and 1200 µL of ELA buffer was added to the pelleted cells. These suspensions were transferred to 2-mL conical microcentrifuge tubes fitted with screw caps (Thermo Scientific,

Waltham, MA, USA) and containing 0.5-mm glass beads (Soda Lime, Bio Spec Products, Inc., Bartlesville, OK, USA). Vigorous shaking of the samples for 20 s using a bead beater disrupted the cell membranes and released the cytoplasmic contents into the solution. Light microscopy observation was used to confirm that all cells were broken. The samples were maintained for 48 h at 4 °C until used in the assay.

Sheep blood in Alsever solution for use in the ELA was kindly provided by I. Manzano (CZ Veterinaria S.A., Porriño, Spain). Erythrocytes were separated from the plasma by centrifugation (400 g, 10 °C, 10 min), washed twice with ELA buffer and then diluted in ELA buffer (1:99) at a final concentration of ~1.7×10^7 red cells mL^{-1}. The ELA was conducted using Riobó's method [63], modified as described by Holland et al. [48].

Each assay was carried out in triplicate in a 96-microwell plate by mixing 150 mL of blood with 150 mL of extract diluted in ELA buffer. Haemolysis controls for 0 and 100% lysis were prepared by mixing 150 µL of erythrocyte suspension with 150 µL of ELA buffer or distilled water, respectively. The microplates were incubated for 24 h at 4 °C after which the whole plate was centrifuged for 10 min at 400 *g* at 4 °C. From each well, 180 µL of supernatant was transferred to a new plate and the absorbance at 405 nm was measured using a microplate reader (BioRad model 550, BioRad Laboratories, Hercules, CA, USA). Measurements from three replicates were averaged.

2.4. Statistical Analysis

An analysis of covariance (ANCOVA) was used to test the effect of Gambierdiscus species and cell abundance on the response variable (percent haemolysis). Post-hoc comparisons between Gambierdiscus species were carried out using Tukey's HSD. Normality, equal variance and the linearity assumptions of ANCOVA were previously checked.

All statistical analyses and graphic representations were performed using the statistical and programming software R 2.1.12 [62], packages 'multcomp' and 'marmap', available through the CRAN repository (www.r-project.org).

3. Results

3.1. Screening of CTX-Like Toxicity Using the Neuro-2a cell-based assay

As predicted, when neuroblastoma cells were exposed to CTX1B standard they did not exhibit toxicity signals (>80%) in O/V− conditions. Conversely when neuroblastoma cells were exposed to CTX1B in O/V+ treatment, the cell viability exhibited a typical sigmoid dose-response curve. The IC_{50} mean for the CTX1B standard was 2.33 ± 1.30 pg CTX1B mL^{-1}. The crude extracts (CE) of nineteen canaries strains and the Cuba strain VGO1397 were tested with the neuro-2a assay. CTX-like toxicity was attributed to microalgal extracts when the addition of a sample extract to neuroblastoma cells exposed to O/V+ treatment resulted in 20–80 % viability decrease. However, the addition of the same extract to neuroblastoma cells exposed to O/V− treatment did not affect the cell viability (>80 % viability). Moreover, enhanced mortality in the wells in O/V− conditions were observed (<80 % of viability) for some strains. This indicates the presence of non-specific toxic compounds other than sodium channel activators that apparently is according to the results of the ELA analysis. These non-specific effects are shown in Figure 2 showing the dose-response curve of 20 *Gambierdiscus* strains tested in the Neuro-2a cell-based assay.

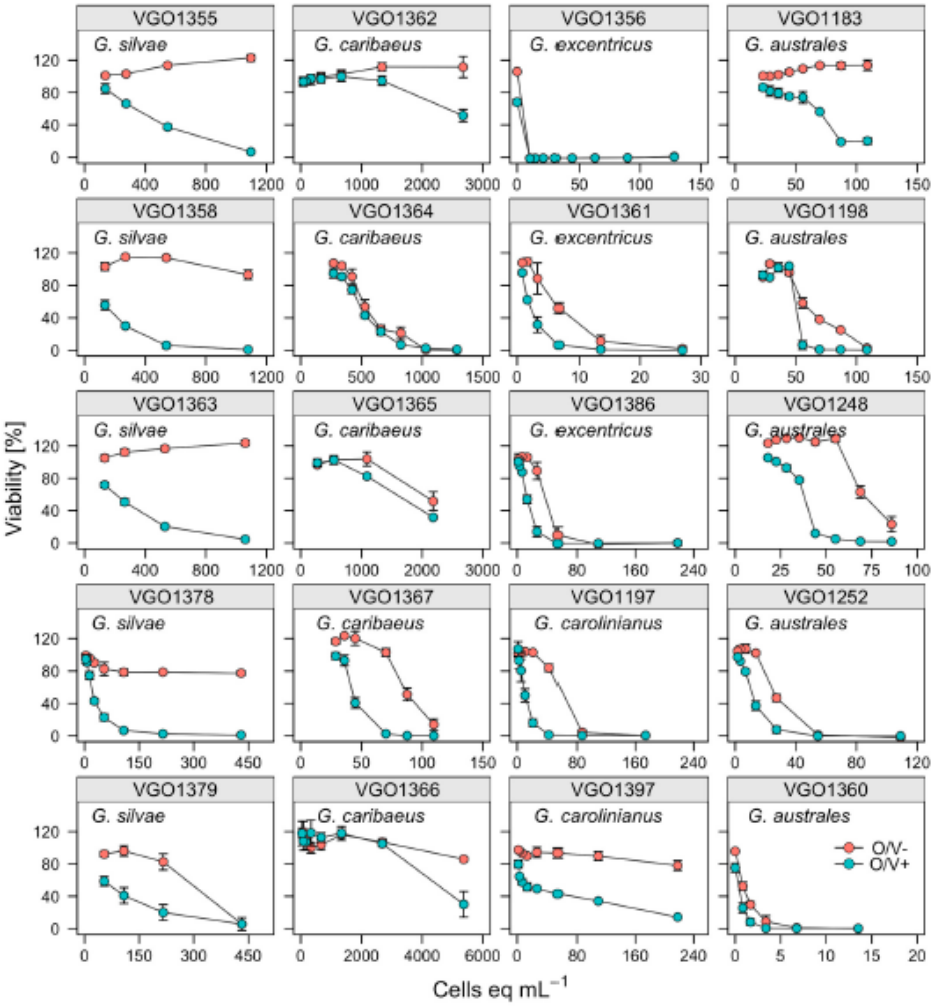

Figure 2. Dose–response curves of treated (O/V + green symbol) and non-treated (O/V−, red symbol) neuroblastoma cells 24 h after their exposure to extracts prepared from 20 *Gambierdiscus* strains.

Neuro-2a cells, exposed to *G. excentricus* extracts (VGO1356, VGO1361, VGO1386) at concentrations ranging between 10.5 and 54 microalgal cells eq. mL^{-1} with O/V−, showed a very low viability (<20%). The activity of *G. australes* extracts presented higher variability in toxicity responses as compared to *G. excentricus*. Four extracts (VGO1198, VGO1248, VGO1252, VGO1360), at concentrations ranging between 80 and 100 cells mL^{-1} in O/V−conditions, affected the cell viability highly. However, extract (VGO1183) at the same concentrations and conditions did not affect the cell

viability. This variability in O/V− conditions could be observed also in *G. caribaeus* and *G. silvae* extracts. For *G. caribaeus*, 3 out of 5 strains affected Neuro-2a viability at levels ranging from 70 to 1000 cells eq. mL^{-1} (VGO1364, VGO1365, VGO1367). For *G. silvae*, 1 out of 4 strains (VGO1379) affected Neuro-2a cell viability at approximately 500 cells eq. mL^{-1} (Figure 2). For the other strains of *G. caribaeus* (VGO1366) and *G. silvae* (VGO1358), no signals of toxicity under O/V− conditions appeared at 3000 cells eq. mL^{-1} and 1000 cells eq. mL^{-1}, respectively (Figure 2).

A quantitative estimation of CTX-like toxicity was possible in only 16 of the 20 sample analysed, despite the very low cell viabilities (<20 %) in the O/V− treatments exposed to VGO1198 and VGO1360 (*G. australes*), VGO1356 (*G. excentricus*) and VGO1397 (*G. carolininanus*), the cell viability plots did not result in a sigmoidal curve (Figure 2). The results for the other strains are expressed as fg CTX1B eq. $cell^{-1}$ (Figure 3). Among these strains, the highest levels of CTX-like toxicity (between 128.2 ± 25.68 and 510.6 ± 134.2 fg CTX1B eq. $cell^{-1}$) occurred in response to *G. excentricus* extracts, followed by VGO1197 *G. carolinianus* extracts (100 ± 18.05 fg CTX1B eq $cell^{-1}$) and *G. australes* and *G. silvae* extracts (from 31.1 ± 0.56 to 107.16 ± 21.88 and from 12.19 ± 0.62 to 76.79 ± 4.97 fg CTX1B eq. $cell^{-1}$, respectively) (Figure 3). The lowest toxicity occurred in response to extracts from the *G. caribaeus* strains (from below the detection limit (1.76) to 2.59 ± 0.5 fg CTX1B $cell^{-1}$ eq.) (Figure 3). However, *G. caribaeus* VGO1367, the only strain whose origin (La Gomera, San Sebastián-Playa la Cueva) differed from that of other strains of the same species (La Gomera Porto-Playa Santiago), had a high CTX-like toxicity (90.37 ± 15.89 fg CTX1B eq. $cell^{-1}$) (Figure 3).

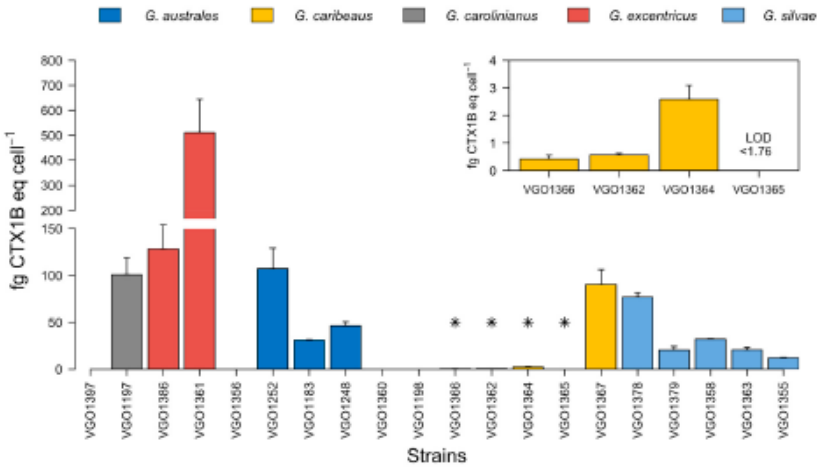

Figure 3. CTX-like toxicity expressed in femtograms of CTX1B equivalents per cells (fg CTX1B eq cell^{-1}) of *Gambierdiscus* strains as determined in a Neuro-2a cytotoxicity assay. Strains with the lowest concentrations are indicated by asterisks (*) and shown in the small bar plot in the inset. Strains VGO1397 (*G. carolininanus*), VGO1356 (*G. excentricus*), VGO1360 and VGO1198 (*G. australes*) could notbe quantified. Bars in the columns represent the standard deviation.

3.2. Toxicity Screening Using the Erythrocyte Lysis Assay

The relationship between cell abundance and haemolysis (%) for each strain of the five Gambierdiscus species (*G. australes*, *G. caribaeus*, *G. carolinianus*, *G. excentricus* and *G. silvae*) is shown in Figure 4A,B. Among the tested strains of *G. excentricus*, cell abundance correlated significantly with haemolysis ($p < 0.05$; $R = 0.67$, Figure 4B). The analysis of covariance (ANCOVA) showed the significant effects of cell abundance and Gambierdiscus species on the percent haemolysis (Table 1). There was no interaction between two explicative variables.

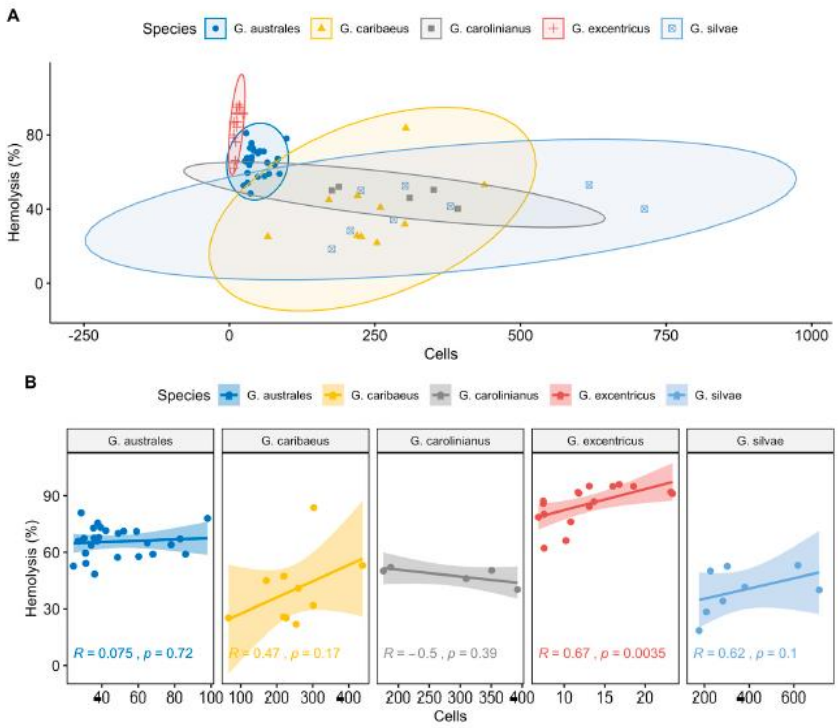

Figure 4. Scatter plot (**A**) and line regression (**B**) of the relationship between cell abundance and haemolysis (%) for each *Gambierdiscus* strain. Ellipses and shaded areas in panels A and B represent the 95% confidence intervals. Correlation coeffcients (R) and p-values are indicated in each line regression plot.

Table 1. Analysis of covariance (ANCOVA) results of the effect of *Gambierdiscus* species and cells on haemolysis (%). Significant differences are indicated with asterisks (*).

Data	Df	Sum Sq	Mean Sq	F value	*p*	
Cells	1	0.93	0.93	79.34	1.41×10^{-12}	***
Species	4	1.08	0.27	22.95	1.53×10^{-11}	***
Residuals	60	0.70	0.01			

Post-hoc Tukey's H comparisons of the percent haemolysis induced by the five *Gambierdiscus* species showed significant differences, with higher MTX like toxicities associated with *G. excentricus* and *G. australes* than with *G. caribaeus*, *G. carolinianus* and *G. silvae* (Table 2).

Table 2. Post-hoc Tukey's H comparisons of the haemolysis (%) induced by five species of *Gambierdiscus* (*G. australes*, *G. caribaeus*, *G. carolinianus*, *G. excentricus* and *G. silvae*). Significant differences are indicated with asterisks (*).

Species	Estimate	Std. error	t-value	*p*	
G. caribaeus-G. australes	−0.33	0.05	−6.2	<0.001	***
G. carolinianus-G. australes	−0.26	0.07	−3.9	0.0018	**
G. excentricus-G. australes	0.21	0.03	6.17	<0.001	***
G. silvae-G. australes	−0.37	0.07	−5.34	<0.001	***
G. carolinianus-G. caribaeus	0.06	0.06	1.08	0.80	
G. excentricus-G. caribaeus	0.54	0.06	9.18	<0.001	***
G. silvae-G. caribaeus	−0.04	0.06	−0.78	0.93	
G.excentricus -G. carolinianus	0.47	0.07	6.59	<0.001	***
G. silvae-G. carolinianus	−0.11	0.06	−1.70	0.42	
G. silvae-G. excentricus	−0.58	0.08	−7.69	<0.001	***

3. Discussion

The Canary Islands are a recent area of ciguatera endemicity [18]. Benthic dinoflagellates belonging to the *Gambierdiscus* genus, specifically those that could produce MTXs and CTXs, seem to be implicated in ciguatera outbreaks in the region and therefore the risk of seafood poisoning. Vulnerable fish species include *Seriola* spp. and *Epinephelus* spp.

Tools to assess the ciguatera risk in different areas based on the toxin detection should be fast and have a low detection limit. In addition, they should be able to detect the different toxins analogues and derivatives. Neuroblastoma cell-based assay accomplishes very well the last two requirements. Neuroblastoma cell-based assay can detect the effect of CTXs and MTXs analogues at low concentrations [49,50,51,52] and also other possible novel toxins with the same effect on sodium channels [53]. The drawback of this is that it cannot attribute the CTX-like or MXT-like effect to one specific molecule [52]. For this, instrumental techniques such as LC-MS/MS or LC-HRMS are required. In the present study, the cell-based assay was applied as a first screening tool to be used for the higher risk species through the comparison of the strains composite CTX-activity and MTX-activity.

Two approaches to evaluate the toxicity of the extracts in neuroblastoma cells were used in this study. The first one is neuroblastoma cell viability evaluation in absence of ouabain and veratridine. Under this approach, cell death may not indicate the presence of a specific type of compound. This is called non-specific toxicity. MTXs production has been described in *Gambierdiscus* species [46,54,55]. The non-specific toxicity in the absence of ouabain and veratridine observed when

testing *Gambierdiscus* extracts could indicate the presence of MTXs. In this case if CTXs or substances that provoke CTX-like activity were present, CTX-like toxicity could not be detected. The second approximation is the evaluation of the cell viability in the presence of ouabain and veratridine. In this case, the presence of substances affecting voltage gated sodium channels will result in cell death, therefore, CTXs or other analogues having a similar mechanism of action will enhance cell mortality. With regard to the CTX standard for these assays, we are aware that many CTXs and precursors of CTXs have been described (Atlantic, India and Pacific) still, our reference molecule in this case is CTX1B and it was used for this purpose in all the experiments.

Our ANCOVA analysis and post-hoc Tukey's H comparisons identified *G. excentricus* and *G. australes*, the two dominant species in the Canary Islands, [23], as producers of the highest levels of MTX like activity (Figure 4 and Table 1 and Table 2). The differences in the MTX-like response between these two species and *G. caribaeus, G. carolinianus* and *G. silvae* were significant ($p < 0.05$; Table 2).

High levels of MTX-like activity by *G. excentricus* strain VGO791 from Tenerife (Canary Islands) were reported by Fraga et al. [45] who estimate for this strain a value of 600 pg MTX eq cell^{-1}. However, according to Pisapia et al. [46], the value estimated for the same strain, determined by ELA, was much lower (80 pg MTX eq cell^{-1}). In addition to the different assay methods, the discrepancies in the results may have been due to the use of a crude extract [45] vs. aqueous methanol extracts after liquid-liquid partitioning [46]. In agreement with Fraga et al. [45], we measured very high levels of MTX - like activity by strain VGO791. The discrepancy with the results of Pisapia, et al. [46] suggest that liquid-liquid partitioning did not achieve a complete separation of MTXs and CTXs. Our evaluation of MTX-like response in 18 other strains of *G. excentricus* from the Canary Islands (Tenerife, La Palma and La Gomera) also showed that MTX-like activity was highest in this species, followed by *G. australes*.

High levels of MTX-like toxicity, similar to those found in *G. excentricus*, were also measured in 30 strains of *G. australes*, from Tenerife, Lanzarote, Gran Canaria, El Hierro, Fuerteventura and La Gomera (Figure 4). These results are in agreement with the MTX-like toxicity determined by ELA for a strain of *G. australes* isolated in Hawaii [48], but not for *G. australes* strains VGO1178, VGO1181 (both from Tenerife, Atlantic Ocean) and CCMP1653 (Hawaii, Pacific Ocean) Pisapia et al. [46]. For the latter three strains, MTX-like toxicity levels were low and the strains were less maitotoxic than the strains of the species *G. silvae, G. carolinianus* and *G. excentricus.* Strains VGO1178 and VGO1181 were also analysed in the present study

and their MTX-like toxicities were much higher than determined by Pisapia et al. [46]. However, the analyses were conducted more than 2 years apart, which together with other (e.g., environmental, methodological) factors may explain the disparity of the results.

MTX-like response was also detected in the other three species of *Gambierdiscus* evaluated in this work, *G. caribaeus, G. silvae* and *G. carolinianus*. The differences between these three *Gambierdicus* species were not significant ($p > 0.05$; Table 2).

Literature reports suggest that MTX is of low oral toxicity and does not accumulate in fish flesh, but this conclusion is yet to be substantiated. Thus, whether MTX is able to induce CP is unknown and need more investigation. A feeding study aimed at assessing MTX accumulation in the tissues of *Pagrus auratus* fed with *G. australes* showed the accumulation of MTX in the tissues of this carnivorous fish, including the viscera, liver and muscle [56]. Kohli et al. [56] reported that MTXs can accumulate in fish muscle. High MTX-type compounds levels were detected in microalgae from this study, especially in *G. excentricus* and *G. australes.* Therefore, a considerable risk of presence of these very dangerous toxins in the flesh fish of *Seriola* spp. and *Epinephelus* spp. in the Canary Islands exists. Investigations of whether MTXs contribute to ciguatera outbreaks must therefore begin with determinations of the potential presence of these toxins in fish and their remnants, if available.

Among the species analysed in the present work, CTX-like response levels were highest in two strains of *G. excentricus* from La Gomera (VGO1361 and VGO1386, Figure 3). These results are in accordance with those reported for this species by other authors. For example, Litaker et al. [44] analysed eight *Gambierdiscus* species using a Neuro-2a assay and found that the most toxic was *G. excentricus*, specifically, a strain from Florida (USA). Also, in the above-described study by Fraga et al. very high levels of CTX-like activity were measured in a strain from Tenerife (VGO791). The high CTX-like response of *G. excentricus* is comparable to that of *G. polynesiensis*, the predominant CTX producer in the South Pacific [2,54].

In contrast to the consistent toxicity characteristics of *G. excentricus*, analyses of the toxicity of *G. australes* have yielded variable results. In our study, of the five analysed species, MTX activity was highest in *G. australes* and *G. excentricus*, but in three strains of *G. australes* CTX levels were ~ten-fold lower than those of the assayed *G. excentricus* strains. Pisapia et al. [46] also found a low range of CTX-like toxicity in three strains of *G. australes* differing in their origin (VGO1178 and

VGO1181 from Canary Islands and CCMP1653 from Hawaii). Chinain et al. [2] reported comparatively low CTX-like toxicity for six *G. australes* strains originating from French Polynesia. Nishimura et al. [57] reported on a Japanese *G. australes* strain (S080911_1) with dichloromethane soluble fraction (DSF)—toxicity (Mousse bioassay, MBA) comparable to that of highly toxic *G. polynesiensis* species.

Only one strain of *G. caribaeus,* VGO1367, had a higher CTX-like toxicity than determined in other strains of the same species. This difference may reflect the different origin of the strains, as also observed in other species. For example, for two strains of *G. excentricus* from different sites (La Gomera Valle Rey-Ch. Condesa and La Gomera-San Sebastián), while high CTX-like toxicities characterised both, the respective values were markedly different. A comparison of those two strains with the published data of *G. excentricus* VGO791, from Tenerife Punta Hidalgo, also revealed considerable differences, as the latter strain has the highest toxicity detected thus far (1050 fg CTX1B cell^{-1}) [45]. For strains of *G. australes* and *G. silvae*, however, the CTX-like response level was not remarkably related to their origin. For *G. carolinianus,* only one strain from the Canary Islands (VGO1197) was available for our study, such that its origin-dependent variability could not be evaluated.

In summary, our findings clearly show differences in the toxicities of different microalgal species and in the distribution of *Gambierdiscus* species between islands of the Canary Islands [22]. Moreover, the differing toxicities of different strains of the same species highlight the need to assess intraspecific and geographic variations in the distribution of CTX and MTX toxicity. This information will facilitate determinations of the risk of ciguatera in the Canary Islands. Nonetheless, other factors must also be taken into account, including the behaviour patterns of the two main fish species linked to ciguatera outbreaks in the Canary Islands (*Seriola* spp. and *Epinephelus* spp.), the fishing effort, age of the fish and the depth of the fishing platform.

The next essential step to improve the knowledge about Ciguatera intoxication is undoubtedly the determination of toxin profiles in *Gambierdiscus* species, mainly *G. excentricus* and *G. australes.* It is also necessary to compare the toxin profiles of fish captured in the same area. This information would be very useful to relate the content of CTXs produced by different *Gambierdiscus* species with their presence in fish captured in a particular area.

4. Conclusions

In this work, CTX-like and MTX-like total toxicities were evaluated in a neuro-2a assay and an ELA, respectively. This approach confirmed previous results of the high-level risk of CP posed by strains of *G. excentricus*. Among the strains estimated quantitatively for CTX-like toxicity, the levels were highest in those of *G. excentricus* (ranging between 128.2 ± 25.68 and 510.6 ± 134.2 fg CTX1B eq cell^{-1}), followed by *G. carolinianus* (100 ± 18.05 fg CTX1B cell^{-1}).

The results of our study seem to demonstrate the possible presence of MTXs in *Gambierdiscus* species from the Canary Islands, mainly *G. australes*. A determination of the presence and concentration of MTXs in fish is needed in order to establish whether safety measures aimed at protecting human health should be implemented. Our characterisation of the toxicity *Gambierdiscus* strains is an important contribution to improving ciguatera risk assessments in the Canary Islands region.

Author Contributions

Conceptualization, A.E.R. and P.R.; formal analysis, A.E.R.; A.T. and P.R..; data curation, P.A.D.; writing—original draft preparation, A.E.R. and P.R..; writing—review and editing, A.T.; I.B.; P.A.D. and J.D.; funding acquisition, I.B. All authors have read and agreed to the published version of the manuscript.

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Conflicts of Interest

The authors declare no conflict of interest.

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3.3. CP HAZARD IDENTIFICATION IN THE MADEIRA ARCHIPELAGO

i. Evaluation of Ciguatoxins in fish from the Madeira Archipelago and identification of CTX analogues

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ABSTRACT

Ciguatera Poisoning (CP) is caused by consumption of fish contaminated with ciguatoxins (CTXs). CP is a typical disease in tropical areas, but presently it is a public concern in some temperate regions, such as Macaronesia (North Eastern Atlantic Ocean). To characterize the fish species that can accumulate CTXs, seventeen fish speciments comprising nine species were captured from coastal waters in the Madeira Archipelago including the Madeira, Desertas and Selvagens islands. Toxicity analysis was performed by screening CTX-like toxicity with the neuroblastoma cell-based assay. Afterwards, the presence of CTX anàlogues in the four most toxic samples was confirmed with liquid chromatography-high resolution mass spectrometry (LC-HRMS). Thirteen fish specimens presented CTX-like toxicity in their liver, but only four of these in their flesh. These CTX-like toxicities were higher than the guidance level of 0.01 µg of CTX1B equiv. kg^{-1}. The liver of one specimen of

Gymnothorax unicolor presented the highest CTX-like toxicity, estimated at 0.270 ± 0.121 µg of CTX1B equiv. kg^{-1} with the CBA. Moreover, CTXs analogues were confirmed with LC-HRMS, for *Muraena augusti* and *G.unicolor*. Confirmation of CTXs analogues by LC-HRMS was obtained in fish from the Selvagens Islands and for the first time in the Desertas Islands. The presence of three CTX analogues was confirmed: C-CTX1, which had been previously described in the area, dihydro-dihydro-CTX2, which is reported in the area for the first time and a putative new CTX *m/z* 1127.6023.

1. Introduction

Ciguatoxins (CTXs) are secondary metabolites produced by *Gambierdiscus* and *Fukuyoa* (dinoflagellates) of benthic microalgae [1]. CTXs are accumulated, transferred to upper levels in the food webs with concomitant biotransformations and may ultimately reach humans thereby causing Ciguatera Poisoning (CP) [1–3]. CP causes a variety of gastrointestinal, cardiovascular and neurological symptoms [4,5]. Fatal poisoning cases are rare [6] and it is very difficult to provide percentage of mortality due to the scarcity of data. The neurological symptoms can be prolonged during months or even years, severely affecting patients [7,8]. Although, the variability of symptoms and the difficult diagnosis hamper the estimation of CP cases though, it is suggested that between 25,000 to 500,000 people are affected each year worldwide [9–11].

CTXs are large lipid-soluble polyethers that activate voltage-gated sodium channels (VGSCs) of cells leading to an increase of intracellular sodium and neuronal excitability [12]. CTXs are tasteless, colourless, odourless and they cannot be eliminated by any food preservation or cooking technique [13,14]. Hence, prevention of CP relies on avoiding consumption of ciguateric seafood. More than 400 species of fish [15] and also a few shellfish species are related to CP cases [16]. Top predators and large fish seem to have the highest CTXs concentrations and are involved more often in CP outbreaks than the herbivorous fish [17–19].

Historically, CP was confined to circumtropical areas including the Caribbean Sea, Pacific Islands and regions in the Indian Ocean (35 °N, 35 °S) [20]. CP cases outside these areas resulted from consumption of imported fish or the return of sick travellers from endemic areas where they had been poisoned [21]. In the last decades, CP has expanded to temperate areas [16]. Among the suspected causes, climate change could have influenced the migratory pattern of fish and the distribution of the CTX-producing microalgae [22]. In Europe, an area with recent

cases of CP from local fish consumption is the Macaronesian region, which is a collection of different Archipelagos in the North Atlantic Ocean: Cabo Verde, Canary Islands, Madeira and Azores Archipelagos. Since 2004, several species of the genus *Gambierdiscu*s have been reported in this area [23–25] and their CTX-like toxicity and production of CTX analogues have been described. In fact, *Gambierdiscus excentricus* was found in the Canary Islands and Madeira Archipelago [23,26], and it is considered after *G. polynesiensis* as one of the species producing the hihgest amounts of CTXs.

The first CP case in the Macaronesian region was reported in 2004, in the Canary Islands (Spain) [27], and since then, numerous cases have been reported [28]. The Canary Islands government implemented an official control program based on the CTX-like toxicity of the classified ciguateric species with specific weights. In the Canary Islands, these fish are analysed for CTX-like toxicity with the neuroblastoma cell-based assay (CBA) [29]. This method has been widely used to detect and quantify the CTX-like toxicity of compounds that target VGSCs [30–33]. Presently, the detection limit of the CBA is below the safety level proposed by the Food Drug Administration (FDA) of the USA [34], which is also being contemplated by the European Food Safety Authority (EFSA). Contrarily, in the Madeira Archipelago (Portugal), an official control to test the CTX-like toxicity of fish has not been implemented, although two precautionary measures are established including fishing ban in Selvagens Islands at depth of 200m and ban on catching amberjacks with more than 10 kg. To date, in the Madeira Archipelago, only fish from the reserve of the Selvagens Islands has been involved in CP [35]. Additionally, the Selvagens Islands are the only location in the Madeira Archipelago where CTX analogues have been confirmed in fish [27,36]. CTX1B, CTX3C and a CTX analogue have been detected by ultraperformance liquid chromatography-mass spectrometry (UPLC-MS) [37] and C-CTX1 was detected by liquid chromatography with tandem mass spectrometry detection (LC-MS/MS) [36] and by high-resolution mass spectrometry (LC-HRMS) [38]. The Madeira Archipelago is not the most northern region in the Macaroneasian area where CTXs have been found in organisms since two starfish species (*Asterozoa*), from Azores Archipelago, which is located between 37°and 40° N latitude and 24° and 32° W longitude, presented CTX analogues by Ultra Performance Liquid Chromatography-Mass Spectrometry-Ion Trap-Time of Flight (UPLC-MS-IT-TOF) and UPLC-MS [39].

Detection of CTXs is difficult basically because of the several molecular congeners and a lack of certified CTXs standards and reference materials. At the moment, there are up to 30 analogues of CTXs. According to FAO (2020), thanks to advances in structural elucidation, they are grouped into four groups: the ciguatoxin 4A group

(including CTX4A and derivatives), the ciguatoxin 3C group (including CTX3C and derivatives), Caribbean ciguatoxin group (including C-CTX1 and derivatives) and Indian ciguatoxin group (including I-CTX1 and derivatives). When defining the CTX analogues present, the extraction procedure and the detection methodologies have to be carefully considered as there is no consensus method, probably due to the wide range of fish species, matrixes and CTX analogues [40]. In the present study, seventeen fish from nine species were obtained from the Madeira Archipelago and they were extracted with two different extraction methods. Further, the evaluation of potential CTX compounds was performed using two different approaches. First, a screening method using the neuro-2a CBA for CTX-like toxicity was applied. In a second step, the identification of CTX compounds in the most toxic samples was performed using LC-HRMS analysis.

2. Materials and methods

2.1. Reagents and equipment

CTX1B (also known as P-CTX-1), 52-epi-54-deoxy CTX1B (known as P-CTX-2 and CTX2) and 54-deoxy CTX1B (also known as P-CTX-3 and CTX3) standard solutions were provided by Pr. Richard J. Lewis (The Queensland University, Australia). CTX1B standard was used for the CBA and the LC-HRMS analysis. 52-epi-54-deoxy CTX1B and 54-deoxyCTX1B standards were used only for LC-HRMS analysis. Neuroblastoma murine cells (neuro-2a) were purchased from ATCC LGC standards (USA). Foetal bovine serum (FBS), RPMI medium, ouabain, veratridine, phosphate buffered saline (PBS), RPMI-1640 medium, thiazolyl blue tetrazolium bromide (MTT) were purchased from Merck KGaA (Germany). Dimethyl sulfoxide (DMSO) and absolute methanol were purchased from Honeywell (Spain) and Chemlab (Spain), respectively. Ultrasonic cell disrupter (Watt ultrasonic processor VCX750, USA) and the Syncore® Polyvap evaporator were purchased from Izasa Scientific (Spain) and Büchi Syncore (Switzerland), respectively. Automated plate spectrophotometer was purchased from Synergy HT, Biotek, (USA).

2.2 Fish samples

Seventeen specimens of nine species of fish were collected between October 2013 and December 2014 from several locations in the coastal waters of Madeira Archipelago, specifically in Desertas Islands and Selvagens Islands (Table 1). Fish were obtained in collaboration with the Parque Natural, Direção Regional das Pescas and local fishermen. For each individual, the following data were recorded: total length, and total fresh weight when caught (Table 1). After capture, individuals were frozen and shipped to IRTA's facilities.

Table 1. List of individuals captured between October 2013 and December 2014 in the coastal waters of Madeira and Selvagens Islands. Species determined by molecular genetics. FB: feeding behaviour (C: carnivorous, O: omnivorous, H: herbivorous); G: gender (F: female, M: male). no det: undetermined.

Code- common name	Date of capture	Species	Location	Weight (g)	Length (mm)	B	G.
1 - Comb grouper	25/09/2014	*Mycteroperca acutirostris*	Desertas Islands	2106	505	C	F
2 - Grey mullet	25/09/2014	*Chelon labrosus*	Desertas Islands	1972	572	O	M
6 - Barred Hogfish	24/05/2014	*Bodianus scrofa*	Selvagens Grande	2.432	500	C	M
13 - Bermuda sea chub	25/05/2014	*Kyphosus sectatrix*	Selvagens Grande	3431	570	O	F
14 - Grey triggerfish	25/05/2014	*Balistes capriscus*	Selvagens Grande	997	388	C	F
25 - Brown moray	31/12/2013	*Gymnothorax unicolor*	Selvagens Grande	1688	894	C	n.d
26 - Brown moray	31/12/2013	*G. unicolor*	Selvagens Grande	1210	838	C	n.d
27 - Brown moray	31/12/2013	*G. unicolor*	Selvagens Grande	1305	812	C	n.d
28 - Brown moray	31/12/2013	*G. unicolor*	Selvagens Grande	721	717	C	n.d
29 - Grey mullet	27/12/2013	*C. labrosus*	Desertas Islands	1896	545	O	M
31 - Parrotfish	27/12/2013	*Sparisoma* sp.	Desertas Islands	412	307	O	F
34 - Black moray	08/11/2013	*Muraena augusti*	Desertas Grande	1669	917	C	M
35 - Black moray	08/11/2013	*M. augusti*	Desertas Grande	1397	838	C	M
36 - Moray eel	08/11/2013	*Muraena helena*	Desertas Grande	1339	893	C	n.d
37 - Moray eel	*08/11/2013*	*M. helena*	Desertas Grande	1680	931	C	F
38 - Moray eel	08/11/2013	*M. helena*	Desertas Grande	3234	1070	C	M
39 - Moray eel	08/11/2013	*M. helena*	Desertas Grande	1193	856	C	M

2.3. Molecular identification of fish

For selected samples where species identification was not confirmed, a genetic analysis was performed using DNA sequences obtained by PCR of the cytochrome oxidase subunit I (COI) gene. Primers from previously published work by Kochzius et al. (2010) [41] were used for the amplification of a fragment of this gene. The

purified product was sent for bi-directional sequencing using the same primers as those used in the amplification. A BLAST analysis was performed and those samples for which an identity score of less than 95% was obtained a further phylogenetic analysis was performed by Maximum Likelihood using MEGA ver7 [42].

2.4 Obtention of crude extracts

Fishes were dissected and flesh and liver of seventeen specimens were obtained (10 ± 0.1 g). Fish tissues were kept at -20 °C until toxin extraction. In order to perform tissue extraction, the sample was heated at 70 °C for 10 min in a water bath. Then, an acetone homogenization (2 mL g^{-1} wet weight of tissue) was performed with an Ultraturrax blender (IKA, T25 Basic, Germany) for 3 min. The homogenate was centrifuged at 3,000 g for 15 min at room temperature, and the supernatant was recovered and filtered using a 0.2 µm PTFE filter (Whatman, Sigma-Aldrich). The homogenization process was repeated twice. The supernatants were pooled and evaporated in a Syncore® Polyvap evaporator (Büchi R-200, Flawil, Switzerland) at 40 ± 5 °C. After these step, two different extraction protocols followed.

Protocol-A: After the evaporation of the acetonic phase of all fish flesh (n=35) and all limpets acetonic extracts , the aqueous residues were further extracted according to protocol A, based on Lewis et al., 2003 [43]. The aqueous extract was dissolved in 5 mL methanol:water (9:1, v:v) and partitioned twice with 5 mL n-hexane. The hexane layers were removed and the aqueous methanol phase was evaporated with the Syncore® Polyvap evaporator at 40 ± 5 °C until dryness. After that, the dried extract was dissolved in 5 mL ethanol:water (1:3, v:v) and partitioned twice with 5 mL diethyl ether. Diethyl ether fractions were pooled, dried, and the resulting residue was suspended in 4 mL of methanol and kept at -20 °C until analysed with the neuro-2a CBA.

Protocol-B: An alternative extraction protocol was implemented following protocol B based on Yogi et al. (2011) [44] and modified by Dr. Jean Turquet [45]. After drying the acetonic phase, the aqueous residue was adjusted with Milli-Q water to 4 mL and diethyl ether was added, mixed by vortex, to initiate a water:diethyl ether partition (1:4, v:v) and kept at room temperature for 24h . Afterward, the diethyl ether phase was recovered and dried under N_2 gas (Turbovap, Zymark corp, Hopkinton, USA) at 40 °C. The dried extract was dissolved in methanol: water (2:8, v:v) and the extract was partitioned twice with n-hexane (1:2, v:v). The hexane layers were discarded, and the methanolic phase was dried with the evaporator at 60 ± 5 °C. Finally, the resulting residues were re-dissolved in 4 mL of HPLC-grade methanol and preserved at -20 °C until analysis with the neuro-2a CBA.

2.5. CTX-like toxicity evaluation by neuro-2a CBA

The evaluation of CTX-like toxicity was performed using the CBA following the procedure described in Caillaud et al., (2012) [31]. Briefly, cells (cell line CCL-131, ATCC LGC standards, USA) were seeded in 96 well-plates at 40,000 cells $well^{-1}$. After 24 h, ouabain and veratridine (O/V) were added to a final concentration at 140 µM and 14 µM respectively, and 10 µL of each sample (serial dilutions of extract or Standard, previously evaporated and resuspended in 5 % FBS RPMI medium) was added to each well in triplicate. CTX1B was used as a standard [46]. Then, neuro-2a cells with or without ouabain and veratridine (O/V+ or O/V-) were exposed to the resuspended serial dilutions of the extract or standard. Previously an evaluation of the maximum concentration of the flesh and liver extracts not causing matrix effects was conducted. Tested concentrations ranged between 0.2 to 12 pg mL^{-1} for CTX1B, 25 to 200 ng equivalents (equiv.) mL^{-1} of flesh and 6.25 to 50 ng equiv. mL^{-1} of liver. Controls consisted of cells exposed to 5 % FBS and not to extracts or standard in both conditions (O/V- and O/V+). All conditions were tested in triplicate. After 24 h of incubation, the cell viability was measured following the MTT colorimetric assay [33]. The CTX-like activity of the extracts was estimated according to the standard dose-response curve following Caillaud et al. (2012) [31] and was expressed as µg equivalents (equiv.) of CTX1B per Kg of tissue. The limit of quantitation (LOQ) of the method was defined as the amount of CTX1B that causes 20 % of cell viability inhibition (IC_{20}) in O/V+ and that can be expressed according to the maximum concentration of tested tissue [31].

2.6. LC-HRMS analysis

For four moray eels, the four most toxic liver extracts (eels with codes 25, 26, 27 and 35 from Table 1) and two fish flesh extracts (eels with codes 26 and 27 from table 1) determined by neuro-2a CBA, an analysis of CTXs was conducted with LC-HRMS. For the analysis of CTXs by LC-HRMS an Orbitrap-Exactive HCD (Thermo Fisher Scientific, Bremen, Germany) mass spectrometer was used equipped with a heated electrospray source (H-ESI II), a Surveyor MS Plus pump and an Accela Open AS auto-sampler kept isothermal at 15 °C (Thermo Fisher Scientific, San Jose, California).

The chromatographic separation was performed on a reversed-phase Hypersil Gold C_{18} (50 mm x 2.1 mm, 1.9 µm) (Thermo Fisher, Scientific, Bremen, Germany) at a flow rate of 250 µL min^{-1}. Mobile phase A was water and B was acetonitrile/water (95:5), both containing 2 mM ammonium formate and 0.1 % formic acid. The gradient elution program for the analysis was: 30 % B 1 min, 30-40 % B 2 min, 40-50 % B 1 min, 50-90 % B 5 min, 90 % B 3 min and return to initial conditions to re-

equilibrate (11 min 30 % B). A 20 µL injection volume was used. The total duration of the method was 25 min. The analyses were carried out in positive electrospray ionization (ESI+) mode as described in Diogène et al. 2017 [47]. The data was processed with Xcalibur 2.2 SP1 software (Thermo Fisher Scientific, Bremen, Germany).

The peaks were extracted from the chromatogram using the exact mass of $[M+H]^+$, $[M+NH_4]^+$ and $[M+Na]^+$ diagnostic ions, the mass accuracy (±10 ppm extraction window), and the retention time window. In addition to HRMS and accuracy parameters for identification, in the present study, to be confident of the identification and the proposed elemental formulae, the following restrictive criteria were applied: elements considered were restricted in accordance with CTXs molecular formulae and adduct signals [C: 55 to 70, H: 64 to 110, O: 11 to 25, N: 0 to 1, and cations (Na): 0 to 1]; the isotopic pattern was matched to theoretical in silico approach and the charge, the ring double bond equivalents (RDBEs) and nitrogen rule were taken into account. Additionally, the mono-isotopic pattern (M+1 ion) of these signals was used to assist in the further confirmation of the toxin's identity as a supplementary identification point. Therefore, in total four diagnostic signals were used for toxin identification. The relative ion intensities between the main signal ($[M+H]^+$, $[M+NH_4]^+$ or $[M+Na]^+$) and their M+1 ions were calculated and matched taking into account a tolerance of 30 % according to the EU Comission SANCO/12571/2013 guidance document [59]. The characteristic isotopic pattern, M+1/M ion ratio, is a robust criterion that for all described CTXs is a theoretical value of 0.6-0.7. The combination of high resolution, mass accuracy and restrictive criteria was crucial for the identification of both targeted and unknown compounds.

3. Results

3.1. Identification of fish by COI sequencing

The approach of identification of fish species using COI sequencing followed the protocol indicated in Kochzius and colleagues [41]. The premise for this approach is that the existing databases should provide reference material for comparison of unknowns. The results of the samples analyzed in this study were not in conflict with the morphological identification. However, for the two species of moray eel, *M. augusti* and *G. unicolor* there was no reference sequence for COI for these two species found in GenBank data. Phylogenetic analyses (data not shown) place these

in their respective genera, but as long branches separate from existing species. The COI sequence data obtained is very likely the first obtained for these two species.

3.2 CTX-like toxicity evaluation by neuro-2a CBA.

None of the fish flesh samples extracted using protocol-A (based on Lewis et al., 2003) [43] exhibit CTX-like toxicity with the CBA. The median of LOQ (± standard deviation, SD) of fish flesh for protocol-A was 0.019 ± 0.014 µg CTX1B equiv. kg^{-1}. After these negative results for CTX-like toxicity, the flesh and liver of seventeen fish were extracted using protocol-B (based on Yogi et al., 2011) [44] and their CTX-like toxicity was evaluated again with the CBA. Table 2 shows the CTX-like toxicity of fish specimens extracted with protocol-B obtained with the CBA. The median of LOQ (± standard deviation, SD) of fish flesh for protocol-B was 0.025 ± 0.038 µg CTX1B equiv. kg^{-1}. Thirteen liver samples and four flesh samples of fish presented CTX-like activity above the LOQ. CTX-like toxicity in liver samples ranged from 0.0096-0.270 µg CTX1B equiv. kg^{-1} and for flesh samples the range was 0.039-0.083 µg CTX1B equiv. kg^{-1}. The specimens that presented CTX-like toxicity in flesh were four moray eels of 3 species, *M. helena, M. augusti* and *G. unicolor*. The most toxic flesh was from a *M. helena* specimen (code 39), with 0.083 ± 0.014 µg CTX1B equiv. kg^{-1}. For those fish presenting toxicity in flesh, their corresponding liver exhibited CTX-like toxicity as well. The CTX-like toxicity was higher in the liver than in the flesh for all fishes. The other fishes with signals of CTX-like toxicity in liver but not in flesh, were: five moray eels (*M. helena, M. augusti* and *G. unicolor*), one comb grouper (*M. fusca*), one barred hogfish (*B. scrofa*), one bermuda sea chub (*K. sectatrix*) and one triggerfish (*B. capriscus*). The highest CTX-like toxicity was estimated for one liver of a moray eel of the species *G. unicolor* (code 35) with 0.270 ± 0.121 µg CTX1B equiv. kg^{-1}. Considering all positive individuals, the linear relationship was assessed between CTX-like toxicity and weight, but the potential correlation was negative with a weak coefficient of correlation (R^2=0.18).

Table 2. Quantitations of CTX-like toxicity using the neuro-2a CBA of samples (flesh and livers) extracted with protocol B. Quantitations and LOQ are expressed as µg CTX1B equiv. Kg^{-1} ± SD.

				FLESH		LIVER	
Code	Species	Location	Weight (kg)	CTX-like toxicity (µg CTX1B equiv. kg^{-1})	LOQ (µg CTX1B equiv. kg^{-1})	CTX-like toxicity (µg CTX1B equiv. kg^{-1})	LOQ (µg CTX1B equiv. kg^{-1})
35*	*M. augusti*	Desertas Islands	1.40	< LOQ	0.013	0.270 ± 0.121	0.044
27*	*G. unicolor*	Selvagens Islands	1.31	0.034 ± 0.006	0.029	0.216 ± 0.051	0.011
25*	*G. unicolor*	Selvagens Islands	1.69	< LOQ	0.005	0.212 ± 0.125	0.041
26*	*G. unicolor*	Selvagens Islands	1.21	0.039 ± 0.001	0.002	0.187 ± 0.023	0.082
39	*M. helena*	Desertas Islands	1.19	0.083 ± 0.014	0.026	0.158 ± 0.022	0.044
34	*M. augusti*	Desertas Islands	1.67	0.065	0.013	0.067 ± 0.022	0.044
14	*B. capriscus*	Selvagens Islands	1.00	< LOQ	0.010	0.060 ± 0.000	0.030
38	*M. helena*	Desertas Islands	3.23	< LOQ	0.013	0.050 ± 0.008	0.044
28	*G. unicolor*	Selvagens Islands	0.72	< LOQ	0.153	0.034 ± 0.000	0.004
6	*B. scrofa*	Selvagens Islands	2.43	< LOQ	0.016	0.015 ± 0.001	0.002
1	*M. fusca*	Desertas Islands	2.11	< LOQ	0.007	0.014	0.008
13	*K. sectatrix*	Selvagens Islands	3.43	< LOQ	0.015	0.011 ± 0.008	0.003
37	*M. helena*	Desertas Islands	1.68	< LOQ	0.03	0.010 ± 0.002	0.004
2	*C. labrosus*	Desertas Islands	1.97	< LOQ	0.020	< LOQ	0.008
29	*C. labrosus*	Desertas Islands	1.90	-	-	< LOQ	0.047
31	*Sparisoma* sp.	Desertas Islands	0.41	-	-	< LOQ	0.178
36	*M. helena*	Desertas Islands	1.34	-	-	< LOQ	0.088

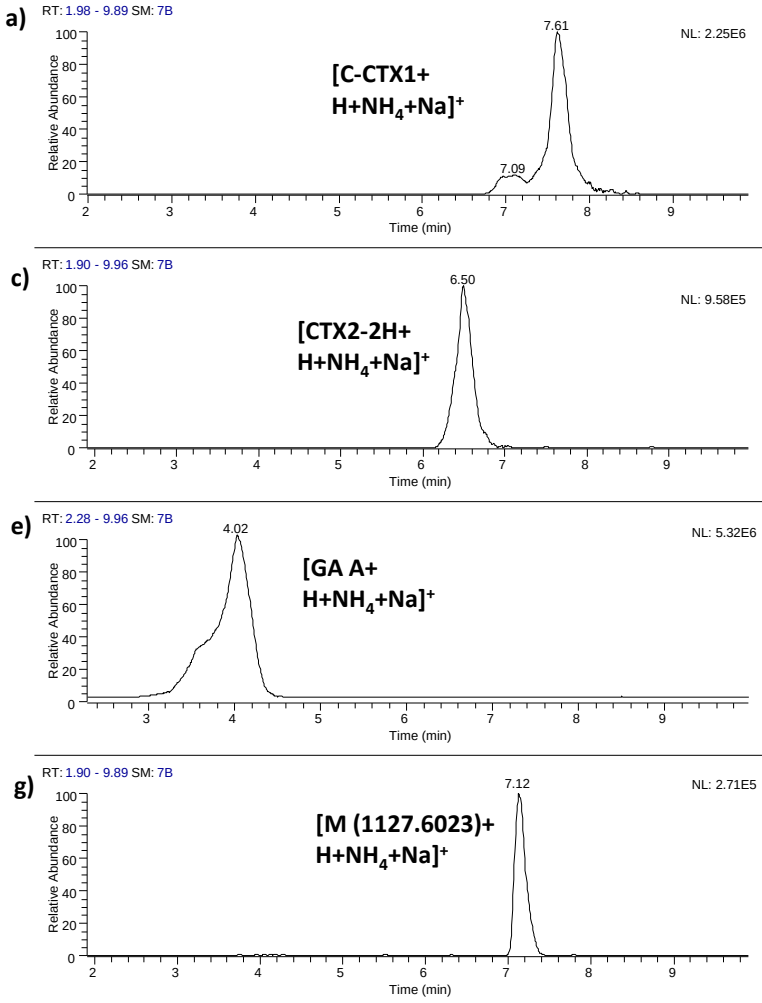
*: liver (25, 26, 27 and 35) and flesh (26, 27) of these individuals were also analysed by LC-HRMS.

3.3. Identification of CTXs by LC-HRMS analysis

After the CBA screening analyses, the extracts of the four most toxic livers corresponding to three *G. unicolor* (codes 25, 26, 27) and one *M. augusti* (code 35) and the corresponding fish flesh samples of fish 26 and 27 were analyzed by LC-HRMS. The spectra of CTXs were dominated by $[M+H]^+$, $[M+NH_4]^+$ and $[M+Na]^+$, which were used for confirmation purposes.

All CTX analogues were confirmed using their measured *m/z*, mass accuracy (Δppm), ring double bond equivalents (RDBEs) and the mono-isotopic pattern (M+1 ion) of the main adduct signal. All results are summarized in Tables S1-S4.

The analogue C-CTX1 ($C_{62}H_{92}O_{19}$) was identified in all four liver samples at a retention time of 7.59±0.05 min by LC-HRMS (Figure 1a and 1b). The identification of this CTX analogue was confirmed detecting *m/z* 1141.6305 $[C_{62}H_{92}O_{19}+H]^+$. The mass accuracy of $[M+H]^+$ matched ppm < 3.9, RDBEs was in all cases 16.5 and the monoisotopic pattern (M+1 ion) ratio of the main signal was between 0.55-0.69 (See Table S2).


a)
RT: 1.98 - 9.89 SM: 7B
NL: 2.25E6
[C-CTX1+ H+NH4+Na]+
7.61
7.09
c)
RT: 1.90 - 9.96 SM: 7B
NL: 9.58E5
[CTX2-2H+ H+NH4+Na]+
6.50
e)
RT: 2.28 - 9.96 SM: 7B
NL: 5.32E6
[GA A+ H+NH4+Na]+
4.02
g)
RT: 1.90 - 9.89 SM: 7B
NL: 2.71E5
[M (1127.6023)+ H+NH4+Na]+
7.12
Relative Abundance
Time (min)

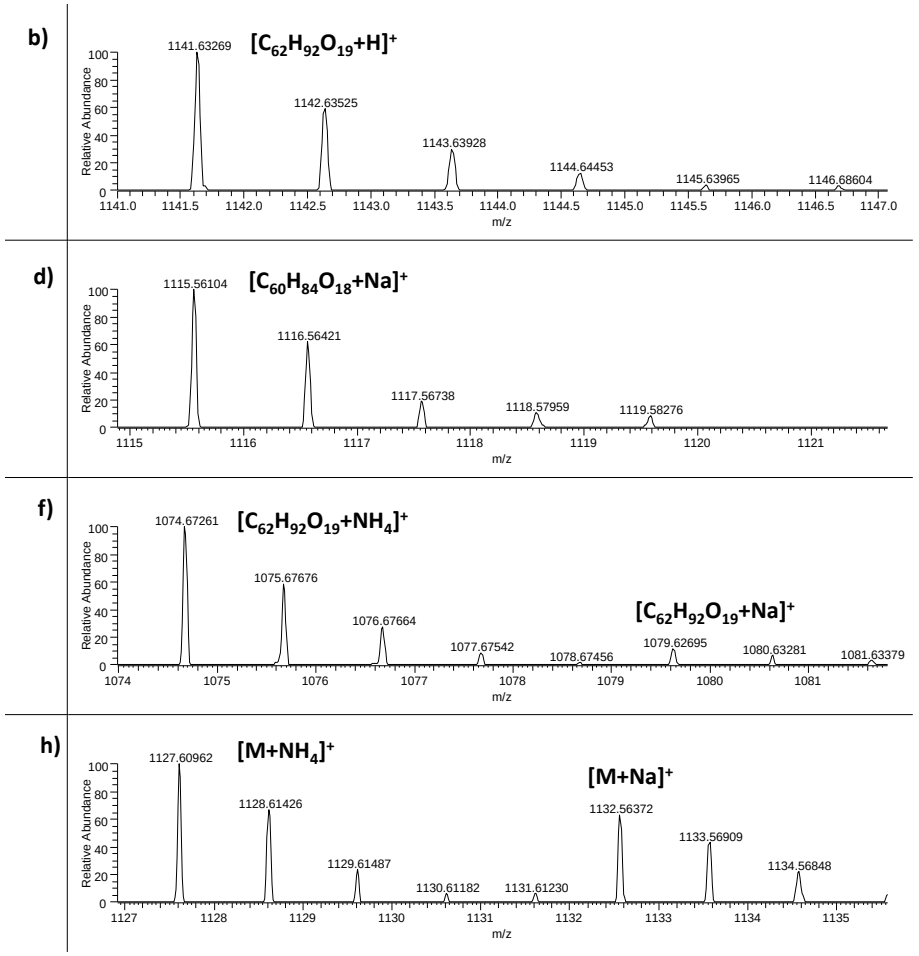


Figure 1. Evidence for the presence of ciguatoxins (CTXs) a) Extracted ion chromatogram of C-CTX1 $[M+H+NH_4+Na]^+$ in liver of fish 26 and (b) HRMS exact mass spectra of CTX1 $[M+H]^+$ at 1141.6305; (c) Extracted ion chromatogram of CTX2-2H $[M+H+NH_4+Na]^+$ in liver of fish 35 and (d) HRMS exact mass spectra of CTX2-2H $[M+Na]^+$ at 1115.5611; and (e) Extracted ion chromatogram of gambieric acid A $[M+NH_4]^+$ in liver of fish 35 and (f) HRMS exact mass spectra of gambieric acid A $[M+NH_4]^+$ at 1074.6724. (g) Extracted ion chromatogram of putative analogue CTX with *m/z* $[M+H+NH_4+Na]^+$ in liver of fish 25 (h) HRMS exact mass spectra of a putative analogue CTX with *m/z* $[M+NH4]^+$ at 1127.6023.

A putative CTX analogue corresponding to CTX2-2H ($C_{60}H_{84}O_{18}$) was identified in four samples (flesh and liver of fish 26, flesh of 27 and liver of 35) at a retention time of 6.62±0.08 min by LC-HRMS (Figure 1c and 1d). To our knowledge, this signal has not been described in any previous study. The identification of this CTX analogue was confirmed detecting *m/z* 1115.5611 $[C_{60}H_{84}O_{18}Na]^+$. The mass accuracy of $[M+Na]^+$ matched ppm < ± 5.5, RDBEs was in all cases 18.5 and the monoisotopic pattern (M+1 ion) ratio of the main signal was between 0.58-0.65 (See table S3).

Gambieric acid A ($C_{59}H_{92}O_{16}$) was identified in two samples (liver of fish 35 and flesh of fish 27) at a retention time of 3.93 ± 0.10 min by LC-HRMS (Figure 1e and 1f). The identification of gambieric acid A was confirmed detecting m/z 1057.6458 $[C_{59}H_{92}O_{16}H]^+$, 1074.6724 $[C_{59}H_{92}O_{16}NH_4]^+$ and 1079.4341 $[C_{59}H_{92}O_{16}Na]^+$. The mass accuracy of $[M+H]^+$ matched ppm < +3.5, RDBEs was 13.5 and the monoisotopic pattern (M+1 ion) ratio of the main signal was 0.59 (See table S3). Additionally, the mass accuracy of $[M+NH4]^+$ and $[M+Na]^+$ matched < +2.4 ppm and < +4.67 ppm, respectively.

A putative new CTX analogue, with a later retention time than CTX-1B on the C_{18}-column, with a *m/z* 1127.6023 $[M+NH_4]^+$ and 1132.5577 $[M+Na]^+$ was identified in all samples (four liver and two flesh) at 7.06±0.05 min by LC-HRMS (Figure 3g and 3h). The isotopic patterns of both signals are identical to the characteristic isotopic pattern of CTXs (M+1/M = 0.55-0.7). The experimental M+1/M ratio was between 0.55-0.66 (see Table S5). Unfortunately, it was not be possible to identify a CTX structural formula for this compound according to theoretical HRMS criteria, unsuitable RDBE data were found, and the nitrogen rule did not fit for the molecular formula proposed by the Xcalibur software.

4. Discussion

Genetic analyses of fish species are a useful tool for establishing records and subsequently implement risk assessment for fish consumption of fish. Many factors can mislead a morphological identification such as the state of conservation of the sample, whether the sample consists on a whole fish or merely a filet, the existence of sexual dimorphism between genders, the ontogenetic differences between juveniles versus adults, and environmental factors affecting size and/or coloration. Molecular analysis can identify species even from partially digested stomach contents. However, the utility of this method relies on a well-annotated database for comparison that includes region-specific haplotypes. In the case of teleost fish species many intensely studied species that are of commercial interest have full

genomes in international databases like EMBL and GenBank. For many wild species, collection of COI sequences is still incomplete. Such seems to be the case for the genera *Gymnothorax* and *Muraena*. This may be true generally for many benthic and deep water fish species and more broadly for all non-commercially exploited species that are not so easily brought to land for study. Most of the species identified morphologically were confirmed using phylogenetic analyses with the exception of *Gymnothorax unicolor* and *Muraena augusti*. Although the long monophyletic branches for each of these sequences obtained suggest that morphological identification is correct, these are the first sequences for COI obtained for these species.

Given the several CP cases described in Madeira, the negative results of CTX-like toxicity with the neuro-2a CBA for all extracted samples using the protocol-A demonstrated that this protocol may be misleading. Flesh and liver of the seventeen fish specimens were extracted for toxin analysis using protocol-B and the CTX-like toxicity of these fish was re-evaluated using the CBA. In contrast to the results using protocol-A, four flesh samples extracted using protocol-B showed CTX-like toxicity. This result suggests that protocol-B is more appropriate to detect CTXs in fish flesh and liver. Previously, several studies had used the extraction protocol-A [31,48] and the results were CTX-like positive. Nevertheless, according to the results of the present study, CTX-like toxicity detection can be enhanced using protocol-B. Additionally, the thirteen positive liver samples compared to the only four positive flesh samples show that the liver is probably a better tissue to reveal the presence of CTXs in fish.

In this study, two liver samples were five times higher than in flesh. This is in accordance with the results in Chan et al. (2011), where CTX concentration in the liver were nine times higher than levels in flesh. Despite this, in the literature, most articles evaluate CTXs only in flesh [49- 52]. This approach may justified by the fact flesh is the most consumed tissue, in some species and regions, the viscera are also consumed [18]. However, the evaluation of liver, in addition to flesh may improve risk assessment studies and may be important as a warning approach in areas where CP may be increasing in intensity and is not yet affecting public health. Besides, evaluating only the flesh would not be the best approach to describe the key ciguateric species in the food webs, and to understand which species may transfer CTXs.

In the present work, the specimens which presented CTX-like toxicity were comb grouper (*M. acutirostris*), bermuda sea chub (*K. sectatrix*), barred hogfish (*B. scrofa*), grey triggerfish (*B. capriscus*) and five moray eels corresponding to 3 species (*M. helena*, *M. augusti* and *G. unicolor*). Even though, CTXs have already been

detected in almost all these species from the Atlantic Ocean [38,53], this study is the first report of CTX-like toxicity for *K. sectatrix* (Fam. *Kyphosidae*) from Macaronesia. Kyphosus species are related to CTXs in the South Pacific and Indic Ocean [55,56]. They are omnivorous and play a very important role as macroalgal consumers in temperate reefs [56,57]. Two flesh samples of K. sectatrix from the Selvagens Islands were analysed previously in Costa et al. (2018) [36] by instrumental methods and no CTX-analogues were detected in this species. In addition, in the current paper, the CTX-like toxicity is analysed for the first time for *C. labrosus* (grey mullet) and *Sparisoma* sp. (parrotfish) from the Madeira Archipelago. In the Pacific and Caribbean Sea, *Chelon* genus is considered ciguateric species [54] and the *Sparisoma* sp. has been involved several times in CP cases [57,58] . In the current study samples of these species did not exhibit CTX-like toxicity.

As previously mentioned, the most toxic extracts for liver and flesh were those of moray eels (Table 2). This is in accordance with the characteristics of moray eels, which are benthic and large carnivorous species. Considering that moray eels have a sedentary behaviour, presence of CTXs in moray eels is a good indicator of CP risk in the area. In the literature, moray eels are the classical species which accumulate high quantities of CTXs. In fact, moray eels have been related to numerous CP cases [18]. It has been mentioned that big fishes contain higher quantities of CTXs than small fishes [34,53]. Quantitations of CTX-like toxicity in liver of the moray eels of the present study were comparable to levels in flesh of the moray eels from the Canary Islands [53]. CTX-like toxicity and weight data of the current study did not show a positive correlation, and the CTX-like toxicity considering all moray eels of the present study are not positively related to their weight. Nonetheless, when the species *G. unicolor* was plotted against their weight, a positive linear correlation was obtained. (R^2=0.89). The genus *Gymnothorax* has already shown a positive correlation between CTX-like toxicity and weight for exemplars from the Pacific Ocean [34].

The European Regulations ban placing products containing CTXs in the market (Commission Regulation (EU) No. 2019/624), but, presently CTX1B equiv. kg^{-1} there are no official methods to detect CTXs neither a legal limit of CTXs in tissues. However, there is a guideline for maximum levels of 0.01 µg of CTX1B equiv. Kg^{-1} and 0.1 µg of C-CTX1 equiv. kg^{-1} in tissue by the United States Food and Drug Administration (US FDA) [60] and the concentration of 0.01 ug equiv. CTX1B. kg^{-1} of fish recommended by EFSA to cover all CTX-group toxins that could be present in fish. In the present study, the estimated quantitations of CTX in the samples positive

for CTX-like toxicity were equal or higher than the established level of 0.01 µg of CTX1B equiv. kg^{-1}.

In most of the assays of the current study, the detection levels below the recommended level of 0.01 µg of CTX1B equiv. Kg^{-1} was not achieved. After the experimental work of the present study, efforts have been made to avoid the interference from residue of the biological matrices which cannot normally be eliminated from extracts. For instance, in Castro et al. (2020) [61] diminished the effects of the biological matrices by incorporating additional cleaning steps.

The CBA provides a composite toxicity expressed in CTX1B equiv. kg^{-1} and does not provide information related to the CTXs present. In this study, six fish specimens from the Desertas Islands showed CTX-like toxicity. This is relevant because the Desertas Islands are close to Madeira, approx. 26 km to the south-east. To the best of our knowledge, all the ciguateric fishes reported from the Madeira Archipelago were found in the Selvagens Islands, which are 260 km south-east far from Madeira. In Reis Costa et al., 2018, none of the nine fish flesh samples (M1-M9) from Madeira Island were positive by LC-MS/MS and, on the other hand CTXs were found in five out of eleven fish samples from the Selvagens Islands.

In this study, the presence of two CTX analogues (C-CTX1 (*m/z* 1141.6305 $[C_{62}H_{92}O_{19}+H]^+$, CTX2-2H (*m/z* 1115.5611 $[C_{60}H_{84}O_{18}+Na]^+$), and a putative new CTX analogue with *m/z* 1127.6023 $[M+NH_4]^+$) were confirmed in flesh and liver extracts in three moray eels (*G. unicolor*) (codes 25, 26 and 27) from the Selvagens Islands by LC-HRMS. These three CTX compounds and gambieric acid-A were also confirmed in the liver of one *M. augusti* (code 35) from the Desertas Islands by LC-HRMS. C-CTX1 has detected before in samples from the Atlantic Ocean by Perez Arellano et al (2005) [27], Reis et al. (2018) [36], Sanchez-Henao et al. (2020) [52] and Estevez et al. (2020) [38]. CTX2 has been found several times in fish and in moray eels (*Lycodontis javanicus, Muraenidae)* from Tarawa in the Republic of Kiribati (central Pacific Ocean) [46,62], albeit it is the forst time that the putative CTX analogue corresponding to CTX2-2H ($C_{60}H_{84}O_{18}$) has been detected. It is necessary to be aware that C-CTX1 and CTX2 have not been detected in microalgae, it is suggested that C-CTX1 and CTX2 are CTX analogues are resulting from fish metabolism. Although, C-CTX1, CTX2 and CTX3 jointly with CTX1B share the same mechanism of action [52,63], the potency among C-CTX1 and the Pacific congeners is suggested to be diferent. C-CTX1 is 10-fold less toxic than CTX-1B by mouse bioassay (MBA) [64].

Gambieric acid A was found at first time from *Gambierdiscus toxicus* from the Gambier Islands in the Pacific Ocean [65]. Like CTXs, gambieric acids bind to the VGSC [66]. However, gambieric acid-A has not been related with CP, since at a dose

of 1 mg kg^{-1} was not toxic in mice via intraperitoneal injection [67]. A gambieric acid, gambieric acid D, was found in fish for the first time by our group, specially in a shark involved in a fatal food poisoning in the Indian Ocean [45]. This is the second time a gambieric acid has been detected in fish.

A putative CTX analogue corresponding to m/z 1127.6023 $[M+NH_4]^+$ and *m/z* 1132.5577 $[M+Na]^+$ was also detected in all liver and flesh samples. This signal could be the one identify in the S. fasciata from the Selvagens Islands by Estevez et al. 2019 identified as C-CTX-1127. It presents a larger retention time than CTX-1B on the C_{18}-column and a similar isotopic pattern in comparison to CTX-1B main signals m/z 1128.6102 $[M+NH_4]^+$ and 1133.5656 $[M+Na]^+$ and an experimental M+1/M ration corresponding to a CTX analogue. However, it was not possible to identify a CTX structural formula for this compound. It is believed that these CTX analogues was the result of fish metabolism.

In accordance with the literature of Otero et al. 2010, Reis Costa et al. 2018, Estevez et al. 2019, Estevez et al. 2019 and 2020 [36,37,39,68], we confirmed the Caribbean ciguatoxin-1 (C-CTX1) to be responsible for ciguatera from the Selvagens and Desertas Islands. We observed similar CTX profile in comparison to previous studies. Otero et al. 2010 [37] described the presence of C-CTX1, CTX-3C and CTX1B and a CTX-analogue *m/z* 1040.6 in both fishes (*S. dumerili and S. fasciata*) from the Selvagens Islands. Reis Costa et al. 2018 [36] confirmed and quantified C-CTX1 in four different species from the Selvagens Islands. (*E. marginatus*, *M. fusca*, *B. scrofa* and *B. carpiscus*). Estevez et al. 2019 [39] described the presence of C-CTX1, C-CTX-1157 and C-CTX-1127 in a *S. fasciata* from the Selvagens Islands. None of the previous studies found gambieric acid A and CTX2-2H in the profile of fishes from the Selvagens Islands.

Table 3 summarizes information found in the literature on CTX-positive fish samples from the Madeira Archipelago, jointly with the results of the current study. In Madeira, the first CP episodes were related to *Seriola* spp. [37] and CTX analogues were confirmed by UPLC-MS in two individuals, *Seriola dumerili* (A1) *and Seriola fasciata* (A2) from the Selvagens Islands, involved in CP outbreaks (Table 3). It is important to note that results on CTX-like toxicity of Otero et al. 2010 [37] cannot be compared with the results of the current study since the estimations were performed using cerebellar granule cells (CGN) which is another functional assay based on cell electrophysiology, and also a CTX3C standard was used (Table 3). Afterwards, Caillaud and collaborators (2012) [31] detected in two individuals of *S. fasciata* (SER2, SER3) from the Selvagem Islands CTX-like toxicity (Table 3). Their neuroblastoma assay was comparable to the assay of the current study. CTX-like toxicity quantitations of *Seriola* spp. from Caillaud et al. (2012) [31] were much

higher over 100-fold higher than the most toxic fish of the present study. It is important to note that toxin contents, can vary depending on the fish species and the geographical location, year and season of capture.

Table 3. Positive samples from the literature and this study originating from the Madeira Archipelago, inlcuding Madeira (M), the Selvagens (SI) and Desertas Islands (D).

Code- Species	**Weight (kg)**	**Location (year)**	**Tissue**	**µg kg^{-1} by functional method (CBA)**	**µg kg^{-1} by LC-MS/MS methods**	**Ref.**
A1 - *Seriola dumerili*	70	SI (2009)	tail flesh	37.3	53.76	[37]
			head flesh	40.6	54.35	
			ventral flesh	45.1	33.29	
			mid flesh	41.7	53.37	
			liver	37.7	48.60	
A2 - *Seriola fasciata*	30	SI (2009)	Tail flesh	40.6	35.29	[37]
SER2 - *Seriola fasciata*	63	SI (2012)	Flesh	6.23 ± 0.713		[31]
SER3 - *Seriola fasciata*	37	SI (2012)	Flesh	4.59 ± 0.988		[31]
S1 - *Epinephelus marginatus*	19.5	SI (2016)	Flesh		0.05	[36]
S2 - *Bodianus scrofa*	2.4	SI (2016)	Flesh		< LOQ (0.0150)	[36]
S3 - *Balisters capriscus*	2.2	SI (2016)	Flesh		< LOQ (0.0150)	[36]
S4 - *Mycteroperca fusca*	4.6	SI (2016)	Flesh		0.25	[36]
S5 - *Serranus atricauda*	0.8	SI (2016)	Flesh		< LOQ (0.0150)	[36]
S6 - *B. scrofa*	1.6	SI (2017)	Flesh		0.11	[36]

S7 - *B. scrofa*	0.8	SI (2017)	Flesh			0.06	[36]
S8 - *B. capriscus*	2.0	SI (2017)	Flesh			0.03	[36]
S9 - *K. sectatrix*	2.3	SI (2017)	Flesh			< LOD (0.0045)	[36]
S10 - *K. sectatrix*	0.5	SI (2017)	Flesh			< LOD (0.0045)	[36]
S11 - *Sphyraena viridensis*	1.6	SI (2017)	Flesh			< LOD (0.0045)	[36]
S12 - *Pagrus pagrus*	4.0	SI (2016)	Flesh			0.76	[68]
S13 - *Seriola fasciata*	37.0	SI (2008)	Flesh			1.4	[68]
B. scrofa		SI (2018)	Flesh			C-CTX1 identified	[38]
1 - *M. fusca*	21	DI (2014)	Liver		0.014 (LOQ 0.008)		This study
6 - *B. scrofa*	2.4	SI (2014)	Liver		0.015 (LOQ 0.002)		This study
13 - *K. sectatrix*	3.4	SI (2014)	Liver		0.011 (LOQ 0.003)		This study
14 - *B. capriscus*	0.99	SI (2014)	Liver		0.060 (LOQ 0.030)		This study
25 - *G. unicolor*	16.9	SI (2013)	Liver		0.212 (LOQ 0.041)		This study
26 - *G. unicolor*	12.1	SI (2013)	Flesh	Liver	0.039 (LOQ 0.002) 0.187 LOQ 0.082		This study
27 - *G. unicolor*	13	S I (2013)	Flesh Liver		0.039 (LOQ 0.002) 0.216 (LOQ 0.011)		This study
28 - *G. unicolor*	0.72	SI (2013)	Liver		0.034 (LOQ 0.004)		This study
34 - *M. augusti*	16.7	DI (2013)	Flesh Liver		0.065 (LOQ 0.013) 0.067 (LOQ 0.044)		This study
37 - *M. helena*	16.8	DI (2013)	Liver		0.010 (LOQ 0.044)		This study
38 - *M. helena*	32.3	DI (2013)	Liver		0.050 (LOQ 0.044)		This study
39 - *M. helena*	11.9	DI (2013)	Flesh Liver		0.083 (LOQ 0.026) 0.158 (LOQ 0.044)		This study

5. Conclusions

The extraction protocol is crucial to extract toxins from fish matrixes. In the fish used in this study form Madeira, the protocol-B described by Yogi et al. (2011) [44] seems to be more suitable to recovery CTX-like compounds than the protocol-A based on Lewis et al. (2003) [43]. As observed in previous studies, the combination of the neuro-2a as a screening assay for CTX-like toxicity followed by a confirmation with LC-HRMS can be a good method for CTX evaluation. The HRMS full scan methodology has proved to be imperative for the reliable detection of putative CTXs, which are not monitored by targeting strategies such as MS/MS. Thirteen out of seventeen fish specimens from the Madeira Archipelago presented CTX-like toxicities, higher than the guidance level of 0.01 µg of CTX1B equiv. kg^{-1}. Although the liver is a tissue that is not often consumed, it is an interesting organ to conduct risk assessment studies and to evaluate the presence of CTX-like compounds in fish, since levels of CTXs are higher than in flesh, according to our study. The detection of CTX-compounds in the liver can contribute as an early warning strategy to better describe in the transfer and biotransformation of CTXs in the food webs in a specific area. Among the ciguateric fish identified by neuro-2a CBA, six specimens were from the Desertas Islands and seven were from the Selvagens Islands. The highest toxicity was found in a liver of *M. augusti* specimen (fish 35) from Desertas Islands. The presence of three CTX analogues (C-CTX1, CTX2-2H and a putative new CTX *m/z* 1127.6023) and gambieric acid A was confirmed by LC-HRMS in some of the analysed samples in this study, for example in the liver of fish 35, *M. augusti* from Desertas Islands. Therefore, the Desertas Islands constitute a new area where ciguateric fish could be harvested and associated with CP cases.

Liquid chromatography coupled to high-resolution mass spectrometry (LC-HRMS) is an emerging and very attractive approach that combines qualitative and quantitative analyses, minimizing the matrix effect, and also reducing the inaccuracies (false positives and negatives). The combination of high resolution, Accurate Mass measurement and restrictive criteria was crucial for identification of both targeted and unknown CTX-compounds. Further work should be performed to obtain the structural characterization of this putative CTX-compounds.

Author contributions:

J.D (Jorge Diogène). and M.C (Mònica Campàs). designed the study. N.G (Neide Gouveia)., C.S (Carolina Santos)., A.M (Antonio Marques)., N.S. (Núria Sagristà) and P.A (Paloma Aguayo). contributed to obtaining and transferring the samples. K.A.

(Karl B. Andree) and A.T. (Àngels Tudó) contributed to genetic analyses. N.S. and P.A. performed the sample preparation. N.S. and A.T. performed the cell-based assays. M.R-A. (Maria Rambla-Alegre) and C.F. (Cintia Flores) performed LC-HRMS interpretation of CTXs in the extracts. J.D., M.R-A., A.T., C.F., M.C., A.M., K.A. and J.C. (Josep Caixach) discussed the results and participated in the writing.

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CHAPTER 4

GENERAL DISCUSSION AND PERSPECTIVES

4. GENERAL DISCUSION AND PERSPECTIVES

Overview

As mentioned above, CP is an emerging risk in Europe. The present thesis has contributed to assessing the risk of ciguatera in Europe in the Balearic Islands (North Western Mediterranean), the Canary Islands and the Madeira Archipelago (Macaronesia, North East Atlantic Sea). These areas are in the spotlight, given that there have been cases of CP, or CTX-producing species have been reported during the last two decades.

This study provides fundamental information about the distribution of potential CTX-producing species and the toxin production of the genera *Gambierdiscus* and *Fukuyoa* in the Balearic and Canary Islands. It includes new toxicological data for *Gambierdiscus* and *Fukuyoa* strains from these areas and identifies new CP risk assessment challenges using microalgae and toxin detection. The study has also examined potential CTXs in fish from the Balearic Islands, Selvagens, Desertas and the Madeira Archipelago, which contribute to the detection of new ciguateric species in the Madeira archipelago and to the description of new potential CTX analogues. In general, these findings underline the need for the implication of those responsible for formulating public health policies and for suitable monitoring of this foodborne illness.

The methods that have been used in this thesis are based on the one hand, on the detection of toxicity in cells (cell-based assays), such as the neuroblastoma cell-based assay (neuro-2a) and the erythrocyte lysis assay (ELA), and on the other, on the detection of the chemical species responsible for the toxicity using immunoassays or liquid mass chromatography-mas spectrometry (LC-MS). As has been discussed in the introduction, CBAs do not confirm the presence of specific molecules. The neuro-2a CBA detects molecules that activate voltage-gated sodium channels (VGSCs), thus providing an overall toxicological response. Nevertheless, it cannot be discounted that it detects compounds other than CTXs that increase intracellular sodium. ELA has been used to detect hemolytic substances in microalgae, which in *Gambierdiscus* and *Fukuyoa* genera are MTXs. Like CBAs, ELAs are highly sensitive [111,86] and provide useful information about the overall toxicity of the sample, which is of the utmost importance to prevent CP from the consumption of seafood. Immunological methods are more specific and sensitive than CBAs [160] but, at present, they can only recognize two types of CTX [159]. For confirmatory purposes, analytical methods such as LC-MS can be used, although the relevant standards must be available. This thesis stresses that toxin profiles of

microalgae or seafood need to be studied with several complementary methods if the risk of CP is to be appropriately assessed.

Below, the key findings and implications of this study are discussed in greater detail.

4.1 Risk hazard evaluation in the Balearic Islands

Presence of *Gambierdiscus* and *Fukuyoa* species

As discussed in the introduction, it has been suggested that harmful benthic microalgae are expanding, among other factors, because of climate change [23,24,70]. Growth rates of *Gambierdiscus* and *Fukuyoa* cells are expected to increase and lead to higher population densities in temperate regions [70,161,162], which may increase the amounts of CTX entering the food webs. Nonetheless, there is little evidence of toxin production in the genera *Gambierdiscus* and *Fukuyoa*. CTXs have only been confirmed for few species [68,101,163], although there are indications that they are produced in the *Gambierdiscus* and *Fukuyoa* genera. For instance, CTX-like toxicity has been found for other species (not only *G. polynesiensis* and *G. excentricus*) using MBA [101,95], CBA [134,135,136], and CTX-like compounds have been detected using RBA [158], immunoassays [160] and LC-MS/MS [49,101].

Nonetheless, the presence of *Gambierdiscus* and *Fukuyoa* species in the areas studied does not necessarily mean that CP will occur. The amounts and profiles of the toxins in food webs can be complex and are determined by numerous factors, including the cell abundance of CTX-producing species, the toxin cell content and the transfer rates, bioaccumulation and metabolic pathways of CTXs in seafood. These factors may vary temporally and spatially. The absence of CP when CTX-producing species were present in the area could be explained by the low production of toxins or their low potency. Besides, toxins transferred or bioaccumulated can be lost at some point in the food webs (e.g. contaminated seafood may not be consumed by predators or toxins may be excreted or detoxified). Moreover, toxins may be transferred and bioaccumulated, but the toxin levels in seafood may not be enough to cause CP or the contaminated fish may simply not be consumed by humans. If they were, the toxin levels and toxicity could be enough to cause poisoning. In the case that concerns us here, in temperate regions of Europe, CP may go underreported due to the lack of historical events (especially in non-endemic areas) or easily be confused with other poisonings.

One example of a region where the *Gambierdiscus* and *Fukuyoa* genera are present but there are no confirmed cases CP is the Red Sea, where *G. belizeanus* and *F.* yasumotoi were reported in 2017 [85], respectively. Another example is the

Mediterranean Sea. Nonetheless, there are several reports of possible cases CP in the Mediterranean Sea, although they have never been confirmed (this is discussed in article ii, chap. 3.1). The Mediterranean is one of the seas that is the most vulnerable to climate change because of its small size [164]. The increase in the sea surface temperature (SST) ranges from 0.24 °C decade^{-1} in the west of the Strait of Gibraltar to 0.51 °C decade^{-1} in the Black Sea [165]. Furthermore, the magnitude and frequency of extreme events are expected to increase throughout Mediterranean Sea [166]. These factors could favour benthic microalgal populations. Other disturbances such as habitat degradation and coral bleaching have also been associated with the increasing and spread in Gambierdiscus populations [167,168]. These disturbances are clearly observed in the Mediterranean Sea [169,170]. Consequently, populations in the Mediterranean could spread and therefore, so could the potential risk of CP.

At the start of this thesis, the presence of the species *Gambierdiscus* and *Fukuyoa* had already been detected in the eastern Mediterranean [84,171], and *F. paulensis* had been described in the western part (the Balearic Islands) [153]. Articles i and ii in chapter 3.1 study which *Fukuyoa* and *Gambierdiscus* species live in the Balearic Archipelago. The present study sheds light on which *Fukuyoa* and *Gambierdiscus* species live in the Balearic Islands and reports the presence of the *Gambierdiscus* species in the western Mediterranean (WM) for the first time. A combination of phylogenetic analysis and morphological data made it possible to identify *G. australes* in samples taken in 2017. This finding was reported in our first article. *F. paulensis* was also found. As a consequence of these findings in the Balearic Islands, additional samplings were taken in the following years (2018-2020), which revealed that both species coexisted in Majorca, Minorca and Formentera, and that they have been present for several years, from 2016 to 2020. This raises concerns about whether there may be CP cases in the future.

Even though *G. australes* is considered to be the most cosmopolitan species within the genus [70], our finding is remarkable because the *Gambierdiscus* genus had never before found at such a high latitude. Previously, although *G. australes* had typically been found in tropical or subtropical zones, but it has also been found in temperate areas: Hawaii, Cook and Gambier Islands, New Zealand [70,172,173], the Sea of China [81], the Sea of Japan [93], the Selvagens Archipelago [174] and the Canary Islands [21]. Besides, recently *G. australes* has been found in the eastern Mediterranean Sea (personal communication of Prof. K. Aligizaki [84]). *F. paulensis* seems to be more confined than *G. australes*, although it has been found in the south west Atlantic Ocean [39] and some Pacific islands [75] (previously reported as *G. yasumotoi*). It is to be expected that *G. australes* and *F. paulensis* will be found in

places at the same latitude and with similar environmental conditions as the Balearic Islands. The environmental data of new locations could be used to predict the current spatial distribution and potential spread of the *Gambierdiscus* and *Fukuyoa* species under future climate scenarios. Besides, the present geographical location can be used to evaluate future translocation to other places by currents or for anthropogenic reasons (e.g. ballast waters).

The new reports of the of the presence of *Gambierdiscus* and *Fukuyoa* species in the Mediterranean and the lack of confirmatory CP cases poses the question of whether *Gambierdiscus* and *Fukuyoa* species are non-native in the Mediterranean Sea. Keeping a registry of non-autochthonous marine species in a particular area is relatively straightforward. This includes fish, crustaceans, molluscs and some macroalgae. By contrast, for microalgae, their small size makes them less apparent. In addition, traditional monitoring of *Gambierdiscus* and *Fukuyoa* species had been under light microscopy (LM). Moreover, the identification of these species by morphological parameters is challenging, and it is not always feasible even using an electronic microscope [39,175]. Therefore, information on non-native microalgal presence often is scarce.

To avoid subjectivity in the identification of microalgae, molecular methods have been developed to identify cells at the species level. Some of them need high DNA concentrations, and consequently, cells have to be isolated and cultivated. During the isolation and culturing process, cells must live and grow under lab conditions and sometimes, this cannot be assured. Then, microalgal biodiversity of the sample may be underestimated. At present, to address this problem, new molecular methods have been developed: one-single cell isolated from the field-collected samples [176], or the semi-quantitative real-time polymerase chain reaction, which can evaluate the diversity and abundance of cells directly from the collected samples [177]. However, the identification of species from unaltered field-collected samples is not straightforward.

It is crucial to bear in mind that almost all samplings in this thesis were performed during September-October, and cell abundance could vary among seasons. Thus, we could not discard the presence of more *Gambierdiscus* or *Fukuyoa* species with low cell abundance in those months. In future works, samplings should be performed in other seasons, and abundances should be contemplated.

The closest place to the Balearic Islands with autochthonous CP is Macaronesia, where several species of *Gambierdiscus* live. Previous studies and the present thesis (chapter 3.2) show that the most frequent species in the Canary Islands are *G. excentricus* and *G. australes*. This raises the question of whether *G. australes*

could have entered the Mediterranean from the Atlantic Ocean by natural processes, currents or ballast waters. In this thesis, these issues have not been addressed, and the molecular markers used are not designed to detect genetic differentiations that could distinguish the source of populations or routes of colonization. In fact, very few studies show marine microalgal population relationships [178,179]. Further research should be done to understand the relationship between populations in the Canary and Balearic Islands. Nevertheless, if *G. australes* or *F. paulensis* are relatively new residents in the Mediterranean Sea, it is only to be expected that more species will enter the Mediterranean and adapt to the conditions they find there. Therefore*, G. excentricus* and other species could colonize the Mediterranean Sea.

Toxin detection in microalgae

Evaluating CP risk is more complicated than evaluating the presence of a certain microalgal species. The same species may have different genotypes, there are low and high CTX-producers [163,174] and toxin profiles can vary with the environmental conditions. At present, it is not possible to the evaluate the toxin content in cells directly from field-collected samples. However, it is possible to use passive systems to detect microalgal toxins in the water column [180,181]. With this approach in the field, there is no need to cultivate the microalgae. Nonetheless, one of the significant problems is that, in most cases, cells release toxins at very low concentrations and the LOD of methods cannot be achieved [180]. Another drawback is that field-collected samples contain other microorganisms and compounds that can interfere with the toxin analysis. In the present thesis to avoid these problems, we isolated single *Gambierdiscus* and *Fukuyoa* cells from field samples and evaluated the toxicity of the strains using monoclonal cultures, which prevent the presence of other species. Likewise, the higher biomass means that toxins can be examined by several methods.

Previous works used analytical methods to find putative CTXs in *G. australes* [49] and *F. paulensis* [71]. However, both species are not considered as high toxin producers [134,136]. Further, previous toxicological studies in mice and neuro-2a CBA for *G. australes* strains showed low CTX-like toxicity in strains from the Pacific Ocean [101,173] and low-intermediate toxicity in strains from the Atlantic Ocean [134,174]. And one *F. paulensis* strain (from the Mediterranean) showed low toxicity [71]. Using LC-MS/MS, this same strain showed MTX, gambieric acid A and potential traces of 54-deoxyCTX1B (also known as P-CTX3 or CTX3) [71]. This is noteworthy because the potential presence of 54-deoxyCTX1B in *F. paulensis* indicates that fish caught in the Balearic Islands may contain CTXs. However, findings were not conclusive because of the low levels of potential 54-deoxyCTX1B

did not allow its confirmation. It is worth noting that *F. paulensis* was only detected on one island of the Balearic Islands and its presence seems to be restricted and exceptional [71]. Therefore, all things considered, the risk of CP in the islands seemed to be low.

In article ii (chap. 3.1), strains of *G. australes* and *F. paulensis* showed CTX-like toxicity using neuro-2a CBA. In comparison to other studies the neuro-2a showed that *F. paulensis* and *G. australes* strains of the present work had, respectively, low (8-16 fg CTX1B equiv. $cell^{-1}$) and medium (1-380 fg CTX1B equiv. $cell^{-1}$) CTX-like toxicity. It should be pointed out that the most toxic *G. australes* strain from the Balearic Islands was comparable in terms of CTX-like toxicity to the strains from the Canary and Selvagens Islands of this thesis and Reverté et al., 2018 [174], where CP is present. Therefore, considering the CTX-like toxicity of the strains, the risk of CP in the Balearic Islands seems to be moderate.

Further analyses were done in collaboration with the Universidade de Vigo and the toxin profile for some *G. australes* and *F. paulensis* strains was investigated by LC-MS/MS (article iii, chap. 3.1), but no CTX was detected (unpublished data). However, in another work that I could participate Gaiani et al. (2020) [160], immunoassays were used in *G. australes* strains (from the Canary Islands) and *F. paulensis* strains (from the Balearic Islands) and strains exhibited CTX analogues. It is important to keep in mind the detection limit (LOD) of each of the approaches used. The LODs of the methodologies were 0.45 ± 0.24 pg CTX1B equiv. mL^{-1} for the CBA, 1.96 and 6.17 pg mL^{-1} for the immunoassays depending on the analogue (CTX1B or 51-OH-CTX3C) [159], and 0.1- 0.4 ng CTX1B mL^{-1} for the LC-MS/MS. Hence, the LOD was much higher for LC-MS/MS than the other methods. Thus, in our samples, the CTX-like toxicity and the CTX analogues in the immunoassays suggest that there are other toxins in the extracts than those that can be detected and quantified by analytical methods.

In article iii (chap. 3.1), in all tested *G. australes*, two molecules were also detected: a putative gambierone analogue and 44-methylgambierone (previously reported as MTX3). We have no further information about p-gambierone other than it was also detected in *G. polynesiensis* strains [163] and 44-methylgambierone (formerly reported as MTX3) is ubiquitous in *Gambierdiscus* spp. though not exclusive to the genus [118]. The toxicological analysis showed that the activity of 44-methylgambierone is more like CTX than MTX, and it is less potent than MTXs [182]. The LD_{50} of 44-methylgambierone in mice by intraperitoneal administration is between 20 and 38 mg kg^{-1} [118], while for MTX1 it is $5 \cdot 10^{-5}$ mg kg^{-1} [183]. It is important to note that the toxicological effect of 44-methylgambierone in neuro-2a CBA being treated with ouabain and veratridine seems to be negligible. At

0.87 ug mL^{-1} no effect on viability was observed (unpublished data from our labotarory). Therefore, 44-methylgambierone does not seem to be responsible for the CTX-like and MTX-like toxicities observed in the neuro-2a CBA of the present study. This is supported by Munday et al. (2017) [101] who found that the levels of toxicity of *Gambierdiscus* spp. extracts in mice did not correlate well to the levels of 44-methylgambierone.

Regarding MTXs, their role in CP is not clear since no MTX has been found in the remains of the meal involved in the CP cases. For this reason, the detection of MTX-like toxicity in the isolated strains is also important. In this thesis, we evaluated the presence of several MTX strains from the Balearic and Canary Islands for neuro-2a using the erythrocyte lysis assay (ELA) (article ii, chap. 3.2) and LC-MS/MS (article iii, chap. 3.1). Almost all of the *G. australes* strains presented MTX-like activity. These results support previous observations that *G. australes* is a potent producer of MTXs [101,173]. However, in article **iii** (chap. 3.1)**,** no MTX1 or MTX4 was detected with LC-MS/MS. Like CTXs, the MTX-like toxicity could be explained by the fact that the LODs for CBAs (neuro-2a and ELA) were lower than the LODs for LC-MS/MS. In the present thesis, however, we were unable to test an MTX standard for CBAs because of the lack of a commercial toxin. In a previous study by Caillaud et al. (2010) [155], with neuro-2a CBA the LOD was 2.7 ng mL^{-1} for MTX1 and much lower than the LOD of this thesis using LC-MS/MS (0.32 µg mL^{-1}).

Interestingly, even though this thesis found that *G. australes* and *F. paulensis* were widely distributed in the islands, and detected CTX-like and MTX-like toxicity for strains from the Mediterranean, there are no confirmed cases of CP in the Mediterranean Sea. The CTX-like toxin that we detected in the strains may not accumulate in the food web or, if it does accumulate, only in small amounts. Another hypothesis is that the toxin produced in lab conditions may not be produced in the environment. To discount this latter situation, more toxins should be analyzed *in situ* and lab assays should be carried out to elucidate the factors involved. It is also possible that CTX-producing microalgae have only been in the Mediterranean for a relatively short time and have not contributed significantly to food webs so the presence of CTXs in fish is not enough to cause CP in consumers.

Toxin detection in fish

In Chapter 3.1, section (iv), we present the unpublished results of the CTX-like toxicity analysis by neuro-2a CBA of the flesh and liver of 36 fish from Majorca and Minorca (the Balearic Islands). The fish were caught during November 2018 and September-October 2019. The specimens caught were edible and typically

ciguateric in other regions. However, none of the tested extracts (flesh and liver) presented signs of CTXs.

Given the fact that the relatively low number of fish in the Balearic Islands were caught in only one season (autumn), future research should examine more individuals caught in different seasons. In the Canary Islands, occurrences of CP and the detection of ciguateric species are more frequent in autumn [184]. However, each region has its own particularities. For instance, in the Canary Islands, the number of cases does not correlate very well with serial temperatures over the years. But over a period of ten years there is a global pattern that cases of CP are more frequent in April and December [184].

4.2 Risk hazard evaluation in the Canary Islands

Presence of *Gambierdiscus* and *Fukuyoa* species

The understanding of CP in the Canary Islands is quite unlike that of the Balearic Islands because several studies on hazard identification have already been published. Previous the present thesis, five species of *Gambierdiscus* had been reported in the Canary Islands: *G. australes*, *G. excentricus*, *G. silvae*, *G. caribaeus* and *G. carolinianus* [21,79,94]. Besides, CTX-like toxicity had been evaluated for three species: *G. excentricus* [79,134,136], *G. australes* and *G. silvae* [136]. These studies showed that the CTX-like toxicity of *G. excentricus* is similar to *G. polynesiensis*, which is the highest producer of CTX.

Furthermore, there have been more than one hundred cases of CP in ten years in the Canary Islands [184] and CTX analogues have been confirmed in fish caught in the Canary Islands [185–188]. To prevent CP, since 2011, the Institute of Animal Health and Food Safety (IUSA) have been using neuro-2a CBA to analyze fish on the market of risk species weighing more than an established threshold. So all fish potentially containing CTXs are removed from the food market. These analyses have helped to identify which fish species are most dangerous for consumption in the Canary Islands.

Chapter 3.2 reports the presence of the *Gambierdiscus* species in several locations in the seven major islands of the Canary archipelago and evaluates their potential contribution to CP cases. In the first article of this chapter, we report the presence of *G. belizeanus* in the Canary Islands (El Hierro) for the first time. Interestingly, it was not detected in previous samplings. Previously, *G. belizeanus* had been found in the Caribbean Sea [68] and recently in the Red Sea [85]. The finding for the first

time of *G. belizeanus* in the Canary Islands (El Hierro) does not change the risk of CP in El Hierro since, as it will discussed below, *G. belizeanus* is not one of the most toxic species. Moreover, and for the first time, the same article shows the presence of *G. australes* in La Palma. These new findings point out that our understanding of which species live in the Canary Islands is still incomplete.

In our studies, *G. australes* and *G. excentricus* were the most common species in the islands. In the Canary Islands, microalgal cell abundances for each species have only been reported for Fuerteventura. Both species were found in Fuerteventura where *G. excentricus* was the most abundant followed by *G. australes* [189]. Thus, they are expected to be the main contributors to CP. Even so, the samples were taken in autumn, a season that may favor populations *G. australes* and *G. excentricus*. As in the Balearic Islands, the evaluation of the presence of *Gambierdiscus* species in our studies is temporally and spatially limited. It will be valuable to have more information about their presence and cell abundances in other seasons.

Rodríguez et al. (2017) [21] reported that the abundances of *Gambierdiscus* cells followed an east-west geographical pattern, and were higher in the east. This is partially consistent with the overall pattern of toxic fish (higher toxicity in the east) in the Canary Islands, which is described in Sanchez-Henao et al. (2019) [190]. However, this pattern of toxic fish should be analyzed for each species of fish and it should be borne in mind that microalgal cell abundances and CTX-like toxicities of strains can be different every year.

Interestingly, even though cases of CP are relatively recent in the Canary Islands (since 2004), in contrast to the Balearic Islands, the high biodiversity of the species seems to indicate that *Gambierdiscus* species in the Canary Islands are the result of ancient colonization [21].

Toxin in microalgae

In the chapter 3.2, the potential contribution to CP for each species from our samplings in the Canary Islands cases was evaluated using the analysis of toxin production. To that, CTX-like and MTX-like toxicity were studied using the neuro-2a CBA and erythrocytes lysis assay (ELA), respectively. Our experiments showed that *G. excentricus* exhibited the highest CTX-like activity. These results are in agreement with the previous studies using neuro-2a CBA [79,134,136], and the analysis by chromatography of Paz et al. (2011) [191] where potential CTXs were detected in *G. excentricus* strains. These results support the hypothesis that *G. excentricus* is the main producer of CTXs and the biggest contributor to CP in the Canary Islands. In addition, the results of this thesis are in agreement with the literature which reports

that overall toxin production of *G. caribaeus*, *G. carolinianus* and *G. belizeanus* is low. For *G. silvae* the CTX-like response is still controversial. Robertson et al. (2018) [192] pointed out that *G. silvae* like *G. excentricus,* was a high CTX producer, which contrasts with the results of the present thesis and the results of Litaker et al. (2017) [136].

Furthermore, the present thesis provides the first data about the toxin production of *G. belizeanus* from the Canary Islands. The toxicity of one strain was evaluated by neuro-2a CBA, and the presence of CTXs was assessed by immunoassays. As a result, *G. belizeanus* presented potential CTX1B and CTX3 analogues and low CTX-like toxicity. This toxicity is in accordance with the previous literature, in which strains from the Red Sea and the Caribbean were considered to be low toxic species [85,136]. At present, *G. belizeanus* is the most unknown in the Canary Islands since it was only found at one sampling point in El Hierro in 2017, and the toxicity was evaluated for only one strain.

Additionally, Estévez et al. (Universidade de Vigo) used LC-MS/MS to evaluate the presence of CTXs for the *G. excentricus* and *G. australes* strains from article iii (chap. 3.1). None of the strains exhibited CTX analogues (unpublished data). This was contrary to the toxicological data from neuro-2a CBA and the results of the immunoassays. As has mentioned above, different methods have different LODs, which are lower in the immunoassays and neuro-2a CBA. Therefore, it is plausible that CTX analogues could be detected by the immunoassays and CBA but not by LC-MS/MS.

4.3 Hazard identification based on fish from the Selvagens Archipelago, Desertas and Madeira Archipelago

Madeira and the Selvagens Archipelago (Portugal) are 400 km north of the Canary Islands (Spain) in Macaronesia (NE Atlantic Ocean). Studies focused on detecting CTX-producing species in Macaronesia have shown that *G. australes* and *G. excentricus* are present in the Selvagens Islands [174] and Madeira [88], respectively. As has been mentioned above, *G. australes* is considered to be a medium toxin producer and *G. excentricus* one of the highest toxin producers. Consequently, a priori without evaluating CTXs in fish, the risk of CP in the Madeira archipelago seems to be high. In fact, in this region, a total of 42 cases of CP occurred between 2008 and 2015 (personal communication by Pedro Reis). Moreover, CTXs have already been found in fish [187] and starfish [193]. However, in contrast to the Canary Islands, CP is practically unknown, and there is little information about the distribution of CTX-producing species, their toxin production, ciguateric species and CTX analogues. Besides, unlike the Canary Islands, Madeira Archipelago have not

official analysis of CTX-like compounds in fish species (before sale) that are considered risky for CP.

In Chapter 3.3, the risk hazard of Madeira, the Selvagens and the Desertas Islands was studied through the CTX-like toxicity of fish using neuro-2a CBA. Two extraction protocols were also compared using neuro-2a CBA, and the assays used flesh and liver. Then the most toxic fish were evaluated to confirm CTX analogues using liquid chromatography-high resolution mass spectrometry (LC-HRMS).

Neuro-2a CBA has been widely used to detect CTX-like toxicity and has constantly proved to be a good screening tool for detecting CTXs in seafood [152,149,54]. The screening evaluation made it possible to discriminate between toxic and non-toxic fish. In this process, fish without CTX-like toxicity could be discarded and, in the subsequent analysis, fish were not examined using instrumental methods. This makes analysis cheaper and faster.

This thesis extends the previous list of potential ciguateric species of Madeira. It also provides information about weight and the geographical point where seafood is from so that it can be used for future restrictions. It is important to note that although some species are cosmopolitan, the marine species in the Atlantic Ocean are not necessarily found in the Pacific and Caribbean Oceans. Consequently, ciguateric species in the tropical CP-endemic areas are not necessarily the same as in the temperate waters. In fact, they could vary from region to region. One species is not necessarily found in the same niche in different areas. Moreover, the composition of species and environmental factors can be different, which can cause changes in interactions throughout the food web. These differences between areas hamper the detection of ciguateric species. However, in the present study, the species that showed CTX-like toxicity had already been related to cases of CP in tropical and subtropical waters.

The species studied are edible, but they are not the most commercial species in the Madeira Archipelago. The most commercial species are big predators such as the black scabbardfish (*Aphanopus carbo*) and tuna (*Thunnus obesus*) (https://estatistica.madeira.gov.pt/). Unlike moray eels, which are large predators too, these species have never been related to CP. A plausible reason for this could be their behavior. Both species are migratory, the scabbardfish is bathypelagic and the tuna is pelagic, whereas moray eels are benthic and sedentary. Morris and co-workers (1990) [194] described a CP incident related to inshore tuna (*Thunnus albacares*) in North Carolina but, as far as we know, this is the only reference to tuna being involved in CP. Therefore, the importance of the ecological niche in the bioaccumulation of CTXs is very evident. For instance, this study has found that

levels of CTXs are ten times higher in moray eels than in other species evaluated. Moray eels are a typical species with CTXs [195]. Further, the bermuda chub (*K. sectatrix*), a positive sample, is also a benthic, omnivorous and sedentary species. This species has already been related to CTXs in the Pacific Ocean [196]. However, this thesis is the first to report CTX-like toxicity in a bermuda chub originating from Macaronesia.

For many years, weight, size, and length have been considered key factors in defining a potential ciguateric fish. However, as has also been mentioned in other studies, we have observed that length and weight are not key factors for CTX-accumulation. For instance, Garobiau et al. (2014) [197] evaluated the CTX-like toxicity for 45 fish species from the Pacific Ocean, and 37 did not show a significant relationship between toxicity and length. Besides, in Costa et al., 2018 [187], CTX content was highest for the island grouper (*Mycteroperca fusca*), which was the heaviest of all individuals from the Selvagens Islands (4 kg). However, in the same study, the two individual *S. dumerili* of 20 and 30 kg, which is a species from Madeira typically related to CP cases, did not exhibit CTXs. Other key factors could be age, since *Mycteroperca fusca* can live for up to forty years; however, the maximum age for *Seriola* spp. is fifteen years. Nonetheless, it seems that behavior is key for CTX-bioaccumulation for *Mycteroperca fusca* is a sedentary species while *Seriola* spp. are migrators. Weight may be an indicator of the presence of CTXs for such species as moray eels [188], but it is controversial. Nevertheless, ciguateric fish in the Canary Islands and Madeira Archipelago are still officially controlled by species and weight.

Falcón (2018) [184], checked the poisonings in the Canary Islands thoroughly, and observed that some cases occurred after fish had been consumed that had been considered weight-safe by the Government of the Canary Islands. Also, Sanchez-Henao et al., 2019 [190], showed that some species with CTX-like activity were below the low limit for weight considered to be safe. The latest articles by Henao et al. [188,190] and article i (chap. 3.3) stress the importance of reviewing the safety thresholds. Moreover, in order to evaluate the risk one species may represent, it is crucial to evaluate all parameters, such as the geographical point of capture, the behavior of the species (migratory, sedentary, diet), the growth model of the species (the relation between age and size), season and year of capture. Besides, information about the absorption, distribution and metabolism of CTXs as well as the excretion of CTXs could be helpful.

By comparing the two extraction protocols, this thesis has shown that the protocol for detecting CTXs reported by Yogi (2011) [198] is better than the one reported by Lewis (2003) [139]. In fact, IRTA (Institute of Agrifood Research and Technology)

communicated this finding to IUSA of the Canary Islands, the official institute there for analyzing ciguatoxins in fish using CBA. IUSA made the corresponding modifications to the toxin extraction protocol according these results [188,190]. Even though, the protocol based on Yogi et al. (2011) [198] obtains higher concentrations of CTX than the one reported in Lewis et al. 2003 [139], the safety guidance level of 0.01 µg of CTX1B kg^{-1} was not achieved for some samples. Therefore, more steps, like another cleaning step in the extraction protocol, should be added if it is to be a CP prevention tool.

Furthermore, in this study, the liver extracts showed more CTXs than the flesh extracts. This has already been observed in other studies [54,182]. Moreover, cases of CP have been observed to be severer after the consumption of viscera [196]. The higher CTX-like toxicity in the liver may identify potential ciguateric individuals better than flesh. Therefore, in order to prevent cases of CP in the official control program, it would be better to analyze the liver not the flesh.

Analytical analysis by LC-HRMS confirmed the presence of CTX in fish from the Madeira Archipelago. The detection of congeners shows which molecules are accumulated in which marine species, and how ciguateric species interact through food webs. Besides, different congeners may be related to different CP symptoms. Thus, the toxin profile analysis could improve the clinical practices. In our study, CTX analogues were detected in fish by LC-HRMS: C-CTX1, CTX2-2H and a putative CTX analogue (the structure of which is not confirmed). C-CTX1 had already been found in samples from the Atlantic Sea by several authors [98,187,188] and CTX2 had already been found in the Pacific Ocean [60]. This article describes CTX2 in the Atlantic Ocean for the first time. Furthermore, a new putative CTX analogue has been detected for the first time in fish captured in the Desertas Islands. It should be pointed out that, after the Azores, the Desertas Islands are the second highest latitude and it is expected that CTXs in seafood can be found in other parts of the world at the same latitudes.

CHAPTER 5

CONCLUSIONS

5. CONCLUSIONS

The findings achieved in this thesis lead to draw the following conclusions:

In the Balearic Islands:

- The genus *Gambierdiscus* is present in the islands and it was described for the first time in the Western Mediterranean Sea.

- Populations of *G. australes* and *F. paulensis* in the Balearic Islands are widespread in the different islands and have been recurrent for several years, from 2016 to 2020, indicating that their presence is not circumstantial and that these populations are well established.

- *G. australes* and *F. paulensis* strains exhibited moderate and low levels of CTX-like toxicity. Morevover, the production of CTX-compounds was confirmed by immunological detection.

- No CTX-like toxicity was obtained in the 36 fish analysed, either in the flesh or the liver.

- At present we estimate that CP risk in the Balearic Islands exists but is low, considering the persistent and wide distribution of *Gambierdiscus* and *Fukuyoa* populations having low CTX-like toxicity, and the lack of toxicity in fish.

In the Canary Islands:

- *G. belizeanus* has been reported for the first time in the Canary Islands, raising from six to seven the number of *Gambierdiscus* species present.

- The *Fukuyoa* genus is not present in the Canary Islands.
- *G. belizeanus* produce CTX-like compounds but exhibited low CTX-like toxicity therefore, its contribution to CP is low.
- Considering the maximum CTX-like and MTX-like toxicity levels, the most contributor species to CP are *G. excentricus* and *G. australes*.

In Madeira Archipelago:

- Fish species of families *Kyphosidae*, *Serranidae*, *Muraenidae*, *Labridae*, *Balistidae* and Scaridae presented CTX-like toxicity.
- *Kyphosus sectatrix* (bermuda sea chup, fam. *Kyphosidae*) is described as a CTX-like species in the Atlantic Ocean for the first time.
- Among the species evaluated, moray eels were the most toxic according to CTX-like toxicity.
- The extraction protocol for CTXs in fish based on Yogi et al. 2011 is more efficient to extract toxins than the protocol of Lewis et al. 2003.
- Levels of CTX-like toxicity were higher in the liver than in the flesh. Therefore, detection of CTX-compounds in the liver can contribute as an early warning strategy to better describe in a specific area the transfer and biotransformation of CTXs in the food webs.
- In both tissues the estimated levels of CTXs were higher than the safety recommendations for EFSA and FDA.
- The finding of new CTX analogue show that it is still lack of knowledge of which compounds could contribute to CP.

CHAPTER 6

REFERENCES

6. REFERENCES

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UNIVERSITAT ROVIRA I VIRGILI
CONTRIBUTION TO THE RISK ASSESSMENT OF CIGUATERA POISONING IN EUROPE (THE BALEARIC AND CANARY ISLANDS AND THE MADEIRA ARCHIPELAGO)
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