DEVELOPMENT OF BIOANALYTICAL DEVICES FOR THE DETECTION OF CIGUATOXINS AND THE CIGUATOXIN PRODUCING GENERA GAMBIERDISCUS AND FUKUYOA

Greta Gaiani

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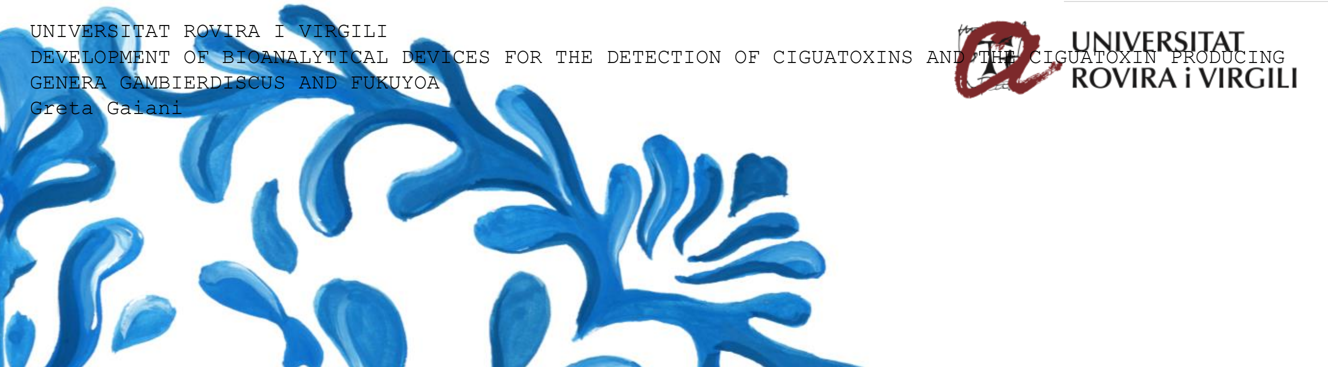

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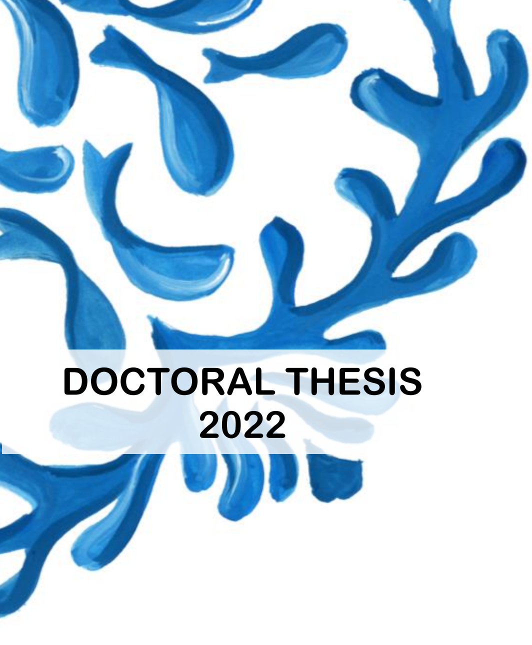

DOCTORAL THESIS
2022

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Doctoral thesis

Development of bioanalytical devices for the detection of ciguatoxins and the ciguatoxin producing genera *Gambierdiscus* and *Fukuyoa*

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Tarragona 2022

I state that the present study entitled **Development of bioanalytical devices for the detection of ciguatoxins and the ciguatoxin producing genera *Gambierdiscus* and *Fukuyoa***, presented by Greta Gaiani for the award of the degree of Doctor, has been carried out under my supervision at the department of Chemical Engineering of this university, and that fulfills all the requirements to be eligible for the International Doctorate Award.

Tarragona, 21st December 2021

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Dr. Mònica Càmpas	Dr. Carles Alcaraz	Dr. Ciara K. O'Sullivan

This thesis is dedicated to my amazing family.
To my mum Barbara, my dad Mauro, my aunt Sonia and my uncle Antonio
who raised me as a free human being.
To my granny Anita
who always understood my weird flow of thoughts.
To my sisters Brigitta and Beatrice
who taught me what unconditional love is.
And especially to my grandparents Maria Gloria and Angelo
taken away by this horrible pandemic.
You were my safe place. Thank you.

Questa tesi è dedicata alla mia meravigliosa famiglia.
A mia mamma Barbara, mio papà Mauro, mia zia Sonia e mio zio Antonio
che mi hanno cresciuto come una persona libera.
A mia nonna Anita
che ha sempre capito il mio confuso flusso di pensieri.
Alle mie sorelle Brigitta e Beatrice
che mi hanno insegnato cosa significa amare incondizionatamente.
E in particolare ai miei nonni Maria Gloria e Angelo
portati via da questa orribile pandemia.
Eravate il mio porto sicuro. Grazie.

Algayarens

Algayarens

Graffiata nel silenzio,

il sole alza lo sguardo

di nere mercanzie

accoglie l'uomo

assediato dall'eterno.

Calas Mortes

Il passaggio della cicogna

sequestro delle ombre.

Il gozzo

smercia linee.

Il faro vive

nel quartiere ebreo.

Calas Mortes

voleva in prestito

"l'intera vita".

Maria Gloria Grifoni. *L'inchiostro dell'uomo.*

Aknowledgments

The common belief is that I talk too much. Unfortunately, this is very close to the reality. Most of the times it happens because I feel like I do not have the words to express myself, and so I end up using too much of them. Although, in this occasion, I know exactly what to say.

First, I would like to thank IRTA and the Universitat Rovira i Virgili for giving me the opportunity to grow as researcher by awarding me the pre-doctoral scholarship Martí Franquès. Additionally, I want to acknowledge the projects EuroCigua (GP/EFSA/ AFSCO/2015/03), CIGUASENSING (BIO2017-87946-C2-2-R), CIGUARISK (PID2019-108781RR-C21) and CELLECTRA (PID2020-112976RB-C21) within which I worked.

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GENERA GAMBIERDISCUS AND FUKUYOA
Greta Gaiani

Summary

Ciguatera fish poisoning (CFP) is a foodborne disease that can cause gastrointestinal, cardiological and neurological symptoms that can last weeks, months or even years and in some cases leads to death. It is caused by the ingestion of fish containing ciguatoxins (CTXs), a group of cyclic polyether lipophilic compounds produced by microalgae of the genera *Gambierdiscus* and *Fukuyoa*, which accumulate into fish flesh and through the food webs. Several fish species are implicated in CFP, and discriminating between contaminated and uncontaminated specimens is an important challenge, since toxic specimens do not look, smell or taste any differently from non-toxic ones. Thus, taking into consideration that an antidote for CFP has not been found yet, the efforts of the scientific community must focus on the prevention, by providing fast and reliable tools for the detection not only of CTXs in fish and algal samples, but also of the *Gambierdiscus* and *Fukuyoa* CTX producing species in the environment.

The main goal of this doctoral thesis is to provide biotechnological tools for the characterization of the risk of CFP in order to promote food safety and human health. Particularly, the major focus is the development of bioanalytical devices for the detection of different *Gambierdiscus* and *Fukuyoa* species and of CTXs. Additionally, this thesis aims at providing fast and reliable strategies to shorten and simplify the sample pretreatment necessary for the analysis of environmental samples. In general terms, this thesis intends to demonstrate the applicability of reliable biotechnological tools, which can be easily implemented in portable devices, paving the way for the *in situ* detection that would speed up monitoring analysis.

In order to achieve this objective, this thesis reports the development of fast extraction techniques for DNA and CTXs with the use of portable devices. Then, on one hand, it shows how primers modified with tails were exploited to perform both recombinase polymerase amplification (RPA) and PCR to simultaneously amplify DNA from the genera *Gambierdiscus* and *Fukuyoa* and from more than one toxin producing species. On the other, the application of antibodies that target four main CTXs belonging to two groups of congeners (CTX1B and CTX3C) is described for their combined or separated detection. At first, the systems were characterized with colorimetric assays, and then they were integrated in the development of electrochemical biosensors for the detection of DNA belonging to two *Gambierdiscus* species at the time or of CTXs. Finally, this thesis provides a description of how the developed systems were applied to the analysis of natural samples, comparing the results to well-established techniques.

This thesis has the following structure:

Introduction. In this chapter, a brief history of CFP is provided. Then, *Gambierdiscus* and *Fukuyoa* distribution is detailed together with the description of their toxic products, CTXs. Additionally, the known methodologies to detect both DNA of *Gambierdiscus* species and CTXs, from traditional tests up to sophisticated instrumental analysis, are listed and commented. Finally, the utility of biosensor in the detection of both DNA and CTXs, and the advantages in developing such tools to help CFP managing are discussed.

Objectives. This section includes the general and specific objectives of this thesis.

Scientific publications. A list of the scientific publications accomplished during the duration of this thesis is provided together with my personal contribution to each of them.

The experimental part of this thesis is divided in two sections, according to the target of the studies:

Section one: *Gambierdiscus* and *Fukuyoa* detection

Chapter 1. The development of three molecular assays for the detection of the *Gambierdiscus* and *Fukuyoa* genera and for *G. australes* and *G. excentricus* species**,** based on the combination of RPA with a sandwich hybridization assay, is here described together with the achievement of a remarkable limit of detection.

Chapter 2. In this chapter, a multiplex-PCR approach, performed with modified primers, is exploited in the development of an electrochemical DNA-based biosensor for the simultaneous detection and discrimination between *G. australes* and *G. excentricus.* Moreover, a rapid DNA extraction technique

is described. Finally, the applicability of the developed technique to the analysis of field samples is investigated.

Section two: CTXs detection

Chapter 3. The development of a biosensor for the detection of CTXs is herein presented. Two capture and a detector antibody were used in a sandwich configuration for the detection of four congeners belonging to two main group of CTXs (CTX1B and CTX3C). Moreover, the applicability of the system is tested on fish samples naturally contaminated with CTXs.

Chapter 4. In this chapter, the deeper analysis of a unique fish sample identified as positive for CTXs with cell-based assay is described. The crude extract belonging to this individual was fractionated and the obtained fractions were screened with the developed biosensor in order to precisely identify the ones with CTXs.

Chapter 5. The application of the developed biosensor to the detection and discrimination between the two main groups of CTXs (CTX1B and CTX3C) in *Gambierdiscus* and *Fukuyoa* pellets, at low cell concentration, is investigated. Moreover, a fast CTXs extraction technique is implemented into the analysis protocol.

General discussion. In this section, a discussion of all the findings obtained in this thesis is provided.

Conclusion. A summary of the general conclusions of this thesis is detailed together with future works and potential applications of the developed techniques.

General introduction

1. Ciguatera fish poisoning

Ciguatera fish poisoning (CFP) is a foodborne disease that can cause gastrointestinal, cardiological and neurological symptoms and may be fatal (Hamilton et al. 2010). The history of CFP is difficult to establish, but it probably dates to ancient times. One of the first mention to fish poisoning in general can be read in Homer's Odyssey (800 B.C.). Additionally, in the poem it is clearly written that heroes were eating fish only if they had no other source of food (*Odyssey,* IV, 368 f.; XII, 330-332) (Figure 1). Evidently, it is impossible to establish if in the poem a CFP event is described, but sure, it makes think there was a general awareness regarding seafood poisoning also in antiquity. Centuries later, Alexander the Great (356-323 B.C) demonstrated this consciousness by forbid his soldiers to eat fish in order to avoid poisonings and consequently a battle loss (Halstead 1988). The first historic record of ciguatera could be the one that, in 1525, involved the intoxication of several crew members of a Spanish fleet that ingested barracuda while anchored in the Gulf of Guinea (Fraga et al 2011). Later, in 1601 another similar episode was described aboard of a vessel that was navigating the south coastal area of Mauritius Island (Indian Ocean). Again, in 1748, a mass mortality event was reported after the consumption of intoxicated fish in the island of Rodrigues (Halstead and Cox 1973) and, in 1774, a fish poisoning incident happened at Vanuatu (Pacific Ocean) and reported by Captain Cook was attributed to ciguateric fish (Doherty 2005). Even though, CFP was reported in the Indian and Pacific Oceans already in the sixteenth century, its name was coined in the Spanish West Indies (Bagnis 1981) much later. In fact, the term "ciguatera" originated in the Spanish Antilles during the eighteenth century and referred to the intoxication caused by the ingestion of *Turbo pica*, a marine snail commonly known as "cigua". In the Caribbean, the first reported episode of CFP dates back to 1862 and happened after the ingestion of parrotfish (Halstead 1967). Of course none of this episodes is officially confirmed as a CFP, and it is necessary to differentiate among intoxication in order to provide the best treatment.

330 καὶ δὴ ἄγρην ἐφέπεσκον ἀλητεύοντες ἀνάγκη,
ἰχθῦς ὄρνιθάς τε, φίλας ὅ τι χεῖρας ἵκοιτο,
γναμπτοῖσ᾽ ἀγκίστροισιν· ἔτειρε δὲ γαστέρα λιμός·

Figure 1. Homer's verse XII, 330-332. *"...and so, due to necessity, they start the hunting of fish and birds and whatever they found, using curved hooks; hunger consumed their stomachs"* (Translation from Greek: Vincenzo Di Benedetto, English adaptation: Greta Gaiani).

Fishes become ciguatoxic when they ingest a sufficient amount of ciguatoxins (CTXs), or their precursors, which are a group of cyclic polyether lipophilic compounds produced by microalgae of the genera *Gambierdiscus* and *Fukuyoa* (Chinain et al. 2021). The grazing activity of herbivores and detritivores constitutes the most known way of entrance of CTXs into the food webs, where they are bio-magnified, bio-transformed and bio-accumulated while transferred within them. In general terms, it is quite difficult to predict where and when a ciguatera outbreak will take place, since more than 400 fish species are believed to be potential vectors (Darius et al. 2021). Additionally, if a fish is identified as toxic, it does not mean that all the other caught at the same moment and in the same place will be toxic as well (Ragelis 1984). Nevertheless, certain species are more likely to contain CTXs and their distribution has been previously documented (Halstead 1988). Moreover, it seems that and also sharks, which flesh ingestion led in some cases to death of numerous consumers (Diogène et al. 2017), and several species of marine invertebrates may be involved in CFP pathways (Darius et al. 2018; Mak et al. 2013; Rongo and van Woesik 2011; Roué et al. 2016; Silva et al. 2015). As if CFP was not already problematic enough, it is almost impossible to distinguish between contaminated and uncontaminated specimens since the toxic ones do not look, smell or taste any differently. Thus, in countries in which CFP is endemic it led from a decrease in fish consumption (Nellis and Barnard 1986) up to drastic modification in dietary habits (Rongo and van Woesik 2011), and management practices ban the sale of certain high-risk species, causing important financial losses (Sanchez-Henao et al. 2019). Furthermore, in 2020 the International Association for Medical Assistance to Travelers (IAMAT) labeled several countries as “ciguatera at-risk destinations”, making CFP a threat also for the tourism sector, important source of income for endemic populations.
Thus, considering that CFP it is quite difficult to diagnose and report, since the symptoms can be easily misunderstood for another food poisoning, and there is no reliable antidote for CFP, the efforts of the scientific community must focus on the prevention, by providing reliable tools for the detection of *Gambierdiscus/Fukuyoa* genera and CTXs in natural environments.

2. *Gambierdiscus* and *Fukuyoa* global distribution with particular focus on the Mediterranean and Macaronesian regions

Until 1995, *Gambierdiscus* was considered as a monotypic taxon with just one species named *G. toxicus* (Adachi and Fukuyo 1979). Up to date, 18 different *Gambierdiscus* species *(G. australes, G. balechii, G. belizeanus, G. caribaeus, G. carolinianus, G. carpenteri, G. cheloniae, G. excentricus, G. holmesii, G. honu, G. jejuensis, G. lapillus, G. lewesii, G. pacificus, G. polynesiensis, G. scabrosus, G. silvae* and *G. toxicus)* (Chinain et al. 1999; Litaker et al. 2009; Fraga et al. 2011; Nishimura et al. 2014; Rhodes et al. 2017; Jang et al. 2018; Kretzschmar et al. 2019) and 4 *Fukuyoa* species (*F. paulensis, F. ruetlzeri, F. yasumotoi, F. koreensis;* Gómez et al. 2015; Li et al. 2021) have been identified. Among them, 14 *(G. australes, G. balechii, G. belizeanus, G. caribaeus,*

G. carolinianus, G. carpenteri, G. excentricus, G. pacificus, G. polynesiensis, G. scabrosus, G. silvae, G. toxicus, F. paulensis and *F. ruetlzeri*) are considered able to produce CTXs (Tester et al., 2020) with different tests and techniques (Chinain et al. 2010a; Fraga et al. 2011; Rhodes et al. 2014; Litaker et al. 2017; Pisapia et al. 2017; Longo et al. 2019; Rossignoli et al. 2020).
Gambierdiscus and *Fukuyoa* genera are originally endemic of the subtropical areas of the world (35°N and 35°S) (Bienfang et al. 2010; Chinain et al. 2021). In more recent times, they have been identified in temperate areas such as Korea, Japan, New Zealand (Jeong et al. 2012; Nishimura et al. 2014; Rhodes et al. 2017), Gulf of Mexico, coast of North Carolina, Brazil (Gómez et al. 2015; Litaker et al. 2009; Litaker et al. 2017) and also the Macaronesia region (Fraga et al. 2011; Kaufmann and Böhm-Beck 2013; Fraga and Rodriguez 2014) and the Mediterranean Sea (Aligizaki and Nikolaidis 2008; Aligizaki et al. 2009; Laza-Martínez et al. 2016; Tudó et al. 2018, 2020). The region that hosts the major diversity of *Gambierdiscus* and *Fukuyoa* species is the Pacific, where the presence of 19 out of the 22 currently recognized species has been reported. Thus, it is not surprising that several archipelagos of this region, such as French Polynesia and Cook Islands, are identified as biodiversity "hotspots" of *Gambierdiscus* (Chinain et al. 2021). In addition, the Caribbean region also presents a huge variety of *Gambierdiscus* and *Fukuyoa* species, and it is quite common to find the co-occurrence of 5 or 6 species (Tester et al. 2013). The fact that *G. excentricus* and *G. silvae*, two of the most CTX-producing species, are not as frequently found as other species draws the attention. The observable disjunt distributional pattern has been related to their thermal tolerance (Chinain et al. 2021). Addionally, still no *G. australes*, which is distribution is global, has been identified in the Caribbean region. Different is the situation of the Indian Ocean, where the distribution of the genera is poorly reported, especially in the coastal areas of Africa. Additionally, most records reported the species as *G. toxicus*, since the identification was mainly performed with microscopy techniques (Turquet 1998; Lugomela 2006). Even if molecular studies identified the presence of few species, like *G. australes* and *G. belizeanus* (Lavenu et al. 2018), more studies are needed to have a correct species composition of this region. Another recently found "hotspot" of *Gambierdiscus* is the Macaronesian region, with the Canary Island hosting the highest biodiversity and the highest number of CTX-producing species (*G. australes, G. caribaeus, G. carolinianus, G. excentricus* and *G. silvae*) (Fraga et al. 2011; Fraga and Rodriguez 2014; Pisapia et al. 2017; Rodríguez et al. 2017; Reverté et al. 2018). Despite the fact that the settlement of a *Gambierdiscus* and *Fukuyoa* species and the finding event can be separated by several decades, there is a general concern that the geographic range of these two genera, and especially of the CTX-producing species, will expand as a consequence of the rise of sea surface temperature (Tester et al. 2010).

Mirador el balcon
Gran Canaria
Canary Islands (Spain)
Photo credit: Francesca Cucchi

According to Parsons et al. (2010), a significant modification in the distribution and the abundances of ciguateric species is to be expected, with some species becoming more dominant over others.

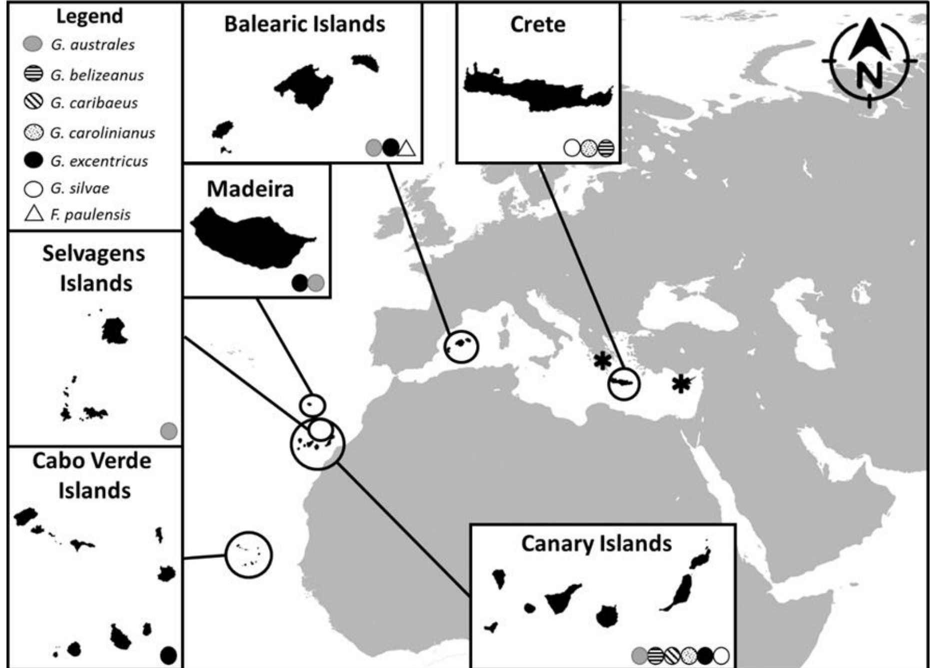


Figure 2. *Gambierdiscus* and *Fukuyoa* distribution in Mediterranean and Macaronesian waters. Symbol * indicates the places (Greece and Cyprus) in which the presence of both *Gambierdiscus* and *Fukuyoa* was reported only at genus level. For the global distribution, see Tester et al. (2018).

3. Ciguatoxins

CTXs are cyclic polyether compounds with a rigid structure formed by 13-14 rings connected with ether bonds. CTXs target the binding site 5 of the voltage-gated Na^+ channels (Lombet et al. 1987), inducing effects at the cellular and physiological levels, such as membrane excitability, release of neurotransmitters (Molgó et al. 1990), increase of intracellular calcium (Molgó et al. 1993) and blockage of voltage potassium channels (Hidalgo et al. 2002). The affinity of the different congeners of CTXs for the binding site on the voltage-dependent Na^+ channels is proportional to their toxicity in mice (Lewis 1994).

Up to date, 34 different CTX congeners have been described and grouped in Pacific (P-CTX) (22 congeners), Caribbean (C-CTX) (12 congeners) and Indian (I-CTX) (no congeners described yet, see below), according to their geographical origin (Longo et al. 2019). CTX1B was the first one to be identified in 1990 by Murata and coworkers (Murata et al. 1990), followed by the description of many other congeners. Additionally, in order to classify the different congeners of P-CTXs, Legrand et al. (1998) proposed to distinguish them into two different groups according to the number of carbons and the structure of the E ring (7 in the CTX1B group and 8 in the CTX3C group) and

to the presence (CTX1B) or absence (CTX3C) of the 4-carbon side chain of the left wing (Figure 3). Afterwards, two CTXs from the Caribbean Sea (C-CTXs) were isolated by Vernoux and Lewis (1997) and identified structurally in 1998 (Lewis et al. 1998). Subsequently, other congeners were identified by Pottier et al. (Pottier et al. 2002). More recently, six Indian Ocean CTXs (I-CTXs) were isolated (Hamilton et al. 2002; Diogene et al 2017), but their structural determination remains undescribed. Alongside with the CTXs bioaccumulation in fish flesh and through the marine food webs, CTXs undergo metabolization processes in fish (Ikehara et al. 2017), resulting in more toxic compounds, as observed in fish samples from the Pacific area (Chinain et al. 2010b). The occurrence of these different toxins in fish and microalgal samples can vary. Nevertheless, P-CTX-1 (CTX1B) is found as dominant in toxin profiles in the carnivorous fishes of the Pacific (Lewis et al. 1991). The toxicity of CTXs in mice (i.p.) is equivalent to an LD_{50} 0.25, 2.3 and 0.9 µg/kg for P-CTX-1, P-CTX-2 and P-CTX-3, respectively (Lewis et al. 1991), classifying them as extremely potent marine toxins. Generally, P-CTXs are more potent than C-CTXs (LD_{50} of 3.6 and 1 µg kg/1 for C-CTX-1 and C-CTX-2) and I-CTXs (5 µg kg/1). In humans, it has been estimated that no more than 1 ng P-CTX-1 per kg of body weight is needed to cause the occurrence of mild CFP symptoms (Lehane and Lewis 2000). Moreover, these toxins are heat resistant, so they cannot be deteriorated by cooking processes (Abraham et al. 2012).

CTX1B : R = OH
54-deoxCTX1B : R = H

CTX3C : R = H
51-hydroxyCTX3C : R = OH

Figure 3. Structure of the two main groups of CTXs congeners: CTX1B and CTX3C.

The United States Food and Drug Administration (US FDA) proposed guidance levels of ≤0.01 µg/kg of CTX1B and ≤0.1 µg/kg of C-CTX-1 equivalent toxicity in fish, and these values represent the only existing suggested

threshold. In fact, New Zealand and Australia provide general guidelines (FZAN 2006) and Japan (MHWL 1953; 2001) banned from the market several species associated with ciguatera. In European markets, no fish product containing CTXs can be sold (Regulation (EC) No. 853/2004), but no regulatory limits have been established and no suggestion regarding the analytical methodology to use is given. Nevertheless, the European Food Safety Authority (EFSA) has adopted the US FDA guidance levels for CTXs Thus, the creation of fast, reliable and easy to use tools for the detection of even small quantities of CTXs in fish and algal samples can be of outmost help for CFP managment.

Cala Macarelleta
Menorca
Balearic Island (Spain)
Photo credit: Greta Gaiani

4. Methods for *Gambierdiscus* and *Fukuyoa* detection

The presence of highly toxic *Gambierdiscus* and *Fukuyoa* species in a given area is likely to contribute to the final toxic profile in fishes. Therefore, document the presence of these ones, that might not be dominant in terms of cell concentration, but whose contribution in the environmental flux of CTXs is noticeable is of extreme importance (Longo et al. 2019). In the Pacific region, *G. polynesiensis* is the species that has the widest range of distribution and is the most CTXs producer (Longo et al. 2019). In the Atlantic, this role is played by *G. australes*, *G. silvae* and *G. excentricus*. The presence of this last species in the Macaronesia region represents a real threat since it is associated with increasing CFP incidences (Pérez-Arellano et al. 2005). Thus, scientists all over the world focused their efforts mainly in the detection of these CTXs producing species in field samples. The most known and used technique is light microscopy, followed by electron microscopy. These strategies are still used, and even if it is possible to differenciate between species with the last one, the required preparation of samples is complicated, time consuming, and requires skilled personnel. Hence, the use of genetic sequencing has increased over the years and nowadays almost mandatory to correctly identify to the species level (Bravo et al. 2019). In fact, in every study concerning the detection of species, especially in environmental samples, the confirmation with sequencing is highly requested to support the findings obtained with other methods. Among the existing sequencing procedures certainly the Sanger has been the most used since its invention in 1977. Consequently, molecular techniques are more and more implied in the identification of microalgal species in field samples. In fact, quantitative polymerase chain reaction (qPCR) has been used on several occasions for the identification and quantification of *Gambierdiscus/Fukuyoa* genera*, G. belizeanus, G. caribaeus, G. carolinianus, G. carpenteri* and *G. ruetzleri, G. australes, G. scabrosus, G. excentricus, G. silvae*, *G. lapillus* and *F. paulensis* targeting the D1-D2 regions (Smith et al. 2017), the D1-D3 region (Vandersea et al. 2012; Litaker et al. 2019; Kretzschmar et al. 2019), or the D8-D10 (Nishimura et al. 2016; Kretzschmar et al. 2019) of the large subunit (LSU) ribosomal gene. This method is strong and reliable, but it requires the use of a thermocycler, is laboratory based and time consuming.

It has to be considered that most of the ciguateric areas are either isolated or with reduced services and, as appears clear from the described methods above, the detection of DNA is a long and expensive task to perform, and needs special facilities and trained personnel. Additionally, an important and critical step of the DNA detection is the sample pre-treatment, which includes the sample conservation, DNA extraction and purification. Therefore, the development of fast and reliable techniques that can shorten and simplify not only the pre-treatment but also the DNA amplification step, avoiding the use of sophisticated equipment and reagents, is of outmost interest. Additionally, the integration of these techniques in portable devices for the *in situ* detection of CTXs producing genera or, even better, directly several toxin producing species simultaneously

would be of extreme interest for the risk assessment of this disease that has no remedy.

5. Methods for ciguatoxin detection

Ciguatera is a threat for human health. Therefore, a huge variety of methods have been developed to detect CTXs and therefore identify contaminated fishes. Up to date, the techniques developed include native tests, animal mortality tests, cell-based assays (CBAs), receptor-binding assays (RBAs), immunological assays and instrumental analysis (such as LC-MS/MS) (Hoffman et al. 1983, Chinain et al. 2010b, Reverté et al., 2014; Diogène and Campàs, 2017; Pasinszki et al. 2020).

The first attempts to detect ciguateric fishes were performed by island communities, whose diets depend mostly on seafood (Chinain et al. 2010b; Darius et al. 2013). These test methods included all kind of traditional tests, such as cooking fish with silver or copper and evaluating the discoloration, rubbing a small piece of the fish on the own skin or mouth and check for itchiness, make an incision on the tail of a dead fish and look for hemorrhagic signs, or considering a fish as toxic if an hour after death (during the *rigor mortis*) its flesh is flaccid. Although most of these tests are considered not specific enough for the screening of fish extracts, Darius et al. (2013) discovered that the bleeding and the *rigor mortis* tests allowed to discard some contaminated fishes from non-contaminated ones. Maybe, if these traditional tests were integrated with more analytical methods, they could help in the management of CFP in areas where the disease is endemic and the resources are limited.

Historically, the use of animal testing for the detection of pollutants or contaminants has been quite common and the research on CTXs makes no exception. In fact, since the toxic activity of these compounds can affect several animals, several animal-based test have been developed using mammals (Bagnis and Fevai 1971), birds (Vernoux et al. 1985), larvae of crustaceans (Granade et al. 1976) and insects (Labrousse and Matile 1996). Even if these tests are quite simple to perform and the results are easy to interpret, such techniques are not sensitive enough, they are time consuming and expensive. Additionally, in recent years, several ethical concerns started to rise around the use of animals for laboratory testing. Even though none of the previously mentioned tests is applied nowadays for the detection of CTXs or other marine toxins, there is one animal-based test that is still in use: the mouse bioassay (MBA) (ANSES). The MBA is useful since it provides a compositive toxicological response, which is very convenient in case of samples with unknow toxicity. Apart from sharing the limitation of the other animal-based tests, it has a limit of detection that is approximately 0.56 ng/g for P-CTX-1B (EFSA 2010), meaning that it does not attain the suggested FDA threshold. Therefore, the scientific community switched to the use of assays based onto mammalian cell, instead of entire animals.

The CBAs developed for CTXs detection are based onto the activity of these toxins on neuronal potassium and voltage-gated sodium channels (VGSCs) (Lewis and Vetter 2016) and involve a huge assortment of cells and tissues, from blood, used for the development of hemolytic assays (Shimojo and Iwaoka 2000), the guinea pig ileums (Endean et al. 1993), the guinea pig atrium (Hokama et al. 1994), frog nerve fibers (Benoit et al. 1986) and crayfish nerve cords (Miller et al. 1986). Despite this variety of available tests, nowadays the most used test is the mouse neuroblastoma cell assay (N2a CBA) (Manger et al. 1993). This test overtook all the others because the necessary material and reagents are commercially available, and the cells are relatively easy to grow. Additionally, the test is easy to perform and interpret. Briefly, it is based on the colorimetric detection of metabolically active N2a cells exposed to CTX in presence of ouabain/veratridine (Manger et al. 1993). The detection of CTXs requires the addition of veratridine, which is a VGSC activator with a different binding site than CTX, and ouabain, a sodium/potassium pump inhibitor. The combined effect of these three substances together increases the concentration of intracellular sodium, which has a negative effect on cell viability and can be measured as a function of CTX concentration. The amount of toxin is measured with the MTT-based CBA, in which the MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) is added in each well, reduced by mitochondrial dehydrogenase activity into a formazan product, which is later solubilized and whose absorbance intensity is proportional to the number of live cells and so inversely proportional to the concentration of CTXs, within a certain range. Even if the limit of detection (LOD) and limit of quantification (LOQ) are different in each experiment, in general they are lower than the FDA suggested threshold. However, a consensus protocol for the MTT-based CBA is still lacking since users throughout the last decade made customized changes to the assay (Viallon et al. 2020). The modifications included almost every aspect of the test, starting from the cell seeding densities, the cell layer viability, the MTT incubation time, up to the ouabain/veratridine treatment. Other N2a CBAs have been developed. For example, in the study of Fairey et al. (1997), they used N2a cells that expressed c-*fos*-Luciferase reporter gene. The c-*fos* is a response gene and a sensitive biomarker that easily localize the effects of toxins. Detection is achieved with luciferase-catalyzed light generation and a luminometer for quantification. Additionally, cell lines other than N2a have been exploited for application in CBA. In particular, the human neuroblastoma cell line SH-SY5Y has been used to develop another fluorescent assay (Lewis et al. 2016). In this test, cells were loaded with a dye containing calcium adsorbed into the cytoplasm, and then incubated with veratridine and subsequently with CTXs. Fluorescence responses to CTXs were measured as the increase of calcium ion influx into cells with a plate reader. These fluorescent assays are not commonly used due to the costs of the fluorescent dye, the need of specialized equipment, and the sensitivity to maitotoxin presence, which can affect enormously the outcome of the test.

La Garita
Gran Canaria
Canary Islands (Spain)
Photo credit: Francesca Cucchi

Indeed, the interfering effects caused by maitotoxins, other toxic compound or the natural matrix itself (i.e. fish or algal extracts) can induce an over or under estimation of the CTXs content. Additionally, the cytotoxicity assays respond similarly to all the toxins that block VGSCs (i.e. brevetoxin) and, therefore, it will be impossible to distinguish one from another.

In order to focus more on the affinity of CTXs for their binding site on the VGSC, RBAs have been developed. Although in an RBA the response is structure-related, the fact that VGSCs are targeted may involve correlation with toxicity. Since CTXs share with brevetoxins the same binding site on the VGSC (i.e. binding site 5) but with a higher affinity, they can be considered as competitors of brevetoxin binding (Fairey et al. 1997, Bottein Dechraoui et al. 2005). Therefore, measuring the competition binding of a radioactively labeled brevetoxin ([3H]-brevetoxin-3) and CTXs for the receptor sites in a membrane can be used to estimate the amount of CTXs in an extract. Hence, the concentration of the labeled brevetoxin (that is maintained constant) should decrease after the addition of CTXs, and a competition dose-response curve can be obtained. This screening method has been widely used, but it is highly sophisticated, making the comparison between laboratories quite complicated. Thus, Díaz-Asencio and coworkers (Díaz-Asencio et al. 2018) made the effort to provide guidance on its quality control checks for the analysis of environmental samples, reaching an LOD of 0.75 ng/g of P-CTX-3C in fish samples in their optimized assay. However, these assays imply the use of radioactive compounds. To avoid the use of instable radioactive compounds, a fluorescence-based RBA has been developed, where CTXs compete with a fluorescently labeled brevetoxin (brevetoxin-2) (McCall et al. 2014). Following this studies, Hardison and coworkers developed a fluorescent RBA using a brevetoxin-2 labeled with BODIPY® (Hardison et al. 2016), which provides a LOD of 0.075 ng/g of P-CTX-3C equivalents. Moreover, a commercial kit for CTXs based on this study has been marketed by Sea Tox Research Inc. (Wilmington, NC, USA https://www.seatoxresearch.com/testing-kits/) and can be used as screening tool for fish extracts.

Despite the undoubtable utility of the kit described above, it does not allow to know which CTXs are inside a sample. The best solution to obtain toxin profiles is to separate the toxins and HPLC is the method to perform this task. Since most CTXs do not have a characteristic chromophore group in their structure (i.e. alternating single and double bounds), they do not strongly absorb radiation over the UV/VIS region, and therefore spectroscopy is not viable for their detection. Indeed, the trials with classical HPLC method that uses UV detector showed not enough sensitivity to detect the presence of low concentrations of CTXs (Caillaud et al. 2010). Therefore, the HPLC with fluorescent detection has been tried, since some CTX congeners have a primary hydroxyl group available for fluorescent labelling. Even if this technique showed better sensitivity than the previous one, it does not detect CTXs at the recommended level (0.01 μg/kg). Additionally, it does not detect CTXs without a primary hydroxyl group (i.e. P-CTX-3C). Therefore, in order to increase the sensitivity and specificity of the system, Lewis and Jones (1997) combined the HPLC

technique with tandem mass spectrometry (HPLC-MS/MS) for the detection of CTXs. Then, Lewis and coworkers (1999) combined an electrospray triple quadrupole mass spectrometer with a gradient reverse-phased HPLC and, with this technique, a limit of detection of 0.04 ppb and 0.1 ppb for P-CTX1 and C-CTX1 was achieved (Lewis et al. 1999). Right after this first trials, LC-MS/MS become one of the most used, if not the most used, technique for detecting and identify CTXs. It must be underlined that CBA is the most used technique to perform sample screening, even though LC-MS/MS is the one that actually confirm the presence of CTXs. Although instrumental analysis techniques are highly sensitive, their application to monitoring programs is hampered by the cost of the machinery, the time needed to prepare the samples for the analysis and the need of highly trained personnel to perform the assays. Additionally, the analysis of CTXs in natural samples is limited by the lack of CTX standards, certified materials and the chemical complexity of the CTX compounds.
These limitations have also hindered the development of immunoassays, based on antibodies (Abs). These assays take advantage from the high specificity of the antigen-antibody reaction. The first group to produce anti-CTXs antibodies was the one of Hokama and coworkers (1977). In their work, they produced an anti-CTX polyclonal Ab (pAb) and labelled it with a radioactive compound to subsequently perform a radioimmunoassay directly on fish tissues from the Hawaiian Islands (Hokama et al. 1977; Kimura et al. 1982). The same pAb was labelled horseradish peroxidase and exploited in an immunoassay also for fish extracts (Hokama et al. 1983). The authors decided to simplify the enzyme immunoassay by formatting it into a faster stick test that did not require any instrumentation (Hokama 1985). These last findings were used to build two commercial kits named Cigua-Check (Hokama 1985; Hokama et al. 1987) and Ciguatect (Park 1995). Although these achievements represent an advance for the development of easy-to-use tests, since no extraction whatsoever was needed to perform the assay, these assays showed cross-reactivity with okadaic acid and brevetoxin (Hokama et al. 1987; Hokama et al. 1989. This cross-reactivity together with the low sensitivity led to false positive and false negative results, respectively (Bienfang et al. 2011). Thus, the only ciguateric fish reported up to date in the Mediterranean, which was analyzed with the Cigua-Check kit (Bentur and Spanier 2007), is still pending of confirmation. Although, in this particular occasion, one of the symptoms was the presence of hallucinations, which is not one of the common symptoms of CFP. Due to the disadvantages represented by the use of these pAbs, Hokama and coworkers (1990) decided to focus on the production of monoclonal antibodies (mAbs), that were subsequently used in a similar system but using colored latex beads for the labelling of the mAbs. Another approach to produce mAbs was based on the use of synthetic haptens instead of natural CTXs. The first work related to the use of such technique is the one of Campora and coworkers (Campora et al. 2008), which developed a sandwich enzyme linked immunosorbent assay ELISA, using one specific Ab for the left wing of P-CTX1B and one specific Ab for the right wing labeled with HRP. No cross-reactivity was observed with other marine toxins such as brevetoxin-3, okadaic acid or domoic acid.

Subsequently, Tsumuraya and coworkers immunized mice with haptens that mimic the left and right wing of the four principal congeners of pacific CTXs, CXT1B, 54-deoxy-CTX1B, CTX3C and 51-hydroxy-CTX3C. The resulting antibodies were used to develop colorimetric sandwich ELISAs. The assays performed with these mAbs demonstrate the high specificity and sensitivity that was expected, showing no cross-reactivity with other marine toxins toxins such as okadaic acid, maitotoxin, brevetoxin A, brevetoxin B (Oguri et al. 2003; Nagumo et al. 2004; Tsumuraya et al. 2006, 2010, 2012). Additionally, the previously described mAbs have been used to develop a fluorescent ELISA, whose LOD was as low as 1 pg/mL for both CTX1B and CTX3C group. Moreover, CTX1B was spiked into a fish extract at the suggested threshold and then detected with the presented technique (Tsumuraya et al. 2018; Tsumuraya and Hirama 2019). Based on this fluorescent technique, a kit named "CTX-ELISATM 1B" for the detection of the CTX1B group of congeners was marketed and can be bought from Fujifilm Wako Corporation (Osaka, Japan).
As previously mentioned for DNA, the CTXs detection is also a difficult achievement, if not the most difficult, in the picture of ciguatera management. The most reliable techniques are based on the use of expensive and delicate equipment that cannot be easily transported in field and requires several extraction and sample purification steps before the analysis, which are long and expensive. Additionally, these analyses need the equipment to be at precise and constant conditions of temperature and pressure, thing that can difficultly be achieved in remote tropical and subtropical areas endemic for ciguatera. Thus, the buildout of fast CTXs extraction techniques combined with the development robust and reliable tools based on molecules with high affinity for CTXs (i.e. antibodies), which would give reliable results in any condition, are extremely needed in the ciguatera management. In fact, such devices would screen samples with low CTXs concentrations in few hours, providing no false positive and no false negative results, without requiring many pre-treatment phases, making easier the ciguatera assessment in isolated endemic areas.

6. Biosensors

Biosensors are a practical and reliable tools to detect biological and chemical hazards. They are composed of a biorecognition element that interacts specifically with a target molecule, and a transducer that converts the biorecognition event into a quantifiable signal, both in intimate contact. The biorecognition element is the one that gives specificity to the system, and it could be an aptamer, enzyme, antibody, oligonucleotide, receptor, whole cell, bacteria, microorganism, animal or vegetal tissue. The transducer can be electrochemical, optical, gravimetric, and thermometric, according to the type of signal they transform in a measurable unit. Even if biosensors represent an interesting and useful tool for the detection of different type of analytes, they have been rarely used to detect DNA of toxin-producing microalgae. This is the case for *Karenia brevis* (LaGier et al. 2007), *Karlodinium armiger* (Magriñá et al. 2019), *Ostreopsis ovata* (Toldrà et al. 2019) and for some species of

Gambierdiscus (G. australes, G. excentricus and *G. silvae), Coolia (C. monotis, C. tropicalis* and *C.* cf. *canariensis), Ostreopsis* genus and *Prorocentrum lima* (Medlin et al., 2020). On the contrary, several biosensors have been developed for the detection of marine toxins, such as surface plasmon resonance immunosensors (palytoxins, tetrodotoxins), surface plasmon resonance receptor-based biosensors (palytoxins), electrochemical immunosensors (tetrodotoxins, okadaic acid, azaspiracids, domoic acid, saxitoxins, palytoxins, brevetoxins), electrochemical enzyme-based sensors (okadaic acid), electrochemical aptasensors (okadaic acid, brevetoxin-2, saxitoxin, tetrodotoxins), electrochemical cell-based biosensors (palytoxin), electrochemiluminescence immunosensors (palytoxins) (for more details see Reverté et al., 2014, Leonardo et al., 2017), although none of them targeted CTXs. The main reason behind the few existence of studies on CTXs has to be found in the considerable lack of standards, and the few existing ones being scarce and expensive. Therefore, the main challenge is to detect CTXs in natural samples at low concentrations, without interference from the matrixes or other marine toxic compounds. Considering the existence of molecular techniques that exploit the use of tailed primers for the development of sandwich hybridization assays for other microalgae detection (as described in Toldrà et al., 2019) and of antibodies with high affinity for CTXs and without cross reactivity with other marine toxins (as demonstrated in Tsumuraya 2006, 2010, 2012), the development of biosensors based on these methods for the detection of *Gambierdiscus* DNA and CTXs can be extremely interesting. In fact, the development of biosensors can act as a step forward in the obtainement of portable devices for the desired *in situ* detection, which would help the quick detection of DNA belonging to CTXs producing species or fishes naturally contaminated with CTXs, even at low concentration, and consequently, the spotting in advance of a possible ciguatera outbreak.

References

Abraham A, Jester ELE, Granade HR, Plakas SM, Dickey RW (2012). Caribbean ciguatoxin profile in raw and cooked fish implicated in ciguatera. Food Chem 131:192-198.

Adachi R, Fukuyo Y (1979). The thecal structure of a marine toxic dinoflagellate *Gambierdiscus toxicus* gen. et sp. nov. collected in a ciguatera-endemic area. Bull Jpn Soc Sci Fish 45:67-71.

Aligizaki K, Nikolaidis G (2008). Morphological identification of two tropical dinoflagellates of the genera *Gambierdiscus* and *Sinophysis* in the Mediterranean Sea. J Biol Res 9:75-82

Aligizaki K, Nikolaidis G, Katikou P, Baxevanis AD, Abatzopoulos TJ (2009) Potentially toxic epiphytic *Prorocentrum* (Dinophyceae) species in Greek coastal waters. Harmful Algae 8:299-311.

Agence nationale de sécurité sanitaire de l'alimentation, de l'environnement et du travail (ANSES) (2015). Contamination des requins, notamment tigre et bouledogue, par des ciguatoxines: occurrence, méthodes analytiques, cas humains rapportés et éléments d'éthologie. Avis de l'Anses Rapport d'expertise collective. Avis de l'Anses Saisine n° 2013-SA-0198.

Bagnis R (1981). The Ciguatera Type Ichtyosarcotoxism: a Complicated Phenomena of Marine and Human Biology. Oceanol. Acta Paris 4:375-387.

Bagnis R, Fevai G (1971). ciguatera feline experimentale a Tahiti. Rev Med Vet Toulouse 122: 629-638.

Benoit E, Legrand AM, Dubois JM (1986). Effects of ciguatoxin on current and voltage clamped frog myelinated nerve fibre. Toxicon 24:357-364.

Bentur Y, Spanier E (2007). Ciguatoxin-like substances in edible fish on the eastern Mediterranean, Clin Toxicol 45: 695-700.

Bienfang P, Oben B, DeFelice S, Moeller P, Huncik K, Oben P, Toonen R, Daly-Engel T, Bowen B (2010). Ciguatera: the detection of neurotoxins in carnivorous reef fish from the coast of Cameroon, Afr J Mar Sci 30:533-540.

Bienfang P, DeFelice S, Dowling A.(2011). Quantitative evaluation of commercially available test kit for ciguatera in fish. Food Sci Nutr 2:594-598.

Bravo I, Rodriguez F, Ramilo I, Rial P, Fraga S (2019). Ciguatera-causing dinoflagellate *Gambierdiscus* spp.(Dinophyceae) in a subtropical region of North Atlantic Ocean (Canary Islands): morphological characterization and biogeography. Toxins 11:423.

Caillaud A, de la Iglesia P, Darius HT, Pauillac S, Aligizaki K, Fraga S, Chinain M, Diogène J (2010). Update on methodologies available for ciguatoxin determination: perspectives to confront the onset of ciguatera fish poisoning in Europe. Mar Drugs 8:1838-1907.

Campora CE, Hokama Y, Yabusaki K, Isobe M (2008). Development of an enzyme-linked immunosorbent assay for the detection of ciguatoxin in

fish tissue using chicken immunoglobulin Y. J Clin Lab Anal 22:239-245.

Chinain M, Faust MA, Pauillac S (1999). Morphology and molecular analyses of three toxic species of *Gambierdiscus* (Dinophyceae): *G. pacificus*, sp. nov., *G. australes*, sp. nov., and *G. polynesiensis*, sp. nov. J Phycol 35:1282-1296.

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

Chinain M, Darius HT, Ung A, Fouc MT, Revel T, Cruchet P, Pauillac S, Laurent D (2010b). Ciguatera risk management in French Polynesia: The case study of Raivavae Island (Australes Archipelago). Toxicon 56:674-690.

Chinain M, Gatti CMI, Darius HT, Quod JP, Tester PA (2021). Ciguatera poisonings: A global review of occurrences and trends. Harmful Algae 102:101873.

Commission Regulation (EU) No. 853/2004 laying down specific hygiene rules for on the hygiene of foodstuffs. Off J Eur Union L139:55.

Darius HT, Drescher O, Ponton D, Pawlowiez R, Laurent D, Dewailly E, Chinain M (2013). Use of folk tests to detect ciguateric fish: a scientific evaluation of their effectiveness in Raivavae Island (Australes, French Polynesia). Food Addit Contam: Part A 30:550-566.

Darius HT, Roue M, Sibat M, Viallon J, Gatti C, Vandersea MW, Tester PA, Litaker RW, Amzil Z, Hess P, Chinain M (2018). Toxicological Investigations on the Sea Urchin *Tripneustes gratilla* (Toxopneustidae, Echinoid) from Anaho Bay (Nuku Hiva, French Polynesia): Evidence for the Presence of Pacific Ciguatoxins. Mar Drugs.

Darius HT; Revel T; Cruchet P; Viallon J; Gatti CMi.; Sibat M; Hess P; Chinain M (2021). Deep-water fish are potential vectors of ciguatera poisoning in the Gambier Islands, French Polynesia. Mar. Drugs 19: 644.

Díaz-Asencio L, Clausing RJ, Rañada ML, Alonso-Hernández CM, Bottein M-YD (2018). A radioligand receptor binding assay for ciguatoxin monitoring in environmental samples: Method development and determination of quality control criteria. J Environ Radioact 192:289-294.

Diogène J, Reverte L, Rambla-Alegre M, Del Rio V, de la Iglesia P, Campàs M, Palacios O, Flores C, Caixach J, Ralijaona C, Razanajatovo I, Pirog A, Magalon H, Arnich N, Turquet J (2017). Identification of ciguatoxins in a shark involved in a fatal food poisoning in the Indian Ocean. Sci Rep 7:8240.

Doherty MJ (2005). Captain Cook on poison fish. Neurology 65:1788-1791.

Endean R, Griffith JK, Robins JJ, Monks SA (1993). Multiple toxins in a specimen of the narrow-barred Spanish mackerel, *Scomberomorus commersoni*. Toxicon 31:195-204.

EFSA Panel on Contaminants in the Food Chain (2010). Scientific Opinion on marine biotoxins in shellfish emerging toxins: Ciguatoxin group. EFSA J. 8:1627.

Fairey ER, Edmunds JSG, Ramsdell JS (1997). A cell-based assay for brevetoxins, saxitoxins, and ciguatoxins using a stably expressed c-*fos*–luciferase reporter gene. Anal Biochem 251:129-132.

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

Fraga S, Rodriguez F (2014). Genus *Gambierdiscus* in the Canary Islands (NE Atlantic Ocean) with description of *Gambierdiscus silvae* sp. nov., a new potentially toxic epiphytic benthic dinoflagellate. Protist 165:839-853.

Food Standards Australia New Zealand (FZAN) (2006). A guide to the Australian Primary Production and Processing Standard for Seafood, Safe Seafood Australia. 2nd edn., Canberra.

Gómez F, Qiu D, Lopes RM, Lin S (2015). *Fukuyoa paulensis* gen. et sp. nov., a new genus for the globular species of the dinoflagellate *Gambierdiscus* (Dinophyceae). PLoS One 10.

Granade HR.; Cheng PC.; Doorenbos NJ.(1976). Ciguatera I: brine shrimp (*Artemia salina* L.) larval assay for ciguatera toxins. J Pharm Sci 65: 1414–1415.

Halstead BW (1967). Poisonous and venomous marine animals of the world. US Government Printing Office, Washington, DC, USA.

Halstead BW, Cox KW (1973). An investigation on fish poisoning in Mauritius.Vol. 4. Imprimerie Commerciale.

Halstead BW (1988). Poisonous and venomous marine animals of the world. Vol 1. Princeton, NJ (USA) Darwin Press.

Hamilton B, Hurbungs M, Vernoux J-P, Jones A, Lewis RJ (2002). Isolation and characterisation of Indian Ocean ciguatoxin. Toxicon 40:685-693.

Hamilton B, Whittle N, Shaw G, Eaglesham G, Moore MR, Lewis RJ (2010). Human fatality associated with Pacific ciguatoxin contaminated fish. Toxicon 56:668-673.

Hardison DR, Holland WC, McCall JR, Bourdelais AJ, Baden DG, Darius HT, Chinain M, Tester PA, Shea D, Flores Quintana HA (2016). Fluorescent receptor binding assay for detecting ciguatoxins in fish. PLoS One 11:e0153348.

Hidalgo J, Liberona JL, Molgó J, Jaimovich E (2002). Pacific ciguatoxin-1b effect over Na^+ and K^+ currents, inositol 1, 4, 5-triphosphate content and intracellular Ca^{2+} signals in cultured rat myotubes. Br J Pharmacol 137:1055-1062.

Hoffman PA, Granade HR, McMillan JP (1983). The mouse ciguatoxin bioassay: a dose-response curve and symptomatology analysis. Toxicon 21:363-369.

Hokama Y, Banner AH, Boylan DB (1977). A radioimmunoassay for the detection of ciguatoxin. Toxicon 15:317-325.

Hokama Y, Abad MA, Kimura LH (1983). A rapid enzyme-immunoassay for the detection of ciguatoxin in contaminated fish tissues. Toxicon 21:817-824.

Hokama Y (1985). A rapid, simplified enzyme immunoassay stick test for the detection of ciguatoxin and related polyethers from fish tissues. Toxicon 23:939-946.

Hokama Y, Shirai LK, Iwamoto LM, Kobayashi MN, Goto CS, Nakagawa LK (1987). Assessment of a rapid enzyme immunoassay stick test for the detection of ciguatoxin and related polyether toxins in fish tissues. Biol Bull 172:144-153.

Hokama Y, Honda SAA, Asahina AY, Fong JML, Matsumoto CM, Gallacher TS (1989). Cross-reactivity of ciguatoxin, okadaic acid, and polyethers with monoclonal antibodies. Food Agr Immunol1:29-35.

Hokama Y (1990). Simplified solid-phase immunobead assay for detection of ciguatoxin and related polyethers. J of clinical laboratory analysis 4:213-217.

Hokama Y, Asahina AY, Titus E, Ichinotsubo D, Chun S, Hong TWP, Shirai JL, Asuncion DA, Miyahara JT (1994). Assessment of ciguateric fish in Hawaii by immunological, mouse toxicity and guinea pig atrial assays. Mem Queensl Mus Brisbane 34:489-496.

IAMAT, 2017. Ciguatera Fish Poisoning. https://www.iamat.org/risks/ciguatera-fish- poisoning. Last accessed 08/11/2021.

Ikehara T, Kuniyoshi K, Oshiro N, Yasumoto T (2017). Biooxidation of Ciguatoxins Leads to Species-Specific Toxin Profiles. Toxins (Basel) 9:205.

Jang SH, Jeong HJ, Yoo YD (2018). *Gambierdiscus jejuensis* sp. nov., an epiphytic dinoflagellate from the waters of Jeju Island, Korea, effect of temperature on the growth, and its global distribution. Harmful Algae 80:149-157.

Jeong HJ, Lim AS, Jang SH, Yih WH, Kang NS, Lee SY, Yoo YD, Kim HS (2012). First Report of the Epiphytic Dinoflagellate *Gambierdiscus caribaeus* in the Temperate Waters off J eju Island, Korea: Morphology and Molecular Characterization. J Eukaryot Microbiol 59:637-650.

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

Kimura LH, Abad MA, Hokama Y (1982). Evaluation of the radioimmunoassay (RIA) for detection of ciguatoxin (CTX) in fish tissues. J Fish Biol 21:671-680.

Kretzschmar AL, Larsson ME, Hoppenrath M, Doblin MA, Murray SA (2019). Characterisation of two toxic *Gambierdiscus* spp. (Gonyaulacales, Dinophyceae) from the Great Barrier Reef (Australia): *G. lewisii* sp. nov. and *G. holmesii* sp. nov. Protist 170.

Labrousse H, Matile L (1996). Toxicological biotest on Diptera larvae to detect ciguatoxins and various other toxic substances. Toxicon 34:881-891.

LaGier MJ, Fell JW, Goodwin KD (2007). Electrochemical detection of harmful algae and other microbial contaminants in coastal waters using hand-held biosensors. Mar Pollut Bull 54:757-770.

Lavenu L, Chomérat N, Díaz-Asencio L, Gerry C, Belmont, C. , Hollanda S, Tunin- Ley A, Dechraoui Bottein MY *Gambierdiscus* from Seychelles: morphology, molecular identification and toxicity. In: 18th International Conference on Harmful Algae 2018. p 497

Laza-Martínez A, David H, Riobó P, Miguel I, Orive E (2016). Characterization of a strain of *Fukuyoa paulensis* (Dinophyceae) from the Western Mediterranean Sea. J Eukaryot Microbiol 63:481-497.

Lehane L, Lewis RJ (2000). Ciguatera: recent advances but the risk remains. Int. J Food Microbiol 61:91-125.

Legrand AM, Teai T,Cruchet P, Satake M,Murata K,Yasumoto T (1998). Two structural types of ciguatoxin involved in ciguatera fish poisoning in French Polynesia. In: Reguera B, Blanco J, Fernandez ML, Wyatt T (eds). VIII International Conference on Harmful Algae. Xunta de Galicia and International Oceanographic Commission of UNESCO, Paris, France, pp 473–475.

Leonardo, S, Toldrà, A and Campàs, M (2017). Trends and prospects on electrochemical biosensors for the detection of marine toxins. In: Diogène J, Campàs M (eds). Recent advances in the analysis of marine toxins, comprehensive analytical chemistry, Elsevier, pp 303-341.

Lewis RJ, Sellin M, Poli MA, Norton RS, MacLeod JK, Sheil MM (1991). Purification and characterization of ciguatoxins from moray eel (*Lycodontis javanicus*, Muraenidae). Toxicon 29:1115-1127.

Lewis RJ (1994). Immunological, biochemical and chemical features of ciguatoxins implications for the detection of ciguateric fish. Mem Queensl Mus Brisbane 34:541-548.

Lewis RJ, Jones A (1997). Characterization of ciguatoxins and ciguatoxin congeners present in ciguateric fish by gradient reverse-phase high-performance liquid chromatography/mass spectrometry. Toxicon 35:159-168.

Lewis RJ, Vernoux J-P, Brereton IM (1998). Structure of Caribbean ciguatoxin isolated from *Caranx latus*. J Am Chem Soc 120:5914-5920.

Lewis RJ, Jones A, Vernoux JP (1999). HPLC/tandem electrospray mass spectrometry for the determination of sub-ppb levels of Pacific and Caribbean ciguatoxins in crude extracts of fish. Anal Chem 71:247-250.

Lewis RJ, Inserra M, Vetter I, Holland WC, Hardison DR, Tester PA, Litaker RW (2016). Rapid extraction and identification of maitotoxin and ciguatoxin-like toxins from Caribbean and Pacific *Gambierdiscus* using a new functional bioassay. PLoS One 11.

Lewis RJ, Vetter I (2016). Ciguatoxin and Ciguatera. In: Gopalakrishnakone P, Haddad VJ, Tubaro A, Kim E, Kem WR (eds). Marine and Freshwater

Toxins, Toxinology. Springer Science+Business Media: Dordrecht, The Netherlands, pp 71-92.

Li Z, Park JS, Kang NS, Chomérat N, Mertens KN, Gu H, Lee KW, Kim KH, Baek SH, Shin K, Han KH, Son MH, Shin HH (2021). A new potentially toxic dinoflagellate *Fukuyoa koreansis* sp. nov. (Gonyaulacales, Dinophyceae) from Korean coastal waters: Morphology, phylogeny, and effects of temperature and salinity on growth. Harmful Algae 109:102107.

Litaker RW, Vandersea MW, Faust MA, Kibler SR, Chinain M, Holmes MJ, Holland WC, Tester PA (2009). Taxonomy of *Gambierdiscus* including four new species, *Gambierdiscus caribaeus*, *Gambierdiscus carolinianus*, *Gambierdiscus carpenteri* and *Gambierdiscus ruetzleri* (Gonyaulacales, Dinophyceae). Phycologia 48:344-390.

Litaker RW, Holland WC, Hardison DR, Pisapia F, Hess P, Kibler SR, Tester PA (2017). Ciguatoxicity of *Gambierdiscus* and *Fukuyoa* species from the Caribbean and Gulf of Mexico. PLoS One 12:e0185776.

Litaker RW, Tester PA, Vandersea MW (2019). Species-specific PCR assays for *Gambierdiscus excentricus* and *Gambierdiscus silvae* (Gonyaulacales, Dinophyceae). J Phycol 55:730-732.

Lombet A, Bidard J-N, Lazdunski M (1987). Ciguatoxin and brevetoxins share a common receptor site on the neuronal voltage-dependent Na^+ channel. FEBS letters 219:355-359.

Longo S, Sibat M, Viallon J, Darius HT, Hess P, Chinain M (2019). Intraspecific variability in the toxin production and toxin profiles of in vitro cultures of *Gambierdiscus polynesiensis* (Dinophyceae) from French Polynesia. Toxins (Basel) 11:735.

Lugomela C (2006). Autecology of the Toxic Dinoflagellate *Gambierdiscus toxicus* Adachi et Fukuyo (Dinophyceae) in Central Coastal Areas of Tanzania. Western Indian Ocean J Mar Sci 5:213-221.

Magriñá I, Toldrà A, Campàs M, Ortiz M, Simonova A, Katakis I, Hocek M, O'Sullivan CK (2019). Electrochemical genosensor for the direct detection of tailed PCR amplicons incorporating ferrocene labelled dATP. Biosens Bioelectron 134:76-82.

Mak YL, Wai T-C, Murphy MB, Chan WH, Wu JJ, Lam JCW, Chan LL, Lam PKS (2013). Pacific ciguatoxins in food web components of coral reef systems in the Republic of Kiribati. Environ Sci Technol 47:14070-14079.

Manger RL, Leja LS, Lee SY, Hungerford JM, Wekell MM (1993). Tetrazolium-based cell bioassay for neurotoxins active on voltage-sensitive sodium channels: semiautomated assay for saxitoxins, brevetoxins, and ciguatoxins. Anal Biochem 214:190-194.

McCall JR, Jacocks HM, Niven SC, Poli MA, Baden DG, Bourdelais AJ (2014). Development and utilization of a fluorescence-based receptor-binding assay for the site 5 voltage-sensitive sodium channel ligands brevetoxin and ciguatoxin. J AOAC Int 97:307-315.

Medlin LK, Gamella M, Mengs G, Serafín V, Campuzano S, J MP (2020). Advances in the Detection of Toxic Algae Using Electrochemical Biosensors. Biosensors 10.

Miller DM, Tindall DR, Tibbs B (1986). Ciguatera-type toxins: Bioassay using crayfish nerve cord In, 1986. (Abstract1103). Fed Proc Abstr 45:344

Ministry of Health W, and Labour (MHWL) (1953). A ban on domestic sales of barracuda. MHWL notification No. 20. vol 20.

Ministry of Health W, and Labour (MHWL) (2001). Handling of ciguatera fish, Office memorandum, by MHWL to heads of quarantine stations.

Molgó J, Cornelia JX, Legrand AM (1990). Ciguatoxin enhances quantal transmitter release from frog motor nerve terminals. Br J Pharmacol 99:695-700.

Molgó J, Shimahara T, Legrand AM (1993). Ciguatoxin, extracted from poisonous morays eels, causes sodium-dependent calcium mobilization in NG108-15 neuroblastoma× glioma hybrid cells. Neurosci Lett 158:147-150.

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

Nagumo Y, Oguri H, Tsumoto K, Shindo Y, Hirama M, Tsumuraya T, Fujii I, Tomioka Y, Mizugaki M, Kumagai I (2004). Phage-display selection of antibodies to the left end of CTX3C using synthetic fragments. J Immunol Methods 289:137-146.

Nellis DW, Barnard GW (1986). Ciguatera: A legal and social overview. Mar Fish Rev 48:2-5

Nishimura T, Sato S, Tawong W, Sakanari H, Yamaguchi H, Adachi M (2014). Morphology of *Gambierdiscus scabrosus* sp. nov.(Gonyaulacales): a new epiphytic toxic dinoflagellate from coastal areas of Japan. J Phycol 50:506-514.

Nishimura T, Hariganeya N, Tawong W, Sakanari H, Yamaguchi H, Adachi M (2016). Quantitative PCR assay for detection and enumeration of ciguatera-causing dinoflagellate *Gambierdiscus* spp.(Gonyaulacales) in coastal areas of Japan. Harmful Algae 52:11-22.

Oguri H, Hirama M, Tsumuraya T, Fujii I, Maruyama M, Uehara H, Nagumo Y (2003). Synthesis-based approach toward direct sandwich immunoassay for ciguatoxin CTX3C. J Am Chem Soc 125:7608-7612.

Park DL (1995). Detection of ciguatera and diarrheic shellfish toxins in finfish and shellfish with ciguatect kit. J AOAC Int 78:533-537.

Parsons ML, Settlemier CJ, Bienfang PK (2010). A simple model capable of simulating the population dynamics of *Gambierdiscus*, the benthic dinoflagellate responsible for ciguatera fish poisoning. Harmful algae 10:71-80.

Pasinszki T, Lako J, Dennis TE (2020). Advances in detecting ciguatoxins in fish. Toxins 12:494.

Pérez-Arellano J-L, Luzardo OP, Brito AP, Cabrera MH, Zumbado M, Carranza C, Angel-Moreno A, Dickey RW, Boada LD (2005). Ciguatera fish poisoning, Canary Islands. Emerg Infect Dis 11:1981.

Pisapia F, Holland WC, Hardison DR, Litaker RW, Fraga S, Nishimura T, Adachi M, Nguyen-Ngoc L, Sechet V, Amzil Z, Herrenknecht C, Hess P (2017). Toxicity screening of 13 *Gambierdiscus* strains using neuro-2a and erythrocyte lysis bioassays. Harmful Algae 63:173-183.

Pottier I, Vernoux J-P, Jones A, Lewis RJ (2002). Characterisation of multiple Caribbean ciguatoxins and congeners in individual specimens of horse-eye jack (*Caranx latus*) by high-performance liquid chromatography/mass spectrometry. Toxicon 40:929-939.

Ragelis EP (1984). Ciguatera seafood poisoning: overview. Seafood Toxin. 262:25-36.

Reverté L, Soliño L, Carnicer O, Diogène J, Campàs M (2014). Alternative methods for the detection of emerging marine toxins: Biosensors, biochemical assays and cell-based assays. Mar Drugs.12:5719-63.

Reverté L, Toldrà A, Andree KB, Fraga S, de Falco G, Campàs M, Diogène J (2018). Assessment of cytotoxicity in ten strains of *Gambierdiscus australes* from Macaronesian Islands by neuro-2a cell-based assays. J Appl Phycol 30:2447-2461.

Rhodes L, Harwood T, Smith K, Argyle P, Munday R (2014). Production of ciguatoxin and maitotoxin by strains of *Gambierdiscus australes*, *G. pacificus* and *G. polynesiensis* (Dinophyceae) isolated from Rarotonga, Cook Islands. Harmful Algae 39:185-190.

Rhodes LL, Smith KF, Murray S, Harwood DT, Trnski T, Munday R (2017). The epiphytic genus *Gambierdiscus* (Dinophyceae) in the Kermadec Islands and Zealandia regions of the Southwestern Pacific and the associated risk of ciguatera fish poisoning. Mar Drugs 15:219.

Rodríguez F, Fraga S, Ramilo I, Rial P, Figueroa RI, Riobó P, Bravo I (2017). Canary Islands (NE Atlantic) as a biodiversity 'hotspot'of *Gambierdiscus*: Implications for future trends of ciguatera in the area. Harmful Algae 67:131-143.

Rongo T, van Woesik R (2011). Ciguatera poisoning in Rarotonga, southern Cook islands. Harmful Algae 10:345-355.

Rossignoli AE, Tudo A, Bravo I, Diaz PA, Diogène J, Riobo P (2020). Toxicity characterisation of *Gambierdiscus* species from the Canary Islands. Toxins (Basel) 12:134.

Roué M, Darius HT, Picot S, Ung A, Viallon J, Gaertner-Mazouni N, Sibat M, Amzil Z, Chinain M (2016). Evidence of the bioaccumulation of ciguatoxins in giant clams (*Tridacna maxima*) exposed to *Gambierdiscus* spp. cells. Harmful Algae 57:78-87.

Sanchez-Henao JA, García-Álvarez N, Fernández A, Saavedra P, Sergent FS, Padilla D, Acosta-Hernández B, Suárez MM, Diogène J, Real F (2019). Predictive score and probability of CTX-like toxicity in fish samples from the official control of ciguatera in the Canary Islands. Sci Total Environ 673:576-584.

Shimojo RY, Iwaoka WT (2000). A rapid hemolysis assay for the detection of sodium channel-specific marine toxins. Toxicology 154:1-7.

Silva M, Rodriguez I, Barreiro A, Kaufmann M, Isabel Neto A, Hassouani M, Sabour B, Alfonso A, Botana LM, Vasconcelos V (2015). First Report of Ciguatoxins in Two Starfish Species: *Ophidiaster ophidianus* and *Marthasterias glacialis*. Toxins (Basel) 7:3740-3757.

Smith KF, Biessy L, Argyle PA, Trnski T, Halafihi T, Rhodes LL (2017). Molecular identification of *Gambierdiscus* and *Fukuyoa* (Dinophyceae) from environmental samples. Mar Drugs 15:243.

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

Tester PA, Vandersea MW, Buckel CA, Kibler SR, Holland WC, Davenport ED, Clark RD, Edwards KF, Taylor JC, Vander Pluym JL (2013). *Gambierdiscus* (Dinophyceae) species diversity in the flower garden banks national marine sanctuary, Northern Gulf of Mexico, USA. Harmful Algae 29:1-9.

Tester PA, Wickliffe L, Jossart J, Rhodes L, Enevoldsen H, Adachi M, Nishimura T, Rodriguez F, Chinain M, Litaker, W (2018). Global distribution of the genera *Gambierdiscus* and *Fukuyoa*. In: Hess P (ed). Harmful Algae 2018 - From ecosystems to socioecosystems. Proceedings of the 18th Intl. Conf. on Harmful Algae. Nantes, International Society for the Study of Harmful Algae, pp 138-143.

Tester PA, Berdalet E, Litaker RW, (2020). Climate change and benthic harmful microalgae. Harmful Algae 91, 101655.

Toldrà A, Alcaraz C, Diogène J, O'Sullivan CK, Campàs M (2019). Detection of *Ostreopsis* cf. *ovata* in environmental samples using an electrochemical DNA-based biosensor. Sci Total Environ 689:655-661.

Tsumuraya T, Fujii I, Inoue M, Tatami A, Miyazaki K, Hirama M (2006). Production of monoclonal antibodies for sandwich immunoassay detection of ciguatoxin 51-hydroxyCTX3C. Toxicon 48:287-294.

Tsumuraya T, Fujii I, Hirama M (2010). Production of monoclonal antibodies for sandwich immunoassay detection of Pacific ciguatoxins. Toxicon 56:797-803.

Tsumuraya T, Takeuchi K, Yamashita S, Fujii I, Hirama M (2012). Development of a monoclonal antibody against the left wing of ciguatoxin CTX1B: thiol strategy and detection using a sandwich ELISA. Toxicon 60:348-357.

Tsumuraya T, Sato T, Hirama M, Fujii I (2018). Highly Sensitive and Practical Fluorescent Sandwich ELISA for Ciguatoxins. Anal Chem 90:7318-7324.

Tsumuraya T, Hirama M (2019). Rationally designed synthetic haptens to generate anti-ciguatoxin monoclonal antibodies, and development of a practical sandwich ELISA to detect ciguatoxins. Toxins 11:533.

Tudó À, Toldrà A, Andree KB, Rey M, Fernández-Tejedor M, Campàs M, Diogène J (2018). First report of *Gambierdiscus* in the Western Mediterranean Sea (Balearic Islands). Harmful Algae News

Tudó À, Toldrà A, Rey M, Todolí I, Andree KB, Fernández-Tejedor M, Campàs M, Sureda FX, Diogène J (2020). *Gambierdiscus* and *Fukuyoa* as potential indicators of ciguatera risk in the Balearic Islands. Harmful Algae 99:101913.

Turquet J (1998). Assemblage of benthic dinofagellates and monitoring of harmful species in Reunion Island, SW Indian Ocean, 1993-1996. Harmful algae:44-47.

Vandersea MW, Kibler SR, Holland WC, Tester PA, Schultz TF, Faust MA, Holmes MJ, Chinain M, Wayne Litaker R (2012). Development of semi-quantitative pcr assays for the detection and enumeration of *Gambierdiscus* species (gonyaulacales, dinophyceae). J Phycol 48:902-915.

Vernoux J-P, Lahlou N, Magras LPH, Greaux JB (1985). Chick feeding test: A simple system to detect ciguatoxin. Acta Tropica 42:235-240.

Vernoux J-P, Lewis RJ (1997). Isolation and characterisation of Caribbean ciguatoxins from the horse-eye jack (*Caranx latus*). Toxicon 35:889-900.

Viallon J, Chinain M, Darius HT (2020). Revisiting the neuroblastoma cell-based assay (CBA-N2a) for the improved detection of marine toxins active on voltage gated sodium channels (VGSCs). Toxins 12:281.

Objectives

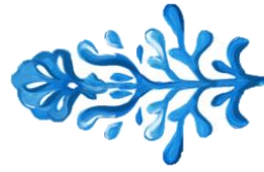
The main objective of this thesis is to provide biotechnological tools for the characterization of the risk of ciguatera in order to promote food safety and protect human health. In particular, the major focus is the development of bioanalytical devices for the detection of different *Gambierdiscus/Fukuyoa* species and of ciguatoxins (CTXs). Additionally, this thesis aims at providing fast and reliable strategies to shorten and simplify the sample pretreatment necessary for the analysis of environmental samples.

With this purpose, the following specific objectives have been established:

- To test portable devices for the development of fast extraction techniques for both DNA and CTXs from low cell concentrations of *Gambierdiscus* and *Fukuyoa*.
- To exploit the advantages of tailed primers in both PCR and fast isothermal amplification to simultaneous obtain amplified products flanked with oligonucleotides tails from DNA of *Gambierdiscus* and *Fukuyoa* genera and two toxin producing *Gambierdiscus* species (*G. australes* and *G. excentricus*).
- To develop a dual biosensor for the simultaneous detection of DNA from the two CTXs-producing species *G. australes* and *G. excentricus* on the same electrode array.
- To develop a biosensor based on antibodies that target specifically four main CTX congeners belonging to two groups (CTX1B and CTX3C) for the combined or separate detection of them.
- To evaluate the applicability of the developed biotechnological tools for the screening of algal samples from laboratory cultures, microalgal field samples and fishes naturally contaminated with CTXs.

Generally, this thesis' intention is to demonstrate the applicability of fast and easy-to-use biotechnological tools that can be easily implemented in portable devices for the screening of natural samples, paving the way for the *in situ* detection of CTXs-producing species and CTXs, speeding up monitoring analysis and spotting of possible ciguatera outbreaks.

Scientific publications



The analysis performed during the development of this thesis resulted in eleven publications, one of which is currently in progress. Five of the publications represent the core of the thesis and are divided in two main sections, (1) *Gambierdiscus* and *Fukuyoa* DNA detection and (2) Ciguatoxin detection, according to the purpose of each of the study performed. Additionally, an annexes section has been added containing the other six publications, which have been accomplished in parallel to the main ones. In the following paragraphs, all the publications are listed, specifying my personal contribution to each of them.

Section 1: *Gambierdiscus* and *Fukuyoa* DNA detection

1. G. Gaiani, A. Toldrà, K.B. Andree, M. Rey, C. Alcaraz, J. Diogène, C.K. O'Sullivan, M. Campàs. Detection of *Gambierdiscus* and *Fukuyoa* single cells using recombinase polymerase amplification combined with a sandwich hybridization assay (2021). *Journal of Applied Phycology,* 33, 2273-2282. (IF 2020: 3.215; Q1).

   Personal contribution:
   - DNA extraction from laboratory cultures pellets and molecular identification;
   - Recombinase polymerase amplification and sandwich hybridization assay;
   - Extraction and detection of DNA from single cells;
   - Design of the DNA combination strategy to assess the applicability of the system;
   - Manuscript writing.

2. G. Gaiani, F. Cucchi, A. Toldrà, K.B. Andree, M. Rey, T. Tsumuraya, C.K. O'Sullivan, J. Diogène, M. Campàs. Electrochemical biosensor for the dual detection of *Gambierdiscus australes* and *Gambierdiscus excentricus* in field samples. First report of *G. excentricus* in the Balearic Islands. (2022). *Science of the Total Environment*, 86:150915. (IF 2020: 7.963, Q1).

   Personal contribution:
   - Application and optimization of the new magnetic beads based DNA extraction protocol optimization;

- Application and optimization of the multiplex PCR protocol;
- Development, optimization and characterization of the magnetic bead-based biosensor for the dual detection of *G. australes* and *G. excentricus*;
- Field samples analysis;
- First report of *G. excentricus* in Balearic Island;
- Manuscript writing.

Section 2: Ciguatoxin detection

3. S. Leonardo, G. Gaiani, T. Tsumuraya, M. Hirama, J. Turquet, N. Sagristà, M. Rambla-Alegre, C. Flores, J. Caixach, J. Diogène, C.K. O'Sullivan, C. Alcaraz, M. Campàs (2020). Addressing the analytical challenges for the detection of ciguatoxin using an electrochemical biosensor. *Analytical Chemistry*, 92:4858-4865. (IF 2020: 6.986, Q1)

   Personal contribution:
   - Analysis of naturally contaminated fish samples with the developed immunoassay and immunosensor;
   - Correlation analysis among the different tests performed;
   - Manuscript writing: section on fish matrix effects and recovery and analysis of fish samples.

4. Multi-approached detection of a ciguateric fish in the Mediterranean Sea. (manuscript in progress)

   Personal contribution:
   - Fish flesh extract fractionation;
   - Magnetic bead-based immunoassay of the extract fractions;
   - Manuscript writing: section of the immunoassay of the extract fractions and map.

5. G. Gaiani, S. Leonardo, A. Tudó, A. Toldra, K.B. Andree, M. Rey, T. Tsumuraya, M. Hirama, J. Diogène, C. K. O'Sullivan, C. Alcaraz, M. Campàs (2020). Rapid detection of ciguatoxins in *Gambierdiscus* and *Fukuyoa* with immunosensing tools. *Ecotoxicology and Environmental Safety*, 204:111004. (IF 2020: 6.291, Q1).

   Personal contribution:
   - Development of a new fast ciguatoxin extraction protocol;
   - Characterization of the system with colorimetric assay;
   - Development of a biosensor for the detection of two main group of CTXs congeners;

- Analysis of laboratory cultures of *Gambierdiscus* and *Fukuyoa* at low cell concentration with the developed immunoassay and immunosensor;
- Manuscript writing.

Annexes section:

6. G. Gaiani, C.K. O'Sullivan, M. Campàs (2019). Magnetic Beads in Marine Toxin Detection: A Review. *Magnetochemistry*, 5, 62. (IF 2019: 1.947, Q2).

   Personal contribution:
   - Manuscript writing.

7. G. Gaiani, J. Diogène, M. Campàs. Addressing ciguatera risk using biosensors for the detection of *Gambierdiscus* and ciguatoxins, in: P.D. Hansen, J.L. Marty, F. Regan, D. Barceló (Eds.), Biosensors for the Marine Environment: Present and Future Challenges, Springer.

   Personal contribution:
   - Manuscript writing.

8. A. Tudó, G. Gaiani, M. Rey, T. Tsumuraya, K.B. Andree, M. Fernández-Tejedor, M. Campàs, J. Diogène (2020). Further Advances of *Gambierdiscus* Species in the Canary Islands, with the First Report of *Gambierdiscus belizeanus. Toxins.* 12(11), 692. (IF 2020: 3.895, Q1).

   Personal contribution:
   - Ciguatoxin extraction from a *G. belizeanus* laboratory culture;
   - Evaluation of the presence of two series of ciguatoxins congeners in the *G. belizeanus* extract with the immunoassay and immunosensor technique previously developed;
   - Manuscript writing: section related to the immunoassay and immunosensor techniques.

9. M. Campàs, M. Alkassar, G. Gaiani, S. Leonardo, M. Rambla-Alegre, J. Diogène (2021). The wide spectrum of methods available to study marine neurotoxins, in: A. Novelli, M.T. Fernandez-Sanchez, M. Aschner and L. G. Costa (Eds.), Marine Neurotoxins Vol 6, Elsevier. pp 275-302.

   Personal contribution:

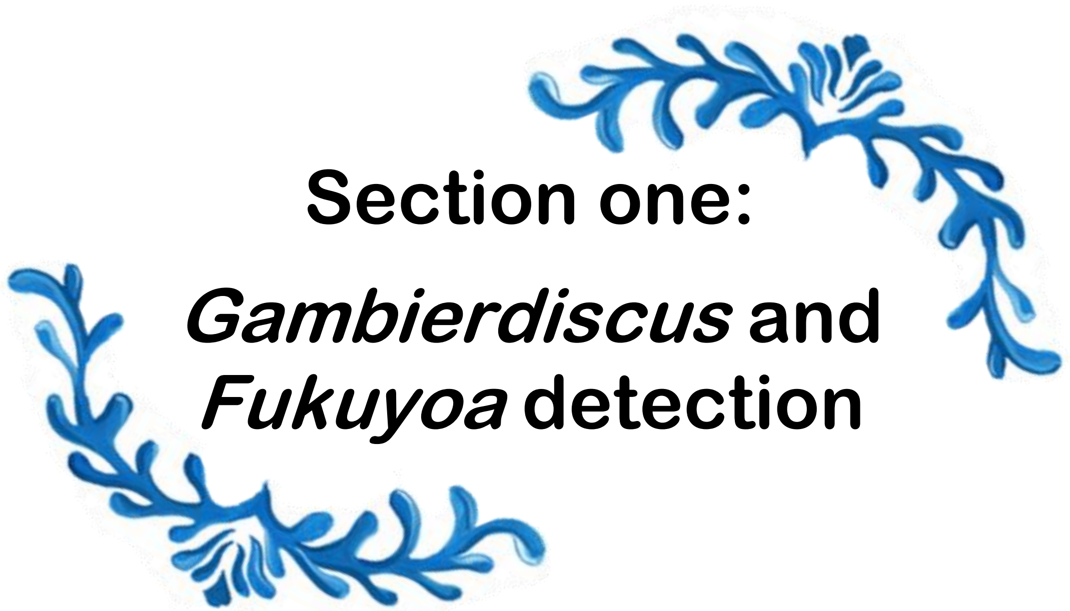
- Manuscript writing: “ciguatoxin case” section.

10. I. Ginés, G. Gaiani, A. Ruhela, V. Skouridou, M. Campàs, L. Masip. Nucleic acid lateral flow dipstick assay for the duplex detection of *Gambierdiscus australes* and *Gambierdiscus excentricus* (2021). *Harmful Algae*, 110:102135. (IF 2020: 4.273, Q1)

    Personal contribution:
    - Preparation of microalgal pellets from laboratory cultures;
    - DNA extraction of the microalgal laboratory cultures pellets;
    - Design of the DNA combination strategy to assess the applicability of the system;
    - Manuscript writing: microalgae-related sections.

11. G. Gaiani, M. Rey, A. Tudó, M. Rambla-Alegre, J. Diogène, M. Campàs, C. Alcaraz (2021). New information about the toxicological profile of the dinoflagellate *Prorocentrum panamense* (Prorocentrales, Dinophyceae) and its global distribution. *Phycological Research.* (Revised version submitted) (IF 2020: 1.675, Q3)

    Personal contribution:
    - DNA extraction and sample preparation for sequencing;
    - Molecular analysis;
    - Manuscript writing.



Section one:

Gambierdiscus and *Fukuyoa* detection

Chapter 1

Detection of *Gambierdiscus* and *Fukuyoa* single cells using recombinase polymerase amplification combined with a sandwich hybridization assay

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Abstract

Dinoflagellates of the genera *Gambierdiscus* and *Fukuyoa* are known to produce several bioactive compounds including the potent neurotoxic ciguatoxins (CTXs), which are able to accumulate in fish and through the food web. When humans ingest fish contaminated with CTXs, it can result in an intoxication named ciguatera. Although not all the currently recognized species are able to produce toxins, *G. australes* and *G. excentricus* have been highlighted to be the most abundant and toxic among the species present in the Atlantic. Even though genus *Gambierdiscus* and *Fukuyoa* are endemic to tropical areas, recently their presence was recorded in subtropical and temperate regions. In this work, the development of three molecular assays for the detection of the *Gambierdiscus* and *Fukuyoa* genera and for *G. australes* and *G. excentricus* species, based on the combination of recombinase polymerase amplification with detection via hybridization, is successfully described. Furthermore, a remarkable limit of detection of a single cell was achieved. Additionally, six different species have been used to check the ability of each primer set to give an amplified product, even in presence of potentially

interfering non-target DNAs. Therefore, these developments provide a rapid and cost-effective strategy for detection of both genera and two of the most toxic species, which will undoubtedly contribute to reliable screening of samples and ciguatera risk assessment, guaranteeing seafood safety and protection of human health.

1. Introduction

Marine dinoflagellates are well-established as toxin producers, and have thus attracted the attention of researchers worldwide, with the epibenthic genera *Gambierdiscus* and *Fukuyoa* being of particular interest due to their ability to produce the potent neurotoxic ciguatoxins (CTXs) (Chinain et al. 2010; Yasumoto et al. 2000; Yogi et al. 2011), maitotoxins (MTXs) (Holmes and Lewis 1994; Murata et al. 1993; Pisapia et al. 2017b), and other bioactive compounds (Nagai et al. 1992; Satake et al. 1993; Watanabe et al. 2013). When *Gambierdiscus* and *Fukuyoa* cells are grazed by herbivorous and detritivorous fish, these toxins accumulate through the food web, potentially reaching humans and causing one of the most common foodborne diseases, known as ciguatera (Begier et al. 2006; Larsson et al. 2019; Lewis 2001; Smith et al. 2017). The presence of *Gambierdiscus* and *Fukuyoa* in tropical areas is well known (Lewis 2001; Stewart et al. 2010; Vandersea et al. 2012). However, in the past decade, *Gambierdiscus* and *Fukuyoa* have been recorded in subtropical and temperate regions, such as the Canary Islands (Fraga and Rodriguez 2014; Fraga et al. 2011; Litaker et al. 2017), Madeira(Kaufmann and Böhm-Beck 2013), the Mediterranean Sea (Aligizaki and Nikolaidis 2008; Aligizaki et al. 2009; Laza-Martínez et al. 2016; Tudó et al. 2018), the Gulf of Mexico(Gómez et al. 2015; Litaker et al. 2017; Litaker et al. 2009), Japan (Nishimura et al. 2014), Brazil (Gómez et al. 2015; Laza-Martínez et al. 2016) and the coast of North Carolina(Litaker et al. 2017; Litaker et al. 2009). The reason behind the increase of these new findings is still unclear. Whether this is due to a worldwide expansion of these genera or because more intense samplings have been performed in the last few years, global warming has most certainly played and will continue to play a role in favor of their proliferation. This will create changes in the diversity and distribution of *Gambierdiscus* and *Fukuyoa* species, resulting in the spread of those species in new areas and potentially increasing the occurrence of ciguatera.

Progress in the field has underlined the existence of 18 species of *Gambierdiscus* (Chinain et al. 1999; Fraga et al. 2011; Jang et al. 2018; Kretzschmar et al. 2019; Litaker et al. 2009; Nishimura et al. 2014; Rhodes et al. 2017) and 3 species of *Fukuyoa* (Gómez et al. 2015). Whilst only some species have been demonstrated to be toxic (*F. paulensis, G. australes, G. caribaeus, G. excentricus, G. pacificus, G. polynesiensis* and *G. toxicus*) (Chinain et al. 2010; Fraga et al. 2011; Gaiani et al. 2020; Litaker et al. 2017;

Longo et al. 2019; Pisapia et al. 2017a; Rhodes et al. 2014; Rossignoli et al. 2020; Sibat et al. 2018), the ability to detect these genera and discriminate among *Gambierdiscus* and *Fukuyoa* species in field samples is of utmost interest. Light microscopy (LM) and electron microscopy allow the identification and discrimination between *Gambierdiscus* and *Fukuyoa* genera, but species identification is almost impossible using those methods. Therefore, the use of genetic sequencing techniques is practically mandatory for the correct identification of *Gambierdiscus* and *Fukuyoa* cells (Bravo et al. 2019). As an alternative approach to save time and resources, molecular assays, based mainly on the use of quantitative polymerase chain reaction (qPCR), have appeared for the identification and quantification of *Gambierdiscus* and *Fukuyoa* genera or species. These assays have been demonstrated to detect *G. belizeanus*, *G. caribaeus*, *G. carpenteri*, *G. carolinianus*, *G. ruetzleri* and *Gambierdiscus* sp. ribotype 2 (Vandersea et al. 2012), *G. australes*, *G. scabrosus*, *Gambierdiscus* sp. type 2 and *Gambierdiscus* sp. type 3 (Nishimura et al. 2016), *Gambierdiscus/Fukuyoa* and *F. paulensis* (Smith et al. 2017), *G. excentricus* and *G. silvae* (Litaker et al. 2019) and *G. lapillus* (Kretzschmar et al. 2019).

PCR-based methods require the use of a thermocycler, often laboratory based, and can require several hours to perform, with this delay resulting in a lengthy period between sampling and the analysis of results. Isothermal DNA amplification techniques may overcome these limitations. These techniques facilitate rapid DNA amplification at a constant temperature, requiring less time and power than conventional PCR. Although handheld PCR-based devices are commercially available, the use of a constant temperature for DNA amplification could simplify the hardware. Among isothermal DNA amplification techniques, recombinase polymerase amplification (RPA) is very convenient as it does not require any initial denaturation step, can be carried out at 22 – 45 °C without any need for tight temperature control, only requires two primers, and can be completed in 15-30 min. In this work, we exploited the use of primers modified with short oligonucleotide tails, which result in double-stranded DNA (dsDNA) amplicons fringed with single-stranded DNA (ssDNA) tails, avoiding the need for denaturation of the amplified products to generate ssDNA for detection by hybridization. The detection is achieved using a sandwich hybridization assay (SHA), where specific surface-anchored thiolated capture probes are complementary to one of the amplicon tails and an enzyme-labelled reporter probe is complementary to the tail in the other extreme (Figure 1). Despite its undeniable advantages, this approach has been

barely used for the detection of marine toxic dinoflagellates (Toldrà et al. 2019a; Toldrà et al. 2019b; Toldrà et al. 2018).

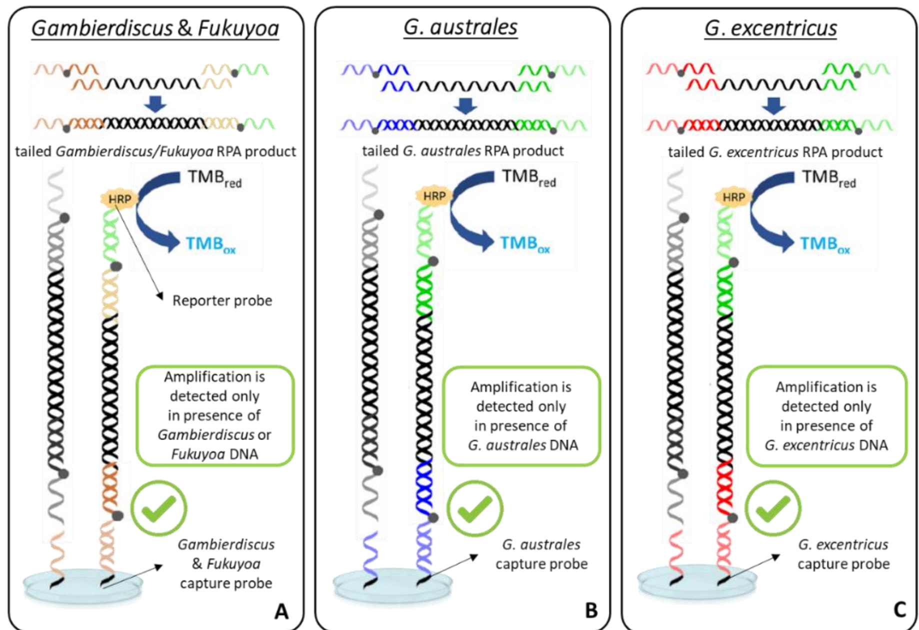

Figure 2 The three systems used in this work. (A) *Gambierdiscus* & *Fukuyoa* primer set amplifies DNA from all the species of both genera and it does not detect DNA from other species. (B) *G. australes* and (C) *G. excentricus* primer sets amplify only their target DNA and they do not detect non-target species or genera

The availability of an assay capable of detecting the presence of all species of the genera *Gambierdiscus* and *Fukuyoa* can provide information of the general composition of a field sample. Additionally, since *G. excentricus* and *G. australes* have consistently demonstrated the ability to produce toxic compounds (Chinain et al. 1999; Gaiani et al. 2020; Pisapia et al. 2017a; Rhodes et al. 2014; Rossignoli et al. 2020), and their range is rapidly expanding (Hoppenrath et al. 2019; Rodríguez et al. 2017), a rapid assay for their simultaneous and discriminable detection will be helpful in assessing the risk of a ciguatera outbreak. Herein, we present the development of three molecular assays based on the RPA-SHA strategy for the detection of the *Gambierdiscus* & *Fukuyoa* genera, and the *G. australes* and *G. excentricus* species. The specificity of the assays has been characterized using clonal cultures of different *Gambierdiscus* species (*G. australes*, *G. balechii*, *G. belizeanus*, *G. caribaeus* and *G. excentricus*) and *F. paulensis*, as well as *Coolia monotis*, *Ostreopsis* cf. *ovata* and *Prorocentrum lima* as non-target genera. The detection and identification of single cells from clonal cultures has been evaluated. Finally, the amplification capacity of the three different primer sets and the discrimination ability of the species-specific primer sets have been proved with several mixtures of DNA from six different target species.

2. Material and methods

2.1. Microalgal cultures

Several microalgal strains were used in this work, obtained from IRTA collection (IRTA-SMM) and the Culture Collection of Microalgae of the Instituto Español de Oceanografía (CCVIEO) in Vigo, Spain (VGO) (Table 1). Clonal cultures were grown in polystyrene flasks containing 500 mL of modified ES medium (Provasoli 1968) prepared with filtered and autoclaved seawater from L'Ametlla de Mar, Spain (salinity adjusted at 36). Cultures were maintained at 24 ± 1 °C under a photon flux rate of 100 µmol m-2 s -1 with a 12:12h light:dark regime. Once the cultures reached the late exponential phase (*ca.* 21 days), 5 µL containing single cells were isolated from some of them. Isolations were performed under the microscope with the aid of a micropipette. The 5-µL drop containing the cell was stored in PCR tubes at -20 °C until DNA extraction. Culture aliquots were fixed with 3% Lugol's iodine and counted using a Kolkwitz chamber (Hydro-Bios, Altenholz, Germany) under an inverted light microscope (Leica DMIL, Spain), following the Sedgwick-Rafter method (Greeson 1977). Additionally, microalgal pellets were obtained by harvesting the entire culture volume in 50 mL tubes and centrifuging at 2,500 rpm for 25 min (Allegra X-15R, Beckman Coulter, Brea, USA). Supernatants were discarded and tubes were stored at -20 °C until DNA extraction.

2.2. DNA extraction

Extraction of genomic DNA from microalgal pellets was performed using a bead beating system and the phenol/chloroform method (Toldrà et al. 2019a). Briefly, cell pellets were re-suspended in 200 µL of lysis buffer (1 M NaCl, 70 mM Tris, 30 mM EDTA, pH 8.6) and moved to an extraction tube containing zirconium beads (0.5 mm in diameter). Subsequently, 25 µL of 10% *w/v* DTAB and 200 µL of chloroform were added for cellular disruption using a Bead Beater-8 (BioSpec, Bartlesville, USA) for 45 s at full speed. Disrupted cells were then centrifuged at 2,300 rpm for 5 min (Eppendorf 5415D, Hamburg, Germany), the aqueous phase was transferred to a fresh tube and DNA was extracted using standard phenol/chloroform method (Sambrook et al. 1989). Precipitation of the DNA was obtained by the addition of 2 volumes of absolute ethanol and 0.1 volumes of 3 M sodium acetate (pH 8.0). The DNA was rinsed with 70% *v/v* ethanol and then dissolved in 50 µL of molecular DNAse/RNAse-free water. Extracted DNA samples (50 µL) were quantified and checked for their purity using a NanoDrop 2000 spectrophotometer (Thermo Fisher Scientific, Madrid, Spain). Extracted DNA was stored at -20 °C until analysis.

Extraction of genomic DNA from single microalgal cells was performed using an Arcturus® PicoPure® DNA Extraction Kit (Thermo Fisher Scientific, Madrid, Spain) following the manufacturer's instructions. Briefly, 155 µL of reconstitution buffer were added to one of the kit vials containing Proteinase K and mixed. Once the pellet had been dissolved, 15 µL of the obtained solution were added to each tube containing a single cell. DNA extraction was performed with a Nexus Gradient Thermal Cycler (Eppendorf, Spain) at 65 °C for 3 h and then 95 °C for 10 min. Extracted DNA was stored at -20 °C until analysis (Tudó et al. 2018).

2.3. Primers and probes

Primers were designed within the D8-D10 (*Gambierdiscus & Fukuyoa*) and D1-D3 (*G. australes* and *G. excentricus*) regions of the 28 S LSU ribosomal DNA (rDNA) gene and synthesized by Biomers (Ulm, Germany). Three primer sets were used: one for *Gambierdiscus* & *Fukuyoa* genera, one for *G. australes*, and one for *G. excentricus*. *G. australes* and *G. excentricus* primer sets shared the same reverse primer. Primers were subsequently modified with oligonucleotide tails to enable direct detection of the RPA product. Each primer set had its individual cognate capture probe, which hybridizes with the corresponding primer tail. The reporter probe was common among all primer sets and hybridizes with all primer tails. The primers are between 23 and 26 bp long and amplify a product of around 150 bp. Tails and probes were tested using Multiple Primer Analyser Software (Themo Fisher Scientific) to confirm absence of cross-reactivity with primers and target sequences. The primers and probes used are listed in Table 2.

Table 1. Microalgae strains used in this study

Species	Strain	Sampling location and year	GenBank accession number	Sequenced region	Source
G. australes	IRTA-SMM-13_07	Selvagem Grande Island, Portugal, 2013	KY564320	D1-D3	Reverté et al. (2018)
G. australes	IRTA-SMM-13_17	Selvagem Grande Island, Portugal, 2013	KY564328	D1-D3	Reverté et al. (2018)
G. australes	IRTA-SMM-16_286	Lanzarote, Spain, 2016	MT119197	D8-D10	Gaiani et al. (2020)
G. australes	IRTA-SMM-17_164	Menorca, Spain, 2017	MG708120	D8-D10	Tudó et al. (2018)
G. balechii	VGO920	Manado, Indonesia, 2007	KX268469	D8-D10	Fraga et al. (2016)
G. belizeanus	IRTA-SMM-13_19	La Réunion, France, 2013	MW350058	D8-D10	This study
G. belizeanus	IRTA-SMM-17_421	El Hierro, Spain 2017	MT379471	D8-D10	Tudó et al. (2020a)
G. caribaeus	IRTA-SMM-17_03	El Hierro, Spain 2017	MT119203	D8-D10	Gaiani et al. (2020)
G. excentricus	IRTA-SMM-17_01	Gran Canaria, Spain, 2017	MT119198	D8-D10	Gaiani et al. (2020)
G. excentricus	IRTA-SMM-17_126	Gran Canaria, Spain, 2017	MT119199	D8-D10	Gaiani et al. (2020)
G. excentricus	IRTA-SMM-17_407	La Gomera, Spain, 2017	MT119200	D8-D10	Gaiani et al. (2020)
G. excentricus	IRTA-SMM-17_428	La Gomera, Spain, 2017	MT119201	D8-D10	Gaiani et al. (2020)
G. excentricus	IRTA-SMM-17_432	La Gomera, Spain, 2017	MT119202	D8-D10	Gaiani et al. (2020)

G. excentricus	VGO791	Tenerife, Spain, 2004	JF303066; JF303075	D1-D3; D8-D10	Fraga et al. (2011)
F. paulensis	IRTA-SMM-17_206	Mallorca, Spain, 2017	MT119204	D8-D10	Tudó et al. (2020b)
F. paulensis	IRTA-SMM-17_211	Menorca, Spain, 2017	MT119205	D8-D10	Tudó et al. (2020b)
F. paulensis	IRTA-SMM-17_220	Menorca, Spain, 2017	MT119206	D8-D10	Tudó et al. (2020b)
F. paulensis	VGO1185	Ubatuba, Brazil, 2013	KM886379	18S; D1-D4; ITS	Gómez et al. (2015)
C. monotis	IRTA-SMM-16_285	Formentera, Spain, 2016	MW328563	ITS	This study
O.* cf. *ovata	IRTA-SMM-16_133	Catalonia, Spain, 2016	MH790463	ITS	Toldrà et al. (2019b)
P. lima	IRTA-SMM-17_47	Lanzarote, Spain, 2017	MW328564	ITS	This study

(Continues from the previous page)

Table 2. Primers with tails and probes used in this study. Tails are underlined.

Name	Sequence (5'-3')
G. australes **Reverse primer**	GTT TTC CCA GTC ACG AC-C3-ATG CAT AAC TCT TCA TTG CCA GTA G
G. excentricus **Reverse primer**	TCT ACA GGC TCG TAT ATG TA-C3-AGC TTG GGT CAC AGT GCA ACA GAG
G. australes **&** ***G. excentricus*** **Forward primer**	TGT AAA ACG ACG GCC AGT-C3-TGC TGC ATG YGG AGA TTC TTT YYT KG
Gambierdiscus **&** ***Fukuyoa*** **Forward primer**	ATA GGC TGG TTC GTA ATC GG-C3-GAY NCG GAC AAG GGG AAT CCG AC
Gambierdiscus **&** ***Fukuyoa*** **Reverse primer**	TGT AAA ACG ACG GCC AGT-C3-GAG AGT CAT AGT TAC TCC CGC CG
G. australes **capture probe**	GTC GTG ACT GGG AAA ACT TTT TTT TTT TTT TT-C3-thiol
G. excentricus **capture probe**	TAC ATA TAC GAG CCT GTA GAT TTT TTT TTT TTT TT-C3-thiol
Gambierdiscus **&** ***Fukuyoa*** **capture probe**	CCG ATT ACG AAC CAG CCT ATT TTT TTT TTT TTT TT-C3-thiol
Reporter probe	HRP-ACT GGC CGT CGT TTT ACA

2.4. Recombinase Polymerase Amplification (RPA)

DNA was amplified with RPA using the TwistAmp Liquid Basic kit (TwistDx Ltd, San Diego, USA). Each reaction contained: 3.1 µL of DNAse/RNAse-free water, 4.5 µL of dNTPs at 1.8 mM, 25 µL of rehydration buffer, 5 µL of Basic E-mix, 2.5 µL of Core reaction mix, 1.2 µL of each primer at 480 nM, 5 µL of genomic DNA at 1 ng/µL or solution with DNA extracted from single cells, and finally 2.5 µL of 14 mM magnesium acetate was used to initiate the RPA reaction. The total volume for each reaction was 50 µL. Non-target controls (NTCs, only DNAse/RNAse-free water) were included in the experimental design. Samples were isothermally amplified for 30 min at 37 °C. Following amplification, RPA products were purified using a GeneJet PCR purification kit (Thermo Fisher Scientific, Madrid, Spain) following the manufacturer´s instructions, ending with 50 µL of DNA in TE buffer after the final elution step.

2.5. Sandwich hybridization assay (SHA)

Three assays were developed: one for the detection of the genera *Gambierdiscus* & *Fukuyoa,* one for the detection of the species *G. australes* and a last one for the detection of the species *G. excentricus.* In these assays, the amplicons obtained in the respective RPA reactions were incubated with the corresponding surface-anchored thiolated capture probe. Thiolated capture probes were prepared in PBS (pH 7.4, 100 mM phosphate, 150 mM NaCl) at a

concentration of 500 nM, and 50 µL of this solution were added to the wells of a maleimide-coated plate (Pierce maleimide-activated microtitre plates from Thermo Fisher Scientific, Madrid, Spain) and incubated overnight at 4 °C on a microplate shaker under gentle agitation. Blocking of the non-functionalised maleimide groups was performed in two different steps, first via the addition of 200 µL of 100 µM 6-mercapto-1-hexanol in Milli-Q water and secondly 200 µL of 5% *w*/*v* skimmed milk in PBS. Subsequently, 45 µL of RPA product was added to the microtiter wells, followed by addition of 50 µL of 10 nM HRP-conjugated reporter probe in PBS containing 0.05% *v*/*v* Tween-20 (PBS-Tween). Three washing steps were performed between each step. All incubations, except for capture probe immobilization, were performed at room temperature for 30 min on a microplate shaker under gentle agitation. Finally, 100 µL of TMB liquid substrate were added and after 10 min, the absorbance was measured at 620 nm using a Microplate Reader KC4 (BIO-TEK Instruments, Winooski, USA). Gene 5 software was used to collect and evaluate the data.

2.6. Molecular identification

To identify microalgae at species level, which had already been recognized at genus level by light microscopy as either *Gambierdiscus* or *Fukuyoa*, the D8-D10 domain of the 28S LSU rDNA gene was amplified by PCR using the pair of primers FD8/RB (5'-GGATTGGCTCTGAGGGTTGGG-3' and 5'- GATAGGAAGAGCCGACATCGA-3') (Chinain et al. 1999). For microalgae cells other than *Gambierdiscus* and *Fukuyoa*, the D2C (5'-CCTTGGTCCGTGTTTCAAGA- 3') (Chomérat et al. 2010), and D1R (5'- ACCCGCTGAATTTAAGCATA-3') (Scholin et al. 1994) primers were used. In both cases, the 25 µL reaction mixtures contained 600 µM dNTP, 2 mM $MgCl_2$, 0.2 µM of each primer, 1 U of Taq polymerase, 5% DMSO and 2 µL of template DNA at 1 ng/µL. For *Gambierdiscus* and *Fukuyoa* cells, the protocol included 45 cycles of amplification (95 °C for 30 s, 60 °C for 45 s and 72 °C for 30 s, followed by an elongation of 10 min at 72 °C) (Gaiani et al. 2020). For the other microalgae, the protocol includes 45 cycles of amplification (94 °C for 30 s, 54 °C for 30 s and 72 °C 4 min, followed by an elongation of 5 min at 72 °C) (Chomérat et al. 2010). Amplifications were carried out in a Nexus Gradient Thermal Cycler (Eppendorf Iberica, Madrid, Spain). PCR reactions were checked using agarose gel electrophoresis and PCR products were then purified with a QIAquick PCR Purification Kit (Thermo Fisher Scientific, Madrid, Spain). Bidirectional sequencing was performed by Sistemas Genómicos, LLC (Valencia, Spain). Sequence reads were edited using BioEdit v7.0.5.2 (Hall 1999) and a consensus sequence for each read was obtained. Sequences were aligned using MAFFT v.7 (Rozewicki et al. 2019)

and the phylogenetic relationships were inferred by Maximum Likelihood (ML) using RaxML v.8 (Stamatakis 2014) and Bayesian Inference (BI) using Mr. Bayes v.3.2.2 (Huelsenbeck and Ronquist 2001). GenBank codes for all the sequences used in this work are listed in Table 1.

3. Results and discussion

3.1. RPA-SHA specificity

The *Gambierdiscus* & *Fukuyoa* primer set was tested with genomic DNA from all the strains used in this work, since the objective was to assess the ability of the system to detect both genera. The *G. australes* and *G. excentricus* primer sets were tested with their target genomic DNA, as well as with genomic DNA from other *Gambierdiscus* species (*G. balechii*, *G. belizeanus* and *G. caribaeus*) and *F. paulensis*. Additionally, all primer sets were tested with genomic DNA from other genera (*C. monotis, O.* cf. *ovata* and *P. lima*) and with NTC.

Since previous works (Toldrà et al. 2019a; Toldrà et al. 2019b) have demonstrated that the purification of RPA products is not always required, a trial without a purification step was first performed. However, there were no differences between samples with target DNA and with non-target genera, non-target species or NTC, with all of them showing very high absorbance values. Thus, despite the fact that the purification step is time consuming, it was included in the procedure to remove undesirable proteins and residual primers, and to avoid non-specific signals.

Results for the RPA-SHA using the three primer sets are shown in Figure 2. The thresholds to discriminate between positive and negative results (i.e. the limits of detection, LODs) were defined as the absorbance values of the NTC plus 10-fold their standard deviations. As can be observed, the *Gambierdiscus* & *Fukuyoa* system provided positive responses (> 0.196 Abs. units) for all *Gambierdiscus* species (*G. australes*, *G. excentricus*, *G. balechii*, *G. belizeanus* and *G. caribaeus*) and for *F. paulensis*, and no response from *C. monotis, O.* cf. *ovata* and *P. lima*. The *G. australes* system provided positive responses (> 0.136 Abs. units) for this species and no responses from all others. Finally, the *G. excentricus* system also provided positive responses (> 0.090 Abs. units) only for the strains belonging to this species.

Comparing the three assays, absorbance values are higher in the system for the detection of the genera *Gambierdiscus* & *Fukuyoa*. This fact could be attributed to a better efficiency provided by primers during the RPA and/or the SHA. It can also be observed that within the same assay, not all strains provide the same absorbance value, this effect being more evident in the system for the detection

of *G. excentricus*. This is likely due to the differences in the rDNA copy number of the samples analyzed. The rDNA copy number can vary between species, strains, geographic origins, and even cell growth phases, and thus sample harvesting times (Galluzzi et al. 2010; Kretzschmar et al. 2019; Nishimura et al. 2016; Vandersea et al. 2012).

All these results confirm the specificity of the primers and the RPA-SHAs for their respective targets. The three systems are not affected by non-target DNA of microalgae that share the same ecological niche as *Gambierdiscus* and *Fukuyoa*, making them suitable for the screening of field samples where microalgae of other genera will be present.

3.2. Detection of single cells

The purpose of this work was to provide a method for the identification of *Gambierdiscus* & *Fukuyoa* genera, and *G. australes* and *G. excentricus* species, rather than a method for the quantification of cell abundances. Nevertheless, the LOD of the technique plays an important role in such identification. When working with clonal cultures, the number of cells used to extract the DNA is not usually a problem, but in field samples, a robust identification system requires the ability to detect even a single cell. Thus, to assess the sensitivity of our systems, DNA was extracted from single cells isolated from the clonal cultures of *Gambierdiscus* and *Fukuyoa* listed in Table 3, and the three RPA-SHAs were performed simultaneously with aliquots of the same extract. Results showed positive responses (above the respective threshold) in the presence of target DNA and no responses from non-target genera or NTC. These results demonstrate the successful detection and identification of individual cells and confirm the specificity already observed in the previous section. In fact, a unique single cell extract was used for the three RPA-SHAs, so the assays are able to detect even less than 1 cell. This is not surprising since the rDNA copy number per cell in *Gambierdiscus* species has been reported to be as high as 4,560-21,500 (Vandersea et al. 2012), or even up to 3,197,000 (Nishimura et al. 2016), probably due to the large cell size and high amount of genomic DNA. Thus, our approach allows the discrimination of the presence/absence of a single cell belonging to the target genera/species.

Table 3. Results of the RPA-SHAs performed with DNA extracted from single cells of clonal cultures. Experiments were performed in duplicate.

Species	Strain	*Gambierdiscus & Fukuyoa* primer set	*G. australes* primer set	*G. excentricus* primer set
G. australes	IRTA-SMM-13_07	+	+	-
G. australes	IRTA-SMM-16_286	+	+	-
G. balechii	VGO 920	+	-	-
G. belizeanus	IRTA-SMM-13_19	+	-	-
G. belizeanus	IRTA-SMM-17_421	+	-	-
G. caribaeus	IRTA-SMM-17_03	+	-	-
G. excentricus	IRTA-SMM-17_126	+	-	+
G. excentricus	IRTA-SMM-17_407	+	-	+
F. paulensis	IRTA-SMM-17_206	+	-	-
F. paulensis	IRTA-SMM-17_211	+	-	-

Sign + (plus) indicates the detection of amplified product and the sign – (minus) indicates the absence.

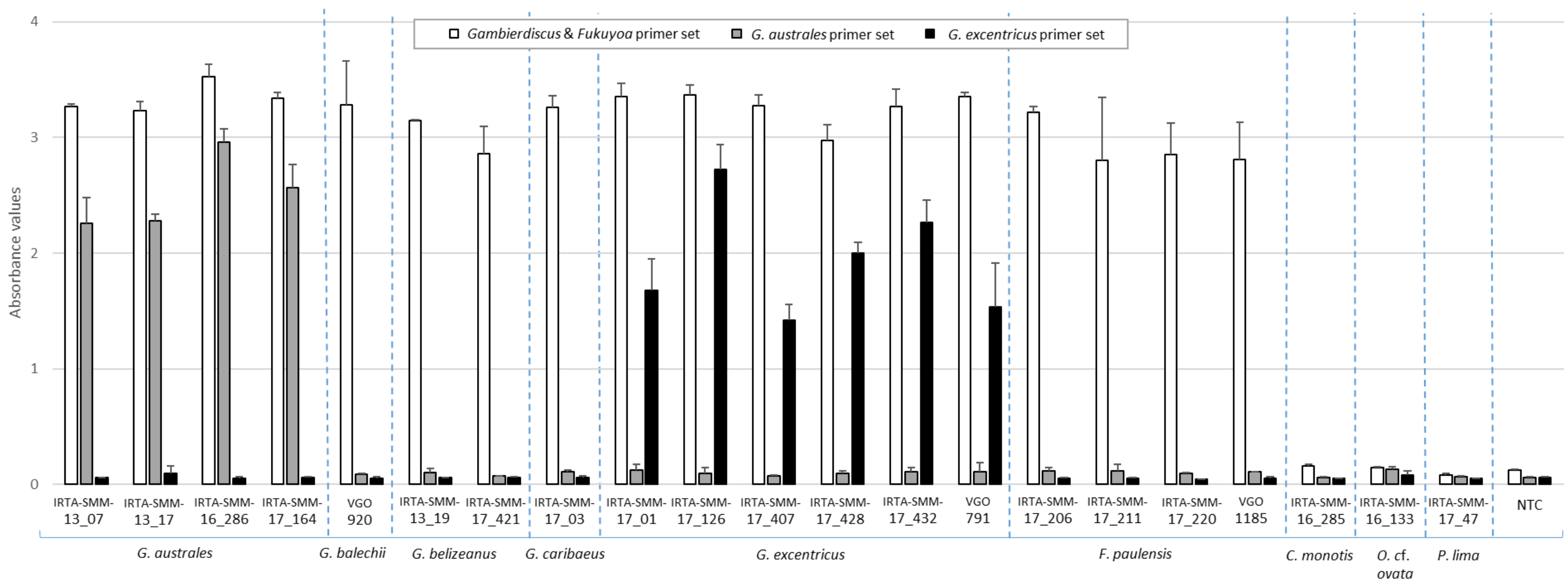

Figure 2. RPA-SHA experiments using genomic DNA extracted from different genera and species and the *Gambierdiscus* & *Fukuyoa* primer set (white), the *G. australes* primer set (grey) and the *G. excentricus* primer set (black). Experiments were performed in triplicate and bars indicate standard deviations. Vertical dashed lines separate species and/or genera

3.3. Detection of DNA combinations

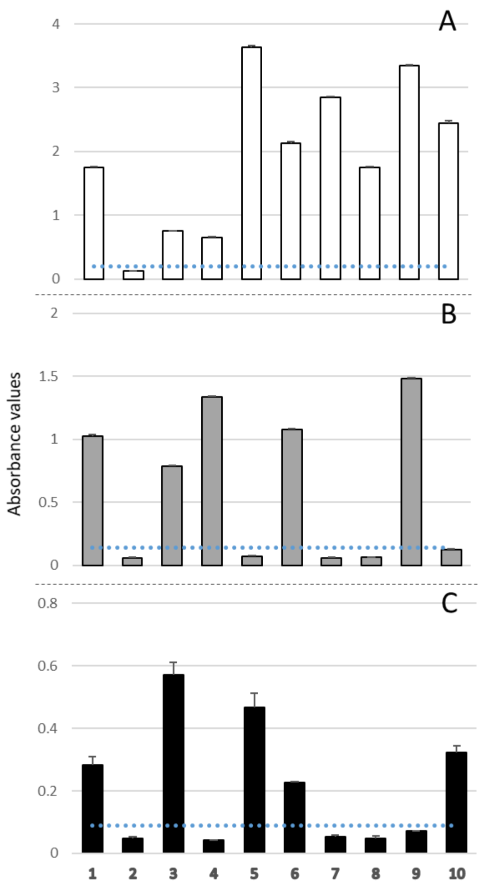

Figure 3. RPA-SHA experiments using combinations of genomic DNA extracted from different genera and species and the (A) *Gambierdiscus* & *Fukuyoa* primer set (white), (B) the *G. australes* primer set (grey) and (C) the *G. excentricus* primer set (black). Dotted lines represent the LOD for each system. Experiments were performed in triplicate and bars indicate standard deviation

In order to assess the ability of the *Gambierdiscus* & *Fukuyoa* primer set to amplify target DNA in the presence of different species, 10 different DNA combinations composed by the target species (five *Gambierdiscus* and one *Fukuyoa*) were tested with the RPA-SHA system (Table 4). All the combinations were prepared at a total DNA concentration of 1 ng/µL, and all the species within each combination were at the same concentration. The results obtained (Figure 3A) demonstrated the ability of the system to amplify and detect the amplicons without any false positives (combination 2). Differences in the absorbance values at 620 nm for each combination were observed. Unlike the results shown in Figure 2, where the strains used for the mixtures provided absorbance values between 2.8 and 3.5, in this experiment some of the combinations provided lower values, with combinations 3 and 4 giving the lowest signals, which could be attributable to the use of microalgal pellets with different rDNA copy number. Nevertheless, since the discrimination between positive and negative results is clear, this experiment shows the reliability and applicability of the RPA-SHA system for *Gambierdiscus* & *Fukuyoa*.

The same DNA combinations were then tested with the *G. australes* (Figure 3B) and the *G. excentricus* (Figure 3C) primer sets, to assess their ability to amplify the corresponding target DNA when mixed with others. The results obtained showed absorbance values higher than the LOD only for the combinations where the target DNA was present. In both cases, absorbance

values were lower than in Figure 2 (3.0 for *G. australes* IRTA-SMM-16_286 and 1.5 for *G. excentricus* VGO791), as expected since 1 ng/µL was the total DNA concentration of the mixtures (i.e. the DNA concentrations for *G. australes* IRTA-SMM-16_286 and *G. excentricus* VGO791 were between 0.16 and 0.5 ng/µL, depending on the combination). It is evident that DNA concentration is not the only crucial parameter, and the presence of non-target DNA may also be playing an important role. Nevertheless, even if the presence of non-target DNA may cause steric hindrance and inhibit the efficiency of the RPA, the experiments demonstrate the robustness of the RPA-SHA systems.

The results obtained with the combination trials demonstrated the ability of the *Gambierdiscus* & *Fukuyoa* primer set to amplify target DNA in the presence of different target species, and the capacity of the *G. australes* and *G. excentricus* primers sets to discriminate the corresponding target DNA in a mixture with DNA from other species. These achievements can be considered as a step forward to the applicability of the systems to screen field samples, even though further studies, beyond the current work, are needed.

Table 4. DNA combinations.

Species	Strain	1	2	3	4	5	6	7	8	9	10
G. australes	IRTA-SMM-16_286	+	-	+	+	-	+	-	-	+	-
G. excentricus	VGO 791	+	-	+	-	+	+	-	-	-	+
G. balechii	VGO 920	+	-	+	+	+	-	+	+	+	+
G. belizeanus	IRTA-SMM-17_421	+	-	+	+	+	-	+	+	+	+
G. caribaeus	IRTA-SMM-17_03	+	-	+	+	+	-	+	+	+	+
F. paulensis	VGO 1185	+	-	-	+	+	-	-	+	-	-

Sign + (plus) indicates presence of the species and sign - (minus) indicates absence.

4. Conclusions

This work reports the successful development and application of the RPA-SHA system for the detection of microalgae of the genera *Gambierdiscus* and *Fukuyoa,* and the discrimination between the species *G. australes* and *G. excentricus*. The method showed a high specificity for the target species and a sufficient LOD for identification of a single cell. Furthermore, the ability of the *Gambierdiscus* & *Fukuyoa* primer set to amplify target DNA in the presence of different species was demonstrated, together with the discriminable capacity of the species-specific primer sets (*G. australes* and *G. excentricus*).

This approach, applied for the first time to microalgae of the genera *Gambierdiscus* and *Fukuyoa*, has several advantages. Firstly, the ability to discriminate these genera from other microalgae is extremely helpful, because the assay is more rapid than traditional light microscopy and does not require taxonomical experts to screen samples. Furthermore, unlike microscopy techniques, the strategy can discriminate between the species *G. australes* and *G. excentricus*, which are known CTX producers. Additionally, the ability to detect a single cell is of extreme importance to avoid false negatives due to the LOD. Moreover, the RPA-SHA system is versatile, and with additional primer optimization, can be utilized for the detection of other toxic microalgal species.

Therefore, the summation of the achievements obtained demonstrate the robustness of the developed system, although further studies are needed to test the applicability for screening of field samples. Undoubtedly, the inclusion of the RPA-SHA system in monitoring programs would be useful to assess the risk of ciguatera, to predict possible outbreaks and consequently to preserve human health.

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References

Aligizaki K, Nikolaidis G (2008). Morphological identification of two tropical dinoflagellates of the genera *Gambierdiscus* and *Sinophysis* in the Mediterranean Sea. J of Biol Res-Thessalon 9:75-82.

Aligizaki K, Nikolaidis G, Katikou P, Baxevanis AD, Abatzopoulos TJ (2009). Potentially toxic epiphytic *Prorocentrum* (Dinophyceae) species in Greek coastal waters. Harmful Algae 8:299-311.

Begier EM, Backer LC, Weisman RS, Hammond RM, Fleming LE, Blythe D (2006). Outbreak bias in illness reporting and case confirmation in ciguatera fish poisoning surveillance in south Florida. Public Health Rep 121:658-665.

Bravo I, Rodriguez F, Ramilo I, Rial P, Fraga S (2019). Ciguatera-Causing Dinoflagellate *Gambierdiscus* spp.(Dinophyceae) in a subtropical region of North Atlantic Ocean (Canary Islands): morphological characterization and biogeography. Toxins 11:423.

Chinain M, Faust MA, Pauillac S (1999). Morphology and molecular analyses of three toxic species of *Gambierdiscus* (Dinophyceae): *G. pacificus*, sp. nov., *G. australes*, sp. nov., and *G. polynesiensis*, sp. nov. J Phycol 35:1282-1296.

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

Chomérat N, Sellos DY, Zentz F, Nézan E (2010). Morphology and molecular phylogeny of *Prorocentrum consutum* sp. nov. (Dinophyceae), a new benthic dinoflagellate from South Brittany (Norwestern France). J Phycol 46:183-194.

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

Fraga S, Rodriguez F (2014). Genus *Gambierdiscus* in the Canary Islands (NE Atlantic Ocean) with description of *Gambierdiscus silvae* sp. nov., a new potentially toxic epiphytic benthic dinoflagellate. Protist 165:839-853.

Fraga S, Rodríguez F, Riobó P, Bravo I (2016). *Gambierdiscus balechii* sp. nov (Dinophyceae), a new benthic toxic dinoflagellate from the Celebes Sea (SW Pacific Ocean). Harmful Algae 58:93-105.

Gaiani G, Leonardo S, Tudó À, Toldrà A, Rey M, Andree KB, Tsumuraya T, Hirama M, Diogène J, O'Sullivan CK, Alcaraz C, Campàs M (2020). Rapid detection of ciguatoxins in *Gambierdiscus* and *Fukuyoa* with immunosensing tools. Ecotoxicol Environ Saf 204:111004.

Galluzzi L, Bertozzini E, Penna A, Perini F, Garcés E, Magnani M (2010). Analysis of rRNA gene content in the Mediterranean dinoflagellate *Alexandrium catenella* and *Alexandrium taylori*: implications for the

quantitative real-time PCR-based monitoring methods. J Appl Phycol 22:1-9.

Gómez F, Qiu D, Lopes RM, Lin S (2015). *Fukuyoa paulensis* gen. et sp. nov., a new genus for the globular species of the dinoflagellate *Gambierdiscus* (Dinophyceae). PLoS One 10.

Greeson PE (1977). Methods for collection and analysis of aquatic biological and microbiological samples. In: Techniques of water-resources investigations of the United States Geological Survey, vol 5. Laboratory analysis. US Department of the Interior, Geological Survey

Holmes MJ, Lewis RJ (1994). Purification and characterisation of large and small maitotoxins from cultured *Gambierdiscus toxicus*. Nat Toxins 2:64-72.

Hoppenrath M, Kretzschmar AL, Kaufmann MJ, Murray SA (2019). Morphological and molecular phylogenetic identification and record verification of *Gambierdiscus excentricus* (Dinophyceae) from Madeira Island (NE Atlantic Ocean). Mar Biodivers Rec 12:16.

Huelsenbeck JP, Ronquist F (2001). MRBAYES: Bayesian inference of phylogenetic trees. Bioinformatics 17:754-755.

Jang SH, Jeong HJ, Yoo YD (2018). *Gambierdiscus jejuensis* sp. nov., an epiphytic dinoflagellate from the waters of Jeju Island, Korea, effect of temperature on the growth, and its global distribution. Harmful Algae 80:149-157.

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

Kretzschmar AL, Larsson ME, Hoppenrath M, Doblin MA, Murray SA (2019). Characterisation of two toxic *Gambierdiscus* spp. (Gonyaulacales, Dinophyceae) from the Great Barrier Reef (Australia): *G. lewisii* sp. nov. and *G. holmesii* sp. nov. Protist 170.

Larsson ME, Harwood TD, Lewis RJ, Swa H, Doblin MA (2019). Toxicological characterization of *Fukuyoa paulensis* (Dinophyceae) from temperate Australia. Phycol Res 67:65-71.

Laza-Martínez A, David H, Riobó P, Miguel I, Orive E (2016). Characterization of a strain of *Fukuyoa paulensis* (Dinophyceae) from the Western Mediterranean Sea. J Eukaryotic Microbiol. 63:481-497.¡

Lewis RJ (2001). The changing face of ciguatera. Toxicon 39:97-106.

Litaker RW, Vandersea MW, Faust MA, Kibler SR, Chinain M, Holmes MJ, Holland WC, Tester PA (2009). Taxonomy of *Gambierdiscus* including four new species, *Gambierdiscus caribaeus*, *Gambierdiscus carolinianus*, *Gambierdiscus carpenteri* and *Gambierdiscus ruetzleri* (Gonyaulacales, Dinophyceae). Phycologia 48:344-390.

Litaker RW, Holland WC, Hardison DR, Pisapia F, Hess P, Kibler SR, Tester PA (2017). Ciguatoxicity of *Gambierdiscus* and *Fukuyoa* species from the Caribbean and Gulf of Mexico. PLoS One 12:e0185776.

Litaker RW, Tester PA, Vandersea MW (2019). Species-specific PCR assays for *Gambierdiscus excentricus* and *Gambierdiscus silvae* (Gonyaulacales, Dinophyceae). J Phycol 55:730-732.

Longo S, Sibat M, Viallon J, Darius HT, Hess P, Chinain M (2019). Intraspecific Variability in the toxin production and toxin profiles of in vitro cultures of *Gambierdiscus polynesiensis* (Dinophyceae) from French Polynesia. Toxins 11:735. doi:https://doi.org/10.3390/toxins11120735

Murata M, Naoki H, Iwashita T, Matsunaga S, Sasaki M, Yokoyama A, Yasumoto T (1993). Structure of maitotoxin. J Am Chem Soc 115:2060-2062.

Nagai H, Murata M, Torigoe K, Satake M, Yasumoto T (1992). Gambieric acids, new potent antifungal substances with unprecedented polyether structures from a marine dinoflagellate *Gambierdiscus toxicus*. J Org Chem 57:5448-5453.

Nishimura T, Sato S, Tawong W, Sakanari H, Yamaguchi H, Adachi M (2014). Morphology of *Gambierdiscus scabrosus* sp. nov.(G onyaulacales): a new epiphytic toxic dinoflagellate from coastal areas of Japan. J Phycol 50:506-514.

Nishimura T, Hariganeya N, Tawong W, Sakanari H, Yamaguchi H, Adachi M (2016). Quantitative PCR assay for detection and enumeration of ciguatera-causing dinoflagellate *Gambierdiscus* spp.(Gonyaulacales) in coastal areas of Japan. Harmful Algae 52:11-22.

Pisapia F, Holland WC, Hardison DR, Litaker RW, Fraga S, Nishimura T, Adachi M, Nguyen-Ngoc L, Sechet V, Amzil Z, Herrenknecht C, Hess P (2017a). Toxicity screening of 13 *Gambierdiscus* strains using neuro-2a and erythrocyte lysis bioassays. Harmful Algae 63:173-183.

Pisapia F, Sibat M, Herrenknecht C, Lhaute K, Gaiani G, Ferron PJ, Fessard V, Fraga S, Nascimento SM, Litaker RW, Holland WC, Roullier C, Hess P (2017b). Maitotoxin-4, a Novel MTX Analog Produced by *Gambierdiscus excentricus*. Mar Drugs 15:220.

Provasoli L (1968). Media and prospects for the cultivation of marine algae. In: Hattori A (ed). Proceedings of the US-Japanese conference, cultures and collection of algae. Japanese Society of Plant Physiology, Hakone (Japan), pp 63-75.

Reverté L, Toldrà A, Andree KB, Fraga S, de Falco G, Campàs M, Diogène J (2018). Assessment of cytotoxicity in ten strains of *Gambierdiscus australes* from Macaronesian Islands by neuro-2a cell-based assays. J oAppl Phycol 30:2447-2461.

Rhodes L, Harwood T, Smith K, Argyle P, Munday R (2014). Production of ciguatoxin and maitotoxin by strains of *Gambierdiscus australes*, *G. pacificus* and *G. polynesiensis* (Dinophyceae) isolated from Rarotonga, Cook Islands. Harmful Algae 39:185-190.

Rhodes LL, Smith KF, Murray S, Harwood DT, Trnski T, Munday R (2017). The Epiphytic Genus *Gambierdiscus* (Dinophyceae) in the Kermadec

Islands and Zealandia Regions of the Southwestern Pacific and the associated risk of ciguatera fish poisoning. Mar Drugs 15:219.

Rodríguez F, Fraga S, Ramilo I, Rial P, Figueroa RI, Riobó P, Bravo I (2017). Canary Islands (NE Atlantic) as a biodiversity 'hotspot'of *Gambierdiscus*: Implications for future trends of ciguatera in the area. Harmful Algae 67:131-143.

Rossignoli AE, Tudo A, Bravo I, Diaz PA, Diogene J, Riobo P (2020). Toxicity Characterisation of *Gambierdiscus* Species from the Canary Islands. Toxins 12:134.

Rozewicki J, Li S, Amada KM, Standley DM, Katoh K (2019). MAFFT-DASH: integrated protein sequence and structural alignment. Nucleic Acids Res 47:W5-W10.

Sambrook J, Fritsch EF, Maniatis T (1989). Molecular cloning: a laboratory manual. Cold spring harbor laboratory press.

Satake M, Murata M, Yasumoto T (1993). Gambierol: a new toxic polyether compound isolated from the marine dinoflagellate *Gambierdiscus toxicus*. J Ame Chem Soc 115:361-362.

Scholin CA, Herzog M, Sogin M, Anderson DM (1994). Identification of group-and strain-specific genetic markers for globally distributed *Alexandrium* (Dinophyceae). II sequence analysis of a fragment of the LSU rRNA gene. J Phycol 30:999-1011.

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

Smith KF, Biessy L, Argyle PA, Trnski T, Halafihi T, Rhodes LL (2017). Molecular identification of *Gambierdiscus* and *Fukuyoa* (Dinophyceae) from environmental samples. Mar Drugs 15:243.

Stamatakis A (2014). RAxML version 8: a tool for phylogenetic analysis and post-analysis of large phylogenies. Bioinformatics 30:1312-1313.

Stewart I, Eaglesham GK, Poole S, Graham G, Paulo C, Wickramasinghe W, Sadler R, Shaw GR (2010). Establishing a public health analytical service based on chemical methods for detecting and quantifying Pacific ciguatoxin in fish samples. Toxicon 56:804-812.

Toldrà A, Jauset-Rubio M, Andree KB, Fernández-Tejedor M, Diogène J, Katakis I, O'Sullivan CK, Campàs M (2018). Detection and quantification of the toxic marine microalgae *Karlodinium veneficum* and *Karlodinium armiger* using recombinase polymerase amplification and enzyme-linked oligonucleotide assay. Anal Chim Acta 1039:140-148.

Toldrà A, Alcaraz C, Andree KB, Fernández-Tejedor M, Diogène J, Katakis I, O'Sullivan CK, Campàs M (2019a). Colorimetric DNA-based assay for the specific detection and quantification of *Ostreopsis* cf. *ovata* and *Ostreopsis* cf. *siamensis* in the marine environment. Harmful Algae 84:27-35.

Toldrà A, Alcaraz C, Diogène J, O'Sullivan CK, Campàs M (2019b). Detection of *Ostreopsis* cf. *ovata* in environmental samples using an electrochemical DNA-based biosensor. Sci Total Environ 689:655-661.

Tudó À, Toldrà A, Andree KB, Rey M, Fernández-Tejedor M, Campàs M, Diogène J (2018). First report of *Gambierdiscus* in the Western Mediterranean Sea (Balearic Islands). Harmful Algae News.

Tudó À, Gaiani G, Varela MR, Tsumuraya T, Andree KB, Fernández-Tejedor M, Campàs M, Diogène J (2020a). Further advance of *Gambierdiscus* species in the Canary Islands, with the first report of *Gambierdiscus belizeanus*. Toxins 12:692.

Tudó À, Toldrà A, Rey M, Todolí I, Andree KB, Fernández-Tejedor M, Campàs M, Sureda FX, Diogène J (2020b). *Gambierdiscus* and *Fukuyoa* as potential indicators of ciguatera risk in the Balearic Islands. Harmful Algae 99:101913.

Vandersea MW, Kibler SR, Holland WC, Tester PA, Schultz TF, Faust MA, Holmes MJ, Chinain M, Litaker RW (2012). Development of semi-quantitative pcr assays for the detection and enumeration of *Gambierdiscus* species (gonyaulacales, Dynophyceae). J Phycol 48:902-915.

Watanabe R, Uchida H, Suzuki T, Matsushima R, Nagae M, Toyohara Y, Satake M, Oshima Y, Inoue A, Yasumoto T (2013). Gambieroxide, a novel epoxy polyether compound from the dinoflagellate *Gambierdiscus toxicus* GTP2 strain. Tetrahedron 69:10299-10303.

Yasumoto T, Igarashi T, Legrand A-M, Cruchet P, Chinain M, Fujita T, Naoki H (2000). Structural elucidation of ciguatoxin congeners by fast-atom bombardment tandem mass spectroscopy. J Am Chem Soc 122:4988-4989.

Yogi K, Oshiro N, Inafuku Y, Hirama M, Yasumoto T (2011). Detailed LC-MS/MS analysis of ciguatoxins revealing distinct regional and species characteristics in fish and causative alga from the Pacific. Anal Chem 83:8886-8891.

Chapter 2

Electrochemical biosensor for the dual detection of *Gambierdiscus australes* and *Gambierdiscus excentricus* in field samples. First report of *G. excentricus* in the Balearic Islands.

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Abstract

Several genera of marine dinoflagellates are known to produce bioactive compounds that affect human health. Among them, *Gambierdiscus* and *Fukuyoa* stand out for their ability to produce several toxins, including the potent neurotoxic ciguatoxins (CTXs), which accumulate through the food web. Once fishes contaminated with CTXs are ingested by humans, it can result in an intoxication named ciguatera. Within the two genera, only some species are able to produce toxins, and *G. australes* and *G. excentricus* have been highlighted to be the most abundant and toxic. Although the genera *Gambierdiscus* and *Fukuyoa* are endemic to tropical areas, their presence in

subtropical and temperate regions has been recently recorded. In this work, the combined use of species-specific PCR primers for *G. australes* and *G. excentricus* modified with short oligonucleotide tails allowed the development of a multiplex detection system for these two toxin-producing species. Simultaneous detection was achieved using capture probes specific for *G. australes* and *G. excentricus* immobilized on maleimide-coated magnetic beads (MBs), separately placed on the working electrodes of a dual electrode array. Additionally, a rapid DNA extraction technique based on a portable bead beater system and MBs was developed, significantly reducing the extraction time (from several hours to 30 min). The developed technique was able to detect as low as 10 cells of both *Gambierdiscus* species and allowed the first detection of *G. excentricus* in the Balearic Islands in 8 out of the 9 samples analyzed. Finally, field samples were screened for CTXs with an immunosensor, successfully reporting 13.35 ± 0.5 pg CTX1B equiv. cell^{-1} in one sample and traces of toxins in 3 out of the 9 samples analyzed. These developments provide rapid and cost-effective strategies for ciguatera risk assessment, with the aim of guaranteeing seafood safety.

1. Introduction

Marine dinoflagellates are known producers of a wide range of toxins. These toxins may be transferred along the food chain and accumulate in the flesh of seafood. Therefore, they can reach seafood consumers resulting in foodborne diseases. Among all the existing foodborne diseases caused by marine toxins, ciguatera is one of the most common in intertropical and nearby areas (Begier et al., 2006; Larsson et al., 2019; Lewis, 2001; Litaker et al., 2017). Ciguatera is caused by the ingestion of fish contaminated with ciguatoxins (CTXs), potent marine neurotoxins that can accumulate in the food webs, rarely in bivalves, echinoderms and crustaceans, and more frequently in herbivorous, detritivorous and carnivorous fish (Kelly et al., 1992; Ledreux et al., 2014; Roué et al., 2016; Silva et al., 2015). CTXs are produced by dinoflagellates of the genera *Gambierdiscus* and *Fukuyoa,* which also produce other bioactive compounds such as maitotoxins (MTXs) (Holmes and Lewis, 1994; Murata et al., 1993; Pisapia et al., 2017b), gambieric acids (Nagai et al., 1992), gambierol (Satake et al., 1993), gambieroxide (Watanabe et al., 2013) and gambierone (Murray et al., 2019; Rodríguez et al., 2015). However, it is not clear yet if these compounds play a role in ciguatera (Kohli et al., 2015). The *Gambierdiscus* and *Fukuyoa* genera are endemic of subtropical areas, although in the past decade they have been found in temperate areas such as Japan (Nishimura et al., 2014), the coast of North Carolina (Litaker et al., 2009), the Gulf of Mexico (Litaker et al., 2017), Brazil (Gómez et al., 2015), the Canary Islands (Fraga and Rodriguez, 2014; Fraga et al., 2011), Madeira (Kaufmann and Böhm-Beck, 2013) and the Mediterranean Sea, first detected in Greece (Aligizaki and Nikolaidis, 2008; Aligizaki et al., 2009) and then a few years later in the Balearic Islands (Laza-Martínez et al., 2016; Tudó et al., 2018). The intensification of monitoring efforts could give the impression of an increase in

the spread of the genera, as it has been demonstrated for the perceived global increase in algal blooms (Hallegraeff et al., 2021). Nevertheless, the global warming trend is supposed to favor the proliferation and expansion of these harmful genera. Therefore, the probable raise in the occurrence of ciguatera poisoning events in new areas increases the threat to human health.

To date, 18 *Gambierdiscus* species (Chinain et al., 1999; Fraga et al., 2011; Jang et al., 2018; Kretzschmar et al., 2019; Litaker et al., 2009; Nishimura et al., 2014; Rhodes et al., 2017), and 3 *Fukuyoa* species have been described (Gómez et al., 2015). Only few species of these genera have demonstrated the ability to produce toxic compounds (*G. australes, G. caribaeus, G. excentricus, G. pacificus, G. polynesiensis, G. toxicus* and *F. paulensis*) (Chinain et al., 2010; Fraga et al., 2011; Gaiani et al., 2020; Litaker et al., 2017; Longo et al., 2019; Pisapia et al., 2017a; Rhodes et al., 2014; Rossignoli et al., 2020; Sibat et al., 2018). Hence, identifying the presence of *Gambierdiscus* and *Fukuyoa* toxin-producing species directly in field samples can be very useful for predicting and assessing the risk of ciguatera outbreaks. Light microscopy and electron microscopy are the techniques most commonly used to identify *Gambierdiscus* and *Fukuyoa*, but they suffer from the drawback that it is almost impossible to achieve species identification using these techniques alone. In fact, the use of genetic sequencing is practically mandatory to correctly assign the species to field sample isolates (Bravo et al., 2019), and to this end, molecular techniques are increasingly used to identify species of interest in field samples. Regarding *Gambierdiscus* and *Fukuyoa*, the quantitative polymerase chain reaction (qPCR), has been used for the identification and quantification of *G. belizeanus, G. caribaeus, G. carolinianus, G. carpenter* and *G. ruetzleri* (Vandersea et al., 2012), *G. australes* and *G. scabrosus* (Nishimura et al., 2016), *Gambierdiscus/Fukuyoa* and *F. paulensis* (Smith et al., 2017), *G. excentricus* and *G. silvae* (Litaker et al., 2019), and *G. lapillus* (Kretzschmar et al., 2019). However, all these techniques require laboratory work, resulting in a time lag between field sampling and species detection. Thus, to shorten the time between these events, researchers have developed molecular-based strategies that could be integrated into portable devices for the *in situ* detection of microalgae (Medlin et al., 2020; Toldrà et al., 2018a; Toldrà et al., 2019b).

In this work, we used species-specific PCR primers for *G. australes* and *G. excentricus* modified with short oligonucleotide tails to create a multiplex detection system for these two toxin-producing species (Figure 1). The species-specific detection was achieved using capture probes of *G. australes* and *G. excentricus* immobilized on maleimide-coated magnetic beads (MBs), and subsequently capturing them separately on the working electrodes of a dual electrode array. One tail of the amplified products binds specifically to the corresponding capture probe and the other to an enzyme-labelled reporter probe. A similar approach was previously used for the detection of other toxic marine dinoflagellates (Toldrà et al., 2019b), and this is the first time that such a strategy is combined with a dual electrochemical biosensor and used for the

simultaneous detection of two toxin-producing *Gambierdiscus* species in field samples. In addition, a rapid DNA extraction technique combining a portable bead beater system and MBs was developed, which reduces the extraction time from several hours to a few minutes, which can be considered as a step forward for the extraction of samples directly in field. Moreover, we used the sandwich immunosensor previously developed by our group (Gaiani et al., 2020), to screen CTX contents directly in field samples. This technique involves the use of monoclonal antibodies (mAbs) specific for CTXs. Specifically, two capture antibodies were used, the 3G8 mAb which has affinity for the left wing of CTX1B and 54-deoxyCTX1B (Tsumuraya et al., 2012), and the 10C9 mAb which has affinity for the left wing of CTX3C and 51-hydroxyCTX3C (Oguri et al., 2003). Moreover, a detector antibody, 8H4 mAb was used for the recognition of the right wing of the four congeners (Tsumuraya et al., 2006).

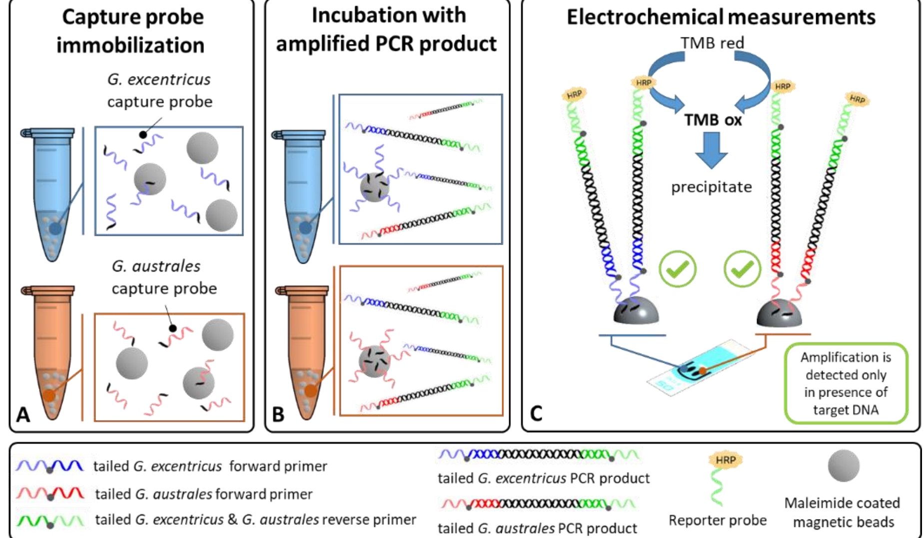


Figure 1. Schematic representation of the strategy developed in this work. (A) Species-specific capture probes were immobilized separately on maleimide-coated MBs and then (B) exposed to PCR products. (C) Detection of tailed *G. australes* and *G. excentricus* PCR products was achieved on each working electrode of a dual electrode array using amperometry.

2. Materials and methods

2.1. Microalgal cultures and field samples

One strain of *G. australes* obtained from the IRTA collection (IRTA-SMM-16_286) and one of *G. excentricus* from the Culture Collection of Microalgae of the Instituto Español de Oceanografía (CCVIEO) in Vigo, Spain (VGO791) were used in this work. Monoclonal cultures were grown in polystyrene flasks containing 500 mL of modified ES medium (Provasoli, 1968) prepared with filtered and autoclaved seawater from L'Ametlla de Mar, Spain (salinity adjusted to 36 psu). Cultures were maintained at 24 ± 1 °C under a photon flux rate of 100 µmol m^{-2} s^{-1} with a 12:12h light:dark regime. Culture aliquots were

fixed with 3% *v/v* Lugol's iodine and counted using a Kolkwitz chamber (Hydro-Bios, Altenholz, Germany) under an inverted light microscope (Leica DMIL, Spain), following the Sedgwick-Rafter method (Greeson, 1977) every second day. Once the cultures reached the early exponential phase (*ca.* 21 days), microalgal pellets of 10^4 cells were prepared splitting accordingly the entire culture volume in 50 mL tubes. The tubes were then centrifuged at 2,500 rpm for 25 min (Allegra X-15R, Beckman Coulter, Brea, USA). Supernatants were discarded and tubes were stored at -20 °C until DNA extraction. A total of 12 samples were collected in Majorca during October 2020 (Table 1). For the sampling, 100-200 g fresh weight of microalgae substrate were mixed with 250 mL of seawater, vigorously shaken and filtered through a 200-µm mesh. Once the 250-mL bottles reached the laboratory, the entire volume was fixed with 3% *v/v* Lugol's iodine solution and 10 mL of the fixed samples were stained with Calcofluor white M2R (Sigma Aldrich, Spain) for identification and counted under UV light using an epifluorescence microscope (LEICA DMLB) with the Utermöhl method (Utermöhl, 1958). Cell abundances were expressed as cell L^{-1}. Fifty millilitre aliquots from each field sample were centrifuged at 2,500 rpm for 25 min. Supernatants were discarded and tubes were stored at -20 °C until DNA extraction.

2.2. DNA extraction methods

Several DNA extraction methods were compared in this work in order to identify the most rapid, efficient and suitable to be used in field analysis. Firstly, extraction of genomic DNA was performed using a bead beating system and the phenol/chloroform/isoamyl alcohol method (Toldrà et al., 2019a). Briefly, cell culture pellets were re-suspended in 200 µL of lysis buffer (1 M NaCl, 70 mM Tris, 30 mM EDTA, pH 8.6) and transferred to 2-mL screw-cap cryotubes containing ca. 20 µg of 0.5-mm diameter zirconium glass beads (BioSpec, USA). Subsequently, 25 µL of 10% *w/v* DTAB and 200 µL of chloroform were added and cellular disruption was performed with a Bead Beater-8 (BioSpec, Bartlesville, USA) for 45 s at full speed. Disrupted cells were then centrifuged at 2,300 rpm for 5 min (Eppendorf 5415D, Hamburg, Germany), the aqueous phase was transferred to a fresh tube and DNA was extracted using a standard phenol/chloroform/isoamyl alcohol method as described in Sambrook et al. (1989). Precipitation of the DNA was then performed by the addition of 2 volumes of absolute ethanol and 0.1 volumes of 3 M sodium acetate (pH 8.0). The DNA was rinsed with 70% *v/v* ethanol and then dissolved in 50 µL of molecular biology grade DNAse/RNAse-free water. This procedure was considered to be the reference method.

Additionally, DNA was extracted from cell culture pellets using the Biomeme Sample Prep Kit for DNA (Biomeme Inc., Philadelphia, USA) using the protocol optimized by Toldrà et al. (2018b), with some minor modifications. Briefly, cell pellets were re-suspended in 250 µL of lysis buffer and moved to 2-mL screw-cap cryotubes containing *ca.* 20 µg of 0.5-mm diameter zirconium glass beads and cell disruption was performed as described above.

Homogenized cell pellets were added to tubes containing 500 µL of Biomeme Lysis Buffer and pumped through a syringe with an ion exchange cartridge attached (5 pumps). Subsequently, samples were washed with 500 µL of Biomeme Protein Wash and 500 µL of Biomeme Salt Wash. Each wash step consisted of a single pumping. Samples were then dried by pumping only air through the columns (*ca.* 50 pumps), and finally the samples were eluted in 250 µL of Biomeme Elution Buffer (5 pumps).

DNA was also extracted from cell culture pellets using the Dynabeads™ DNA DIRECT™ Universal Kit (Thermo Fisher, Barcelona, Spain), following the manufacturer's instructions with some minor modifications. Briefly, cells were first re-suspended in 100 µL of lysis buffer, transferred to 2-mL screw-cap cryotubes containing *ca*. 10 µg of 0.5-mm diameter zirconium glass beads, and bead beating was carried out as for the previous extraction methods. This procedure was also tested without the bead beating step. Subsequently, the disrupted cells were moved to new tubes and 200 µL of Dynabeads™ fully resuspended in lysis buffer (provided by Thermo Fisher) were added to each sample with a rapid pipetting action, and the protocol was then followed as recommended by the manufacturer. After 5 min, tubes were placed on a magnet and the supernatant was discarded. The tubes were then removed from the magnet and 200 µL of Washing Buffer (1X) were rapidly pipetted into each tube. Again, tubes were placed on the magnet and supernatant was discarded. The washing step was repeated twice. After discarding the supernatant, tubes were removed from the magnet and DNA/Dynabeads™ complexes were re-suspended and homogenized (by pipetting) in 30 µL of resuspension Buffer. DNA was eluted off the Dynabeads™ by incubation at 65 °C for 5 min. Tubes were placed one last time on the magnet and the eluted DNA was transferred to new tubes. With the aim of moving closer to DNA extraction that could be carried out in the field, the bead beating step was also performed with a TerraLyzer (Zymo Research, USA), a portable bead beater. A 2-mL screw-cap cryotube containing *ca.* 20 µg of 0.5-mm diameter zirconium glass beads (BioSpec, USA) was again used for each sample, and bead beating was performed for 1 min instead of 45 s.

The genomic DNA obtained with the different techniques was quantified and checked for purity by measuring the absorbance at 260/280 using a NanoDrop 2000 spectrophotometer (Thermo Fisher Scientific, Spain), and subsequently stored at -20 °C until analysis.DNA from field samples was extracted from one of the 50 mL tubes, by resuspending the pellets in 1 mL of seawater, and then taking 500 µL to be processed with the chosen technique (Terralyzer and MBs). The remaining 500 µL were used for CTX extraction.

2.3. DNA amplification

In this study, three different primers previously developed by our group (two reverse primers specific for *G. australes* and *G. excentricus* and a common

forward primer) (Gaiani et al., 2021) were used. Primers were designed within the D1-D3 region of the 28S LSU ribosomal DNA (rDNA) gene and synthesized by Biomers (Ulm, Germany). Particularly, species-specific reverse primers were modified with oligonucleotide tails that bind to their corresponding species-specific thiolated capture probes. The forward primer was also modified with a tail that hybridizes with the reporter probe containing a horseradish peroxidase (HRP) enzyme as label (Table S1). The primers are between 24 and 26 bp long and amplify a product of around 150 bp. Tails and probes were tested using Multiple Primer Analyser Software (Themo Fisher Scientific) to confirm absence of cross-reactivity with primers and target sequences.

DNA was amplified using the Invitrogen Taq DNA kit (Thermo Fisher Scientific, Madrid, Spain). In the amplification of just one target DNA with its corresponding pair of primers (single PCR reactions), each reaction mixture contained 0.5 µL of 0.2 µM of each primer, 3 µL of 600 µM dNTP, 5 µL of PCR Buffer 1X (-Mg), 2 µL of 2 mM $MgCl_2$, 0.2 µL of 1 U of Taq polymerase, 2 µL of template DNA, and DNAse/RNAse-free water up to 50 µL. The amplification reactions in presence of the three primers (multiplex PCR reaction) contained 0.5 µL of 0.2 µM of each reverse primer and 1 µL of 0.4 µM of the forward primer, all the other reagents were kept at the same concentrations, and DNAse/RNAse-free water was added up to 50 µL. Non-target controls (NTCs, only DNAse/RNAse-free water) were included in the experimental design. To optimize the system, 1 µL of DNA (1 ng μL^{-1}) of each target species was used. After optimization, the amplification protocol was as follows: 95 °C for 5 min, 40 cycles of 95 °C for 30 s, 59 °C for 30s and 72 °C for 30s, terminated by a final elongation at 72 °C for 5 min. Amplifications were carried out in a Nexus Gradient Thermal Cycler (Eppendorf Iberica, Madrid, Spain). PCR products were then purified using a GeneJet PCR purification kit (Thermo Fisher Scientific, Madrid, Spain) following the manufacturer´s instructions, resulting in 50 µL of DNA in TE (Tris-acetate-EDTA) buffer following the final elution step. The size of the products from the PCR reactions were checked with agarose (2% *w/v*) gel electrophoresis.

2.4. Colorimetric assay

Thiolated capture probes were prepared in 100 mM phosphate, 150 mM NaCl, pH 7.4, at a concentration of 500 nM and 50 µL were incubated in each well of a maleimide-coated plate (Pierce maleimide-activated microtitre plates from Thermo Fisher Scientific, Madrid, Spain). A first blocking of the non-functionalised maleimide groups was performed via the addition of 200 µL of a 100 µM 6-mercapto-1-hexanol solution dissolved in Milli-Q water. A secondary blocking was executed with 200 µL of 5% *w/v* skimmed milk in PBS, to avoid non-specific adsorption. Subsequently, 45 µL of PCR product was exposed to the immobilized capture probes, followed by addition of 50 µL of 10 nM HRP-conjugated reporter probe in washing buffer (100 mM phosphate, 150 mM NaCl, 0.05% *v/v* Tween-20, pH 7.4). Three washing steps

were performed between each step. Capture probe immobilization was performed overnight at 4 °C, whereas all the other incubations were performed at room temperature for 30 min. For all the incubation steps, a microplate shaker was used, to obtain a constant gentle agitation. Finally, 100 µL of TMB (3,3',5,5'-tetramethylbenzidine) Liquid Substrate System for ELISA (Sigma-Aldrich, Tres Cantos, Spain) were added and after 10 min, the absorbance was measured at 620 nm using a Microplate Reader KC4 (BIO-TEK Instruments, Winooski, USA). Gene 5 software was used to collect and evaluate the data. Colorimetric measurements were performed in duplicate.

2.5. Electrochemical biosensor

For the electrochemical biosensor, 5 µL of PureCube maleimide-activated MagBeads (Cube Biotech, Monheim, Germany) were transferred to a tube to be used as immobilization substrates for each of the capture probes. Fifty microlitres of the thiolated capture probe (500 nM in 100 mM phosphate, 150 mM NaCl, pH 7.4) were added and incubated overnight at 4 °C. Afterwards, 50 µL of 6-mercapto-1-hexanol solution (100 µM in 100 mM phosphate, 150 mM NaCl, pH 7.4) were added to block non-functionalised maleimide groups. Subsequently, conjugates were suspended in 5 µL of washing buffer. MB-capture probe conjugates (4.5 µL) were placed in new tubes and the supernatant was discarded with the aid of a magnetic stand. PCR product (45 µL) was then added, followed by the addition of 90 µL of 10 nM HRP-labelled reporter probe, diluted in washing buffer. Samples were washed three times after each step. All steps were performed for 30 min and under tilt agitation at room temperature (apart from capture probe immobilization).

For the electrochemical measurements, 10 µL of the oligocomplexes with the *G. australes* capture probe were captured on one of the working electrodes of a dual screen-printed carbon Dropsens electrode array (DRP-X1110) with a customized magnetic support underneath, and 10 µL of the oligocomplexes with the *G. excentricus* capture probe were captured on the other electrode. TMB Enhanced One Component HRP Membrane Substrate (100 µL) (Sigma-Aldrich, Tres Cantos, Spain) was added and incubated for 10 min, followed by application of -0.2V *vs.* Ag for 5 s. The reduction current was measured by amperometry using an Autolab (Methrom, Madrid, Spain). Nova 2.1.4 software was used to collect and evaluate the data. Electrochemical measurements were performed in triplicate.

2.6. DNA extraction and sequencing from single cells isolated in field samples

Single cells from field samples were isolated as described in our previous work (Gaiani et al., 2021). Extraction of genomic DNA from these single microalgal cells was performed using an Arcturus PicoPure DNA Extraction Kit (Thermo Fisher Scientific, Spain) following the manufacturer’s instructions. Briefly, 155 µL of reconstitution buffer were added to one of the provided vials with lyophilized proteinase K and mixed. Once dissolved, 10 µL of the solution were

added to each tube containing single cells isolated from field samples and identified as *Gambierdiscus* with light microscopy. DNA extraction was then achieved with a Nexus Gradient Thermal Cycler (Eppendorf, Spain) by incubating at 65 °C for 3 h ending with a step at 95 °C for 10 min. Extracted DNA was stored at -20 °C until analysis. The D1-D3 domain of the 28 S rDNA gene was amplified using *G. excentricus* primers (Table S1) in the single PCR mode as described in Section 2.3. The PCR reactions of single cell DNA preparations were executed in a total volume of 25 µL containing 600 µM dNTP, 2 mM $MgCl_2$, 0.2 µM of each primer, 1 U of Taq polymerase, 5% *v/v* DMSO, and 2 µL of the DNA extracted from single cells. Amplifications were performed in a Nexus Gradient Thermal Cycler (Eppendorf, Spain) and included 45 cycles of amplification following a three-step protocol (95 °C for 30 s, 60 °C for 45 s and 72 °C for 30 s). Each PCR reaction was checked by agarose (2% *w/v*) gel electrophoresis. PCR products of 150 bp were purified with QIAquick PCR Purification Kit and bidirectionally sequenced (Sistemas Genomicos, LLC, Valencia, Spain). Forward and reverse sequence reads were edited using BioEdit v7.0.5.2 (Hall, 1999), and the consensus sequences obtained were checked for similarities with the NCBI BLAST function.

2.7. Ciguatoxin extraction and detection

For the extraction of ciguatoxins from field samples, the remaining 500 µL of the 1-mL pellet resuspension used for DNA extraction were processed according to the protocol described in our previous work (Gaiani et al., 2020). Briefly, this volume was centrifuged, and supernatant was discarded and 1 mL of MeOH was then added to each tube and re-suspended pellets were transferred to 2-mL screw-cap cryotubes containing ca. 50 µg of 0.5 mm diameter zirconium glass beads. Subsequently, bead beating was conducted for 3 runs of 40 s each and extracts were then centrifuged at 3,700 rpm for 1 min and transferred to glass vials. Extracts were stored at -20 °C until analysis.

Analyses of extracts were performed as described in our previous work (Gaiani et al., 2020). Briefly, Dynabeads M-270 Carboxylic Acid MBs (Invitrogen, Life Technologies S.A., Alcobendas, Spain) were first activated with an EDC and NHS solution and then incubated with the capture mAb 3G8 (left wing of CTX1B and 54-deoxyCTX1B) or 10C9 (left wing of CTX3C and 51-hydroxyCTX3C) (Tsumuraya and Hirama, 2019). After incubation, the mAb-MB conjugates were washed, and an equal volume of both was placed into new tubes, exposed to microalgal extract (previously evaporated and suspended in PBS-Tween) or CTX1B standard (for the construction of the calibration curve). A blocking step was then performed in PBS-Tween-BSA. The conjugates were then incubated with a biotinylated 8H4 mAb, which 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. Electrochemical measurements were performed on the working electrodes of an 8-electrode array, following addition of TMB and H_2O_2, and measuring the

reduction current using amperometry (-0.2 V *vs.* Ag for 5 s). Measurements were performed in triplicate.

Table 1. *Gambierdiscus* cell abundances of samples from Majorca obtained with the developed biosensor and light microscopy following the Utermöhl method.

Sampling point	Sample code	Macrophyte substrate	Biosensor			Light microscopy
			G. australes **(cell L^{-1})**	***G. excentricus*** **(cell L^{-1})**	**Total (cell L^{-1})**	***Gambierdiscus*** **spp. (cell L^{-1})**
Cala Gat	2020-ME-886*	*Posidonia oceanica*	ND	ND	ND	ND
Platja Canyamel	2020-ME-906*	*Posidonia oceanica*	ND	ND	ND	ND
Portocolom	2020-ME-946*	*Posidonia oceanica*	ND	ND	ND	ND
Platja Canyamel	2020-ME-914	*Corallina elongata* *Digenea simplex*	484	176	660	5,800
Cala Anguila	2020-ME-930	*Digenea simplex* *Corallina elongata* *Jania adhaerents*	349	109	458	7,800
Cala Anguila	2020-ME-934	*Corallina elongata*	ND	280	280	700

Cala Llombards	2020-ME-966	*Cladostephus spongiosus*	515	108	623	2,700
Cala Llombards	2020-ME-970	*Halopteris scoparia Jania adhaerents*	1,181	58	1,239	36,100
Cala Galiota	2020-ME-986	*Halopithys incurva*	428	ND	428	3,200
Cala Galiota	2020-ME-990	*Halopithys incurva*	4,824	1,883	6,707	1,000
Cala Galiota	2020-ME-994	*Posidonia oceanica*	536	163	699	100
Cala Mosques	2020-ME-1034	*Posidonia oceanica* (rizoma)	2,536	273	2,809	2,300

(Continues from the previous page

*Samples used for the control trial without *Gambierdiscus* spp. ND: not detected*)*

3. Results and discussion

3.1. Optimization of PCR and DNA extraction methods

The DNA used for this experiment was extracted from pellets obtained from 50 mL of microalgal cultures and using the phenol/chloroform/isoamyl alcohol method. The Dynabeads™ DNA DIRECT™ Universal Kit procedure was also tested without the bead beating step, to check if it was possible to furtherly reduce time and machinery to perform the extraction. However, *Gambierdiscus* are known to be armored microalgae, and the disruption of their thecae can be a difficult task to perform.

PCR conditions were optimized with the colorimetric assay on microtiter plates, and the final optimization led to the results shown in Figure 2. Absorbance values in the presence of target DNA at 1 ng μL^{-1} and the corresponding capture probe were always higher than the NTC absorbance values, indicating that the system is able to discriminate between the presence and absence of target DNA. In the presence of both *G. australes* and *G. excentricus* DNA, absorbance signals were also observed and clearly distinguished from the NTC. The absorbance values for the amplification of *G. australes* DNA in the presence of *G. excentricus* DNA were higher than those obtained for the amplification of *G. excentricus* DNA in the presence of *G. australes* DNA. This effect, also observed in our previous work (Gaiani et al., 2021), seems to indicate that the *G. australes* primers are more efficient than the *G. excentricus* ones when both species are present.

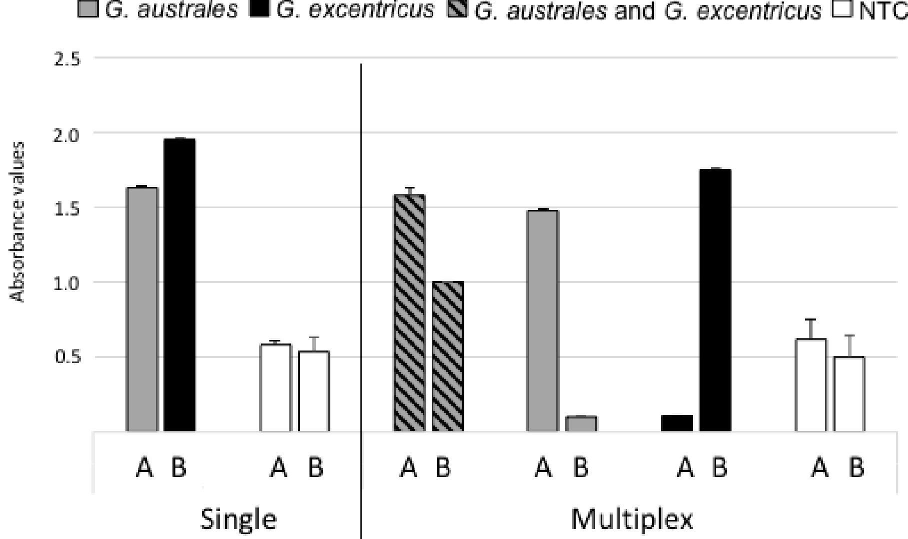


Figure 2. Absorbance values of the PCR-SHA on microtiter plates using single and multiplex PCR. Measurements were performed in triplicate and bars indicate standard deviations. Black line separates results obtained with the single and multiplex strategies. **A**: *G. australes* capture probe; **B**: *G. excentricus* capture probe.

Using the optimized PCR, the different DNA extraction protocols (described in Section 2.2) were evaluated. For this test, 1,000 cells from *G. australes* IRTA-SMM-16_286 and 1,000 cells from *G. excentricus* VGO791 were extracted with each technique. This concentration of cells was chosen as a compromise between a low number of cells and the possibility to obtain sufficient good-quality DNA for the amplification. In this experiment, 1 µL of each extracted DNA was amplified with the multiplex PCR protocol, and the amplified product was then exposed to the *G. australes* and *G. excentricus* capture probes. For all protocols and for both species, higher absorbance values were observed in the presence of the amplified product obtained from a target DNA with its corresponding capture probe (i.e., *G. australes* target DNA with *G. australes* capture probe, and *G. excentricus* target DNA with *G. excentricus* capture probe) than in the presence of the non-corresponding capture probe (Figure 3). Comparing the extraction techniques, the use of the TerraLyzer with MBs was the only one that provided absorbance values close to the ones obtained with the phenol/chloroform/isoamyl alcohol method for both species, which in this experiment is considered as the standard method. This method is also advantageous because the bead beating step with the TerraLyzer can be performed in the field, since it is a portable device with a compact charging system, and also because the use of MBs significantly reduces the DNA extraction time (from several hours to 30 min).

It is known that rDNA copies per cell can vary according to species, strain, geographic origin and growth phase (Galluzzi et al., 2010; Kretzschmar et al., 2019; Nishimura et al., 2016; Vandersea et al., 2012). Hence, to minimize differences due to the copy number, the *G. australes* and *G. excentricus* strains chosen for this experiment came from the same geographic region (Canary Islands) and cells were harvested at the same growth phase (i.e., exponential). However, the *G. excentricus* always provided lower absorbance values than the *G. australes*. Even though we tried to minimize as much as possible the effect of the rDNA copy number, the performance of the assay may be affected by this factor.

In fact, these results are in agreement with those previously obtained with the recombinase polymerase amplification (RPA), which showed different absorbance values between several *G. australes* and *G. excentricus* strains at the same DNA template concentration (Gaiani et al., 2021). The chosen rapid DNA extraction technique (Terralyzer and MBs) enables the procurement of DNA quality and quantity equivalent to that obtained using the reference method, moving towards the realization of an *in situ* DNA extraction method.

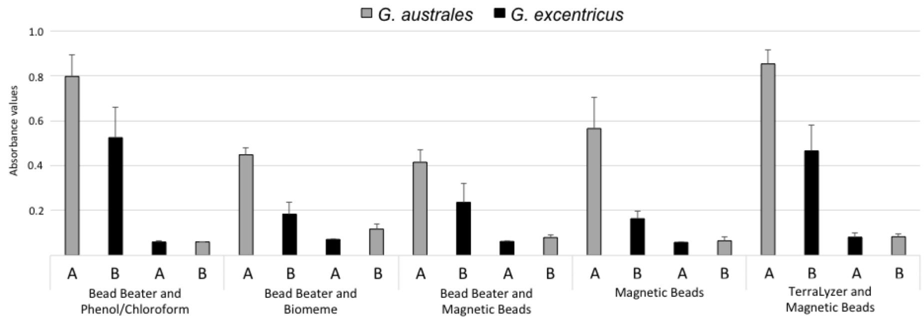


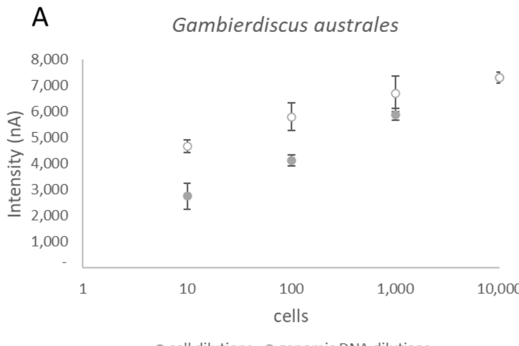
Figure 3. Absorbance values of the PCR-SHA (single) on microtiter plates using 10^3 cells and different DNA extraction methods. Measurements were performed in triplicate and bars indicate standard deviations. **A**: *G. australes* capture probe; **B**: *G. excentricus* capture probe.

3.2. Electrochemical biosensor for DNA detection

Using the optimized PCR conditions, calibrations curves for *G. australes* and *G. excentricus* were constructed using the dual biosensor and genomic DNA extracted from 10^4, 10^3, 10^2 and 10 cells of each species as well as dilutions of genomic DNA extracted from a sample containing 10^4 cells (using the Terralyzer and MBs for the DNA extraction). The precipitation of TMB_{ox} was chosen, since it has been reported as an efficient electrochemical substrate (del Río et al., 2014). In our configuration, the HRP-labelled reporter probe hybridizes with the tail of the amplified product, which is hybridized with the *G. australes* or *G. excentricus* capture probe on the MBs. In the presence of HRP in the system, the addition of TMB Enhanced One Component HRP Membrane Substrate produces a stable electroactive precipitate at the surface of the electrode that does not dissolve in aqueous buffer, as TMB used for colorimetric ELISA normally does (Sánchez et al., 2016). In this way, it was possible to differentiate between the presence of amplified product on one electrode and the absence on the other, without cross-reactivity.

Results showed higher reduction current intensity with increasing concentrations of DNA for both DNA extracted from cells as well as dilutions of genomic DNA, as expected. The analysis of dilutions of *G. australes* genomic DNA resulted in higher absorbance values in comparison to the corresponding extracted cells (Figure 4A). It is important to take into account that in the calibration curve obtained from the cell dilutions, there is an extraction step for each point of the curve. The efficiency of this extraction step may be compromised by the number of cells, and may be lower when cells are more dispersed in the lysis buffer. However, on the other hand, the analysis of dilutions of *G. excentricus* genomic DNA and extracted cells resulted in closer absorbance values between equivalent concentrations (Figure 4B). We postulate that the cells of this strain and culture may be easier to disrupt in comparison to the *G. australes* ones, and thus the effect of the efficiency of the extraction is less notable. For both *G. australes* and *G. excentricus*, the strategy

facilitated successful extraction of DNA, allowing detection of a small number of cells (10).



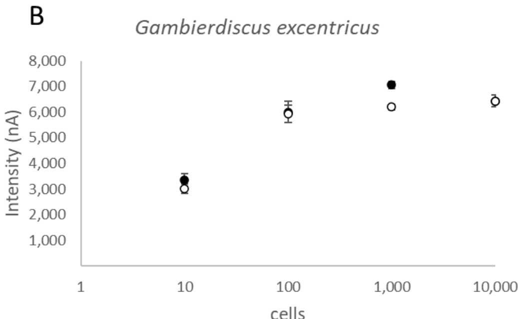


Figure 4. Calibration curves obtained from the extraction of 10, 10^2, 10^3 and 10^4 cells (grey/black) and genomic DNA dilutions from 10^4 cells (white) using the dual biosensor (multiplex). Measurements were performed in triplicate and bars indicate standard deviations. A: *G. australes*; B: *G. excentricus*.

Subsequently, to demonstrate the ability of the dual biosensor to simultaneously detect both species, nine mixtures of cells (Table 2) were prepared and DNA was extracted with the TerraLyzer and MBs, amplified with PCR (multiplex), and analyzed with the biosensor. The results obtained demonstrate that reduction currents above the background are observed when the target amplified products are exposed to the corresponding capture probes. The system allows discrimination between amplified products belonging to *G. australes* and *G. excentricus* species. Negligible signals were observed in the absence of both targets, i.e. mix 9 (Figure 5). As observed in previous experiments, the presence of the non-target amplified product affects the detection of the target ones. Indeed, the analysis of the mixes in which only one of the two target species is present (mixes 1 and 4 for *G. australes* and mixes 7 and 8 for *G. excentricus*) gave higher current intensity values in comparison with the mixes with the same amount of target cells, but in the presence of different concentrations of the other species (mixes 2, 3, 5 and 6). The observed differences could be attributed to a better affinity of the primers for the target region of *G. australes,* since its presence seems to hinder more the detection of *G. excentricus* than the contrary even though, when only *G. excentricus* is present (mixes 7 and 8), current intensities are higher than for *G. australes* (mixes 1 and 4). Again, as observed in the previous experiment (Figure 4), it seems that *G. excentricus* cells are easier to lyse than those of *G. australes*. Therefore, a better extraction efficiency of *G. excentricus* cells can also be the explanation for the differences observed. However, as mentioned above, the rDNA copy number cannot be excluded as one of the reasons contributing to these differences. Moreover, the presence of

G. australes cells has a higher effect on the detection of *G. excentricus* than the contrary (mixes 2 and 6). Nevertheless, at an equal concentration of cells (mixes 3 and 5), the intensity values are similar, indicating that, even if the detection is to some extent influenced by the non-target species, the system recognizes both. In summary, since the specificity of the primers allows them to amplify target DNA even in the presence of non-target species belonging to the same genus (as demonstrated in this work and in Gaiani et al., 2021), the system is suitable for the screening of field samples.

Table 2. *G. australes* and *G. excentricus* cells amount for each mix.

Mix number	*G. australes* cells	*G. excentricus* cells
1	10^3	0
2	10^3	10^2
3	10^3	10^3
4	10^2	0
5	10^2	10^2
6	10^2	10^3
7	0	10^2
8	0	10^3
9	0	0

3.3. *G. australes* and *G. excentricus* DNA detection in field samples

To evaluate the applicability of the TerraLyzer and MBs protocol combined with the dual biosensor for the analysis of field samples, a preliminary experiment was performed using several dilutions (pure, 1:10, 1:100, 1:1,000) of DNA extracted from samples in which no *Gambierdiscus* sp. had previously been detected with light microscopy (2020-ME-886, 2020-ME-906, 2020-ME-946 in Table 1), but other microalgae were present (Table S2). Results demonstrate that the presence of other genera of microalgae did not give current intensity values higher than the limit of detection (LOD). The amount of *Gambierdiscus* spp. cells, if any, was below the LOD for both *G. australes* and *G. excentricus*. Subsequently, 10^2, 10^3 and 10^4 *G. australes* cells were spiked into those field samples. Samples spiked with *G. excen*tricus cells were prepared in a similar manner. DNA was again extracted with the TerraLyzer and MBs protocol and PCR amplification was performed with several dilutions of the extracted DNA (pure, 1:10, 1:100, 1:1,000). Results were very similar to those obtained in the construction of the calibration curves, but at 1:1,000 DNA dilutions, indicating that the field sample matrix affects the detection of the target species, in agreement with that observed by Nishimura et al. (2016).

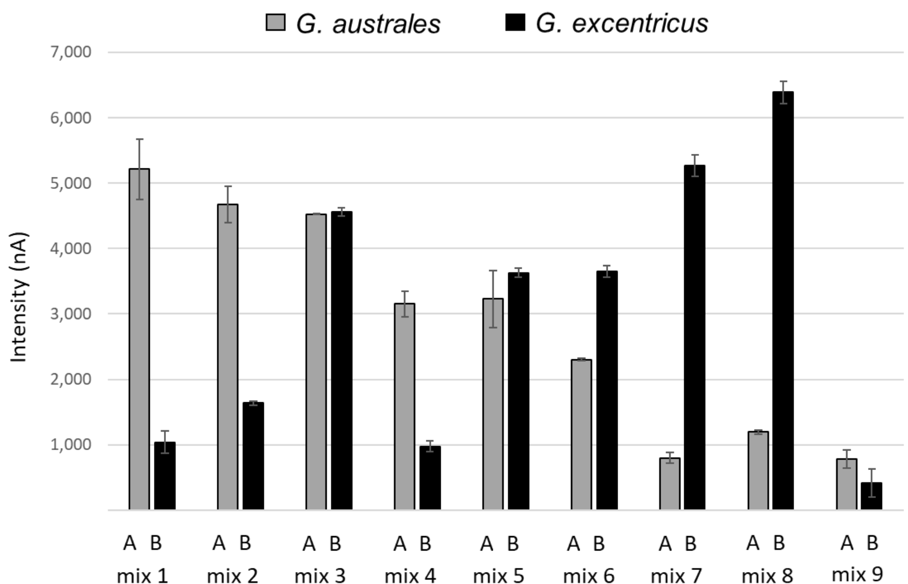


Figure 5. Current intensity values of the PCR-SHA (multiplex) on the dual electrode using mixes with different amounts of *G. australes and G. excentricus* cells. Measurements were performed in triplicate and bars indicate standard deviations. **A**: *G. australes* capture probe; **B**: *G. excentricus* capture probe.

Subsequently, DNA was extracted from 9 field samples from Majorca in which *Gambierdiscus* spp. had previously been detected with light microscopy, using the TerraLyzer and MBs protocol (Table 1). DNA was diluted 1:1,000, multiplex PCR was performed, and the amplified products were analyzed with the dual biosensor. Cell abundancies were estimated using the calibration curves of cell dilutions. Results showed an overall higher estimated abundance of *G. australes* cells rather than *G. excentricus* (Table 1), with the exception of sample 2020-ME-934 in which *G. australes* was not detected. Furthermore, *G. excentricus* was not detected in sample 2020-ME-986. In general (6 out of 9 samples), the cell abundances estimated with our strategy are lower than the ones obtained with light microscopy (apart from sample 2020-ME-990, 2020-ME-994 and 2020-ME-1034, in which the difference in cell abundance estimation does not go over one order of magnitude), similar to that reported by Vandersea et al. (2012). This may be attributable to the rDNA copy number of the field samples cells differing with that obtained in cultured cells, and this can result in an over/under estimation of the real cell abundances in a sample (Galluzzi et al., 2010; Andree et al. 2011). Additionally, it should be taken into consideration that other species may also be present in the samples and their identification based on morphological features by light microscopy is almost impossible due to the similarities among species of this genus (Litaker et al., 2009). Recently, another biosensor for the detection of different toxin-producing microalgae, including *G. australes* and *G. excentricus,* was developed (Medlin et al., 2020), where they used an approach similar to our system with synthetic DNA or RNA of *G. australes* and *G. excentricus.* The

LOD achieved by Medlin and coworkers was close to 1 pM of RNA, but they did not provide a corresponding quantification of cell abundance. Nevertheless, for the other dinoflagellates targeted in their study, the number of cells corresponding to 1 pM of RNA ranged from 10 to 444 cultivated cells, so it is probable that the LOD for *Gambierdiscus* species is in that range, and thus, similar to our results. While the technique presented by Medlin and coworkers is faster, as there is no PCR step, the analysis of genomic DNA/RNA or the screening of field samples was not demonstrated. Additionally, RNA has a highly labile nature, thus detecting it from fixed field samples cells can be problematic (Loukas, et al., 2017). Therefore, despite its limitations, the strategy developed by our group can be considered as a successful step towards practical application in the field, with the developed biosensor allowing the simultaneous discrimination between *G. australes* and *G. excentricus,* both of which are known toxin-producing microalgae species, making the tool suitable for monitoring and research programs. Moreover, *Gambierdiscus* cell abundances in field samples can reach more than 1,000,000 cells per g wet weight algae (Chinain et al., 1999; Litaker et al., 2010; Vandersea et al., 2012), but CTX production has also been detected at very low cell abundances (80.4 ± 56.9 cells per g *Dictyota*, Liefer et al. (2021)). Therefore, the ability to detect low *Gambierdiscus* cell abundances in field samples is of utmost importance to provide timely warnings of possible ciguatera outbreaks, thus enabling informed management decisions.

3.4. First report of *Gambierdiscus excentricus* in Balearic Islands

To date only one species of *Gambierdiscus*, *G. australes*, has been described in the Balearic archipelago (Tudó et al., 2018, Tudó et al., 2020a). Our results obtained from the screening of field samples with the developed biosensor revealed the presence of DNA belonging to *G. excentricus*. Therefore, to have a further confirmation, several single cells were isolated from field samples, and the DNA was extracted and sequenced. Results showed that, among the analyzed cells, 5 belonged to *G. excentricus* species (2 from Cala Galiota, 2 from Platja Canyamel and 1 from Camp de Mar, see Figure 6). As significant as this discovery might seem, it is not entirely surprising since *Gambierdiscus* species have been found in cohabitation in several studies of other locations (Nishimura et al., 2016; Vandersea et al., 2012; Tester et al., 2020; Tudó et al., 2020b). Particularly, in the Canary Islands *G. excentricus* is usually accompanied by the presence of *G. australes* (Tudó et al., 2020b). Nevertheless, *G. excentricus* was the first species identified and described in the Canary Islands (Fraga et al., 2011), and only some years later, *G. australes* was identified in field samples together with the description of another new species, *G. silvae* (Fraga and Rodriguez, 2014). Something similar happened in the Mediterranean, where the presence of *Gambierdiscus* was recorded for the first time in Crete (Aligizaki and Nicolaidis, 2008) (the species was not assigned then, even if the authors stated that it was a "*G. toxicus* type"). Later on, G. *carolinianus* (Holland et al., 2013), *G.* cf. *belizeanus* and *G. silvae* (Aligizaki et al., 2018) were found and identified again in Crete waters. In 2018, Tudó and

coworkers reported the presence of *G. australes* in the Balearic Islands (Tudó et al, 2018; Tudó et al., 2020a), and the detection of another species in Majorcan waters was somewhat expected. The reason behind the delay in detecting different species is still unclear. It can either be due to an increase in the monitoring effort, which can cause a perceived increase in the reports of *Gambierdiscus* spp. outside of their endemic area (as Hallegraeff et al. (2021) demonstrate for the perceived global increase in algal blooms), or to the true recent introduction of *Gambierdiscus* spp. In this last scenario, coastal ship traffic could play an active role as a vector of introduction, as this has already been found to be responsible for the transport of various genera of harmful dinoflagellates (Butron et al., 2011; Roy et al. 2012). Another hypothesis could involve the transport through plastics or other types of substrates.

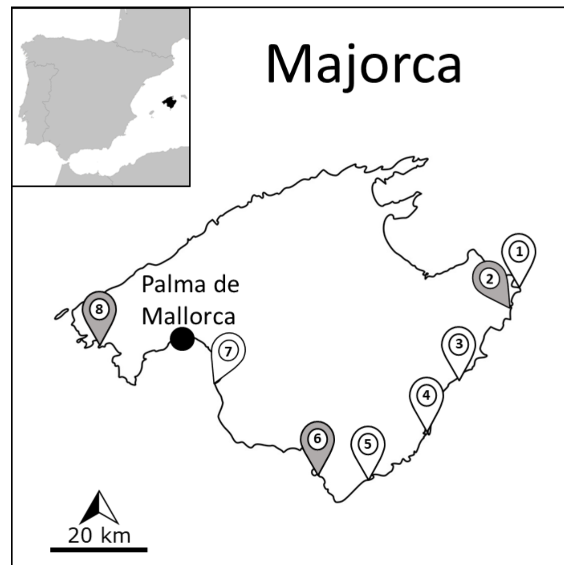


Figure 6. Sampling points of Majorca. (1) Cala Gat, (2) Platja Canyamel, (3) Cala Anguila, (4) Portocolom, (5) Cala Llombards, (6) Cala Galiota, (7) Cala Mosques and (8) Camp de Mar. Grey color indicates the points from which *G. excentricus* single cells have been isolated and identified.

3.5. Ciguatoxin detection in field samples

Analysis with our previously developed immunosensor with the combined 3G8 and 10C9 capture antibodies revealed the presence of quantifiable CTX1B equivalents in 1 (2020-ME-970 from Cala Llombards) out of the 9 analyzed samples. Three samples (2020-ME-990 and 2020-ME-994 from Cala Galiota and 2020-ME-1034 from Cala Mosques) showed very low CTX1B equivalents, which were not quantifiable, since the values were above the LOD but below the LOQ. Recently, Liefer and coworkers (2021) suggested considering a cell toxin quota (pg CTX equiv. cell^{-1}) rather than cell abundances to investigate the presence of CTX, since in their studies CTX detection mostly occurred in the presence of low abundances of *Gambierdiscus* cells. Liefer et al. (2021) converted the mouse units (one mouse unit = 18 ng of CTX3C for Pacific samples and 72 ng of C-CTX-1 for Caribbean samples) of the mouse bioassay (MBA) data obtained in previous works to CTX toxin quotas (whose ranges were 0.03-1 (Bagnis et al., 1980), 0.05-1.35 (Bagnis et al., 1990), 0.09-3.60 (Chinain et al., 1999), 1.14-5.14 (McMillan et al., 1986), 0.23 (Holmes et al., 1994), 0.96-1.42

(Yasumoto et al., 1979) and 24 pg CTX equiv. cell^{-1} (Withers, 1983)), in order to make the comparison among different studies easier to interpret. In recent years, due to the ethical controversy and the lack of specificity of the MBA, other tests have been used to detect CTX in field samples such as the Radioligand Receptor Binding Assay (RBA) (Chinain et al., 2020; Darius et al., 2007) (which results ranged respectively from 0.5-13.5 and 0.85-3.90 pg of CTX1B equiv. cell^{-1}) and the *in vitro* neuroblastoma cell-based assay (Neuro2a) (Liefer et al., 2021; Pawlowiez et al., 2013) (which results ranged respectively from 0-12.62 and 0.03 ± 0.004 pg of CTX1B equiv. cell^{-1}). In our study, sample 2020-ME-970 showed 13.35 ± 0.5 pg CTX1B equiv. cell^{-1}, a value that is comparable to the results obtained in the studies mentioned above, indicating that our rapid and reliable strategy is suitable for the analysis of field samples. The CTX contents obtained in the analysis of laboratory cultures are usually much lower than those obtained from field samples. In fact, CTX contents in laboratory cultures of *G. australes* isolates from Majorca analyzed with the Neuro2a assay ranged from 1.38 to 381 fg CTX1B equiv. cell^{-1} (Tudó et al., 2020a). Regarding *G. excentricus*, to date there are no studies regarding the toxicity of strains from Majorca (or the Balearic Islands in general) since it has not yet been isolated and cultured. However, the data available for cultured strains of this species from other regions presented a CTX production comparable to that obtained in our study (0.47 pg CTX3C equiv. cell $^{-1}$ (Litaker et al., 2017) and 1.43 pg CTX3C equiv. cell $^{-1}$ (Pisapia et al., 2017a)). Undoubtedly, laboratory studies of cultured *Gambierdiscus* are essential to better understand the ecotoxicological behavior of this toxin-producing genus, but these artificial systems cannot completely mimic the complex interactions that occur in a natural system. It must be underlined that, even if the cell toxin quota value obtained from the analysis of Balearic Island samples is comparable to the ones obtained in the Great Caribbean region, it has to be considered as a preliminary result, and further studies are definitely needed to better investigate the risk of a future ciguatera outbreak.

4. Conclusion

In this study, the development of the first dual biosensor for the simultaneous detection of *G. australes* and *G. excentricus* in field samples is presented. Additionally, a protocol for the rapid extraction of DNA, based on a portable bead beater and MBs, is developed and successfully tested on microalgal cultures and field samples. Using the strategy presented in this work, it has been possible to detect *G. excentricus* in the Balearic Islands, the presence of which had not yet been reported. Therefore, the developed strategy could be implemented in monitoring systems to identify new areas of expansion of these two toxin-producing species, preventing the occurrence of a ciguatera intoxication event.

Furthermore, for the first time, an electrochemical immunosensor is exploited for the detection of CTXs in a field sample extract, and the results obtained are similar to those previously observed in the Great Caribbean Region. This result

underlines the need for rapid and easy-to-use tools to monitor the Mediterranean Sea for CTXs in order to correctly manage potential ciguatera outbreaks.

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Supplementary material

Table S1. Primers with tails and probes used in this study. Tails are underlined.

Name	Sequence (5'-3')
G. australes **reverse primer**	GTT TTC CCA GTC ACG AC-C3-ATG CAT AAC TCT TCA TTG CCA GTA G
G. excentricus **reverse primer**	TCT ACA GGC TCG TAT ATG TA-C3-AGC TTG GGT CAC AGT GCA ACA GAG
G. australes & G. excentricus **forward primer**	TGT AAA ACG ACG GCC AGT-C3-TGC TGC ATG YGG AGA TTC TTT YYT KG
G. australes **capture probe**	GTC GTG ACT GGG AAA ACT TTT TTT TTT TTT TT-C3-thiol
G. excentricus **capture probe**	TAC ATA TAC GAG CCT GTA GAT TTT TTT TTT TTT TT-C3-thiol
Reporter probe	HRP-ACT GGC CGT CGT TTT ACA

Table S2. Microalgae abundances other than *Gambierdiscus* in the samples used in this work.

Sampling point	Sample code	*Fukuyoa* sp. (cell L^{-1})	*Ostreopsis* sp. (cell L^{-1})	*Prorocentrum* sp. (cell L^{-1})	*Coolia* sp. (cell L^{-1})
Cala Gat	2020-ME-886	ND	12,252	32,672	34,714
Platja Canyamel	2020-ME-906	ND	2,700	9,700	7,000
Platja Canyamel	2020-ME-914	ND	61,260	55,134	106,184
Cala Anguila	2020-ME-930	ND	38,798	47,266	51,050
Cala Anguila	2020-ME-934	ND	12,252	14,294	18,378
Portocolom	2020-ME-946	ND	1,000	1,000	400
Cala Lombards	2020-ME-966	ND	ND	2,900	900
Cala Llombards	2020-ME-970	ND	ND	6,126	34,714
Cala Galiota	2020-ME-986	ND	ND	18,378	12,252
Cala Galiota	2020-ME-990	ND	200	2,542	22,462
Cala Galiota	2020-ME-994	ND	700	ND	100
Cala Mosques	2020-ME-1034	ND	100	500	1,000

ND: not detected

References

Aligizaki K, Nikolaidis G (2008). Morphological identification of two tropical dinoflagellates of the genera Gambierdiscus and Sinophysis in the Mediterranean Sea. J Biol Res-Thessalon 9: 75-82.

Aligizaki K, Nikolaidis G, Katikou P, Baxevanis AD, Abatzopoulos TJ (2009). Potentially toxic epiphytic *Prorocentrum* (Dinophyceae) species in Greek coastal waters. Harmful Algae 8:299-311.

Aligizaki K., Iliadou M., Kappas I., Arsenakis M (2018). Is the eastern Mediterranean a "*Gambierdiscus* biodiversity hotspot"? New data from Greece and Cyprus. In: Hess P (ed). Abstract book of the 18th International Conference on Harmful Algae. IFREMER, Nantes, 493 pp.

Andree KB, Quijano-Scheggia S, Fernández M, Elandaloussi LM, Garcés E, Camp J, Diogene J (2011). Quantitative PCR Coupled with melt curve analysis for detection of selected *Pseudonitzschia* spp. (Bacillariophyceae) from the Northwestern Mediterranean Sea. Appl Environ Microbiol 77: 1651-1659.

Bagnis R, Chanteau S, Chungue E, Hurtel JM, Yasumoto T, Inoue A (1980). Origins of ciguatera fish poisoning: a new dinoflagellate, *Gambierdiscus toxicus* Adachi and Fukuyo, definitively involved as a causal agent. Toxicon 18: 199-208.

Bagnis R, Legrand A-M, Inoue A. Follow-up of a bloom of the toxic dinoflagellate *Gambierdiscus toxicus* on a fringing reef of Tahiti. In: Graneli E, Sundstrom B, Edler L, Anderson DM (eds). Toxic Marine Phytoplankton. Elsevier, New York, USA, 1990, pp 98-103.

Begier EM, Backer LC, Weisman RS, Hammond RM, Fleming LE, Blythe D (2006). Outbreak bias in illness reporting and case confirmation in ciguatera fish poisoning surveillance in south Florida. Public Health Rep 121:658-665.

Bravo I, Rodriguez F, Ramilo I, Rial P, Fraga S (2019). Ciguatera-Causing Dinoflagellate *Gambierdiscus* spp. (Dinophyceae) in a subtropical region of North Atlantic Ocean (Canary Islands): morphological characterization and biogeography. Toxins 11:423.

Butrón A, Orive E, Madariaga I. Potential risk of harmful algae transport by ballast waters: The case of Bilbao Harbour (2011). Mar Pollut Bull 62(4): 747-757.

Chinain M, Faust MA, Pauillac S (1999). Morphology and molecular analyses of three toxic species of *Gambierdiscus* (Dinophyceae): *G. pacificus*, sp. nov., *G. australes*, sp. nov., and *G. polynesiensis*, sp. nov. J Phycol 35:1282-1296. doi:https://doi.org/10.1046/j.1529-8817.1999.3561282.x

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

Chinain M, Gatti CM, Ung A, Cruchet P, Revel T, Viallon J, Sibat M, Varney P, Laurent V, Hess P, Darius HT (2020). Evidence for the range expansion of ciguatera in French Polynesia: a revisit of the 2009 mass-poisoning outbreak in Rapa Island (Australes Archipelago). Toxins 12 :759.

Darius HT, Ponton D, Revel T, Cruchet P, Ung A, Tchou Fouc M, Chinain M (2007). Ciguatera risk assessment in two toxic sites of French Polynesia using the receptor-binding assay. Toxicon 50: 612-626.

del Río JS, Yehia Adly N, Acero-Sánchez JL, Henry OYF, O'Sullivan CK (2014). Electrochemical detection of *Francisella tularensis* genomic DNA using solid-phase recombinase polymerase amplification. Biosens Bioelectron 54: 674-678.

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

Fraga S, Rodriguez F (2014). Genus *Gambierdiscus* in the Canary Islands (NE Atlantic Ocean) with description of *Gambierdiscus silvae* sp. nov., a new potentially toxic epiphytic benthic dinoflagellate. Protist 165:839-853.

Gaiani G, Leonardo S, Tudó À, Toldrà A, Rey M, Andree KB, Tsumuraya T, Hirama M, Diogène J, O'Sullivan CK, Alcaraz C, Campàs M (2020). Rapid detection of ciguatoxins in *Gambierdiscus* and *Fukuyoa* with immunosensing tools. Ecotoxicol Environ Saf 204:111004.

Gaiani G, Toldrà A, Andree KB, Rey M, Diogène J, Alcaraz C, O'Sullivan CK, Campàs M (2021). Detection of *Gambierdiscus* and *Fukuyoa* single cells using recombinase polymerase amplification combined with a sandwich hybridization assay. J Appl Phycol 33:2273–2282.

Galluzzi L, Bertozzini E, Penna A, Perini F, Garcés E, Magnani M (2010). Analysis of rRNA gene content in the Mediterranean dinoflagellate *Alexandrium catenella* and *Alexandrium taylori*: implications for the quantitative real-time PCR-based monitoring methods. J Appl Phycol 22:1-9.

Gómez F, Qiu D, Lopes RM, Lin S (2015). *Fukuyoa paulensis* gen. et sp. nov., a new genus for the globular species of the dinoflagellate *Gambierdiscus* (Dinophyceae). PLoS One 10.

Greeson PE (1977). Methods for collection and analysis of aquatic biological and microbiological samples. In: Techniques of water-resources investigations of the United States Geological Survey, vol 5. Laboratory analysis. US Department of the Interior, Geological Survey

Hall TA (1999). BioEdit: a user-friendly biological sequence alignment editor and analysis program for Windows 95/98/NT. Nucleic Acids Symp Ser 41: 95–98.

Hallegraeff GM, Anderson DM, Belin C, Bottein M-YD, Bresnan E, Chinain M, Enevoldsen E, Iwataki M, Karlson B, McKenzie C, Sunesen I, Pitcher GC, Provoost P, Richardson A, Schweibold L, Tester PA,

Trainer VL, Yñiguez AT, Zingone A (2021). Perceived global increase in algal blooms is attributable to intensified monitoring and emerging bloom impacts. Commun Earth & Environ 2: 117.

Holmes MJ, Lewis RJ (1994). Purification and characterisation of large and small maitotoxins from cultured *Gambierdiscus toxicus*. Nat Toxins 2:64-72.

Holmes MJ, Lewis RJ, Sellin M, Street R (1994). The origin of ciguatera in Platypus Bay, Australia. Mem Qld Mus 34: 505-512.

Jang SH, Jeong HJ, Yoo YD (2018). *Gambierdiscus jejuensis* sp. nov., an epiphytic dinoflagellate from the waters of Jeju Island, Korea, effect of temperature on the growth, and its global distribution. Harmful Algae 80:149-157.

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

Kelly AM, Kohler CC, Tindall DR (1992). Are crustaceans linked to the ciguatera food chain? Environ Biol Fishes 33: 275-286.

Kohli GS, Farrell H, Murray SA (2015). *Gambierdiscus*, the cause of ciguatera fish poisoning: An increased human health threat influenced by climate change. In: Botana LM, Louzao C, Vilariño N (eds). Climate change and marine and freshwater toxins. De Gruyter, Berlin (Germany), pp 273-312.

Kretzschmar AL, Larsson ME, Hoppenrath M, Doblin MA, Murray SA (2019). Characterisation of two toxic *Gambierdiscus* spp. (Gonyaulacales, Dinophyceae) from the Great Barrier Reef (Australia): *G. lewisii* sp. nov. and *G. holmesii* sp. nov. Protist 170.

Larsson ME, Harwood TD, Lewis RJ, Swa H, Doblin MA (2019). Toxicological characterization of *Fukuyoa paulensis* (Dinophyceae) from temperate Australia. Phycological Res 67:65-71.

Laza-Martínez A, David H, Riobó P, Miguel I, Orive E (2016). Characterization of a strain of *Fukuyoa paulensis* (Dinophyceae) from the Western Mediterranean Sea. J Eukaryot Microbiol 63:481-497.

Ledreux A, Brand H, Chinain M, Bottein M-YD, Ramsdell JS (2014). Dynamics of ciguatoxins from *Gambierdiscus polynesiensis* in the benthic herbivore *Mugil cephalus*: Trophic transfer implications. Harmful Algae 39:165-174.

Lewis RJ (2001). The changing face of ciguatera. Toxicon 39: 97-106.

Liefer JD, Richlen ML, Smith TB, DeBose JL, Xu Y, Anderson DM, Robertson A (2021). Asynchrony of *Gambierdiscus* spp. abundance and toxicity in the U.S. Virgin Islands: implications for monitoring and management of ciguatera. Toxins 13.

Litaker RW, Vandersea MW, Faust MA, Kibler SR, Chinain M, Holmes MJ, Holland WC, Tester PA (2009). Taxonomy of *Gambierdiscus* including four new species, *Gambierdiscus caribaeus*, *Gambierdiscus carolinianus*, *Gambierdiscus carpenteri* and *Gambierdiscus ruetzleri* (Gonyaulacales, Dinophyceae). Phycologia 48:344-390.

Litaker RW, Vandersea MW, Faust MA, Kibler SR, Nau AW, Holland WC, et al. Global distribution of ciguatera causing dinoflagellates in the genus *Gambierdiscus*. Toxicon 2010; 56: 711-30.

Litaker RW, Holland WC, Hardison DR, Pisapia F, Hess P, Kibler SR, Tester PA (2017). Ciguatoxicity of *Gambierdiscus* and *Fukuyoa* species from the Caribbean and Gulf of Mexico. PLoS One 12:e0185776.

Litaker RW, Tester PA, Vandersea MW (2019). Species-specific PCR assays for *Gambierdiscus excentricus* and *Gambierdiscus silvae* (Gonyaulacales, Dinophyceae). J Phycol 55:730-732. doi: https://doi.org/10.1111/jpy.12852

Longo S, Sibat M, Viallon J, Darius HT, Hess P, Chinain M (2019). Intraspecific variability in the toxin production and toxin profiles of in vitro cultures of *Gambierdiscus polynesiensis* (Dinophyceae) from French Polynesia. Toxins (Basel) 11:735.

Loukas CM, McQuillan JS, Laouenan F, Tsaloglou MN, Ruano-Lopez JM, Mowlem MC (2017). Detection and quantification of the toxic microalgae *Karenia brevis* using lab on a chip mRNA sequence-based amplification. J Microbiol Methods 139: 189-195. DOI: https://doi.org/10.1016/j.mimet.2017.06.008.

McMillan JP, Hoffman PA, Granade H (1986). *Gambierdiscus toxicus* from the Caribbean: a source of toxins involved in ciguatera. Mar Fish Rev 48: 48-52.

Medlin LK, Gamella M, Mengs G, Serafín V, Campuzano S, Pingarrón JM (2020). Advances in the detection of toxic algae using electrochemical biosensors. Biosensors 10.

Murata M, Naoki H, Iwashita T, Matsunaga S, Sasaki M, Yokoyama A, Yasumoto T (1993). Structure of maitotoxin. J Am Chem Soc 115:2060-2062.

Murray JS, Selwood AI, Harwood DT, van Ginkel R, Puddick J, Rhodes LL, Rise F, Williams AL (2019). 44-Methylgambierone, a new gambierone analogue isolated from *Gambierdiscus australes*. Tetrahedron Lett 60: 621-625.

Nagai H, Murata M, Torigoe K, Satake M, Yasumoto T (1992). Gambieric acids, new potent antifungal substances with unprecedented polyether structures from a marine dinoflagellate *Gambierdiscus toxicus*. J Org Chem 57: 5448-5453.

Nishimura T, Sato S, Tawong W, Sakanari H, Yamaguchi H, Adachi M (2014). Morphology of *Gambierdiscus scabrosus* sp. nov.(G onyaulacales): a new epiphytic toxic dinoflagellate from coastal areas of Japan. J Phycol 50:506-514.

Nishimura T, Hariganeya N, Tawong W, Sakanari H, Yamaguchi H, Adachi M (2016). Quantitative PCR assay for detection and enumeration of ciguatera-causing dinoflagellate Gambierdiscus spp.(Gonyaulacales) in coastal areas of Japan. Harmful Algae 52:11-22.

Oguri H, Hirama M, Tsumuraya T, Fujii I, Maruyama M, Uehara H, Nagumo Y (2003). Synthesis-based approach toward direct sandwich immunoassay for ciguatoxin CTX3C. J Am Chem Soc 125: 7608-12.

Pawlowiez R, Darius HT, Cruchet P, Rossi F, Caillaud A, Laurent D, Chinain M (2013). Evaluation of seafood toxicity in the Australes archipelago (French Polynesia) using the neuroblastoma cell-based assay. Food Addit Contam Part A Chem Anal Control Expo Risk Assess 30: 567-86.

Pisapia F, Holland WC, Hardison DR, Litaker RW, Fraga S, Nishimura T, Adachi M, Nguyen-Ngoc L, Sechet V, Amzil Z, Herrenknecht C, Hess P (2017a). Toxicity screening of 13 *Gambierdiscus* strains using neuro-2a and erythrocyte lysis bioassays. Harmful Algae 63:173-183.

Pisapia F, Sibat M, Herrenknecht C, Lhaute K, Gaiani G, Ferron PJ, Fessard V, Fraga S, Nascimento SM, Litaker RW, Holland WC, Roullier C, Hess P (2017b). Maitotoxin-4, a Novel MTX Analog Produced by *Gambierdiscus excentricus*. Mar Drugs 15:220.

Provasoli L (1968). Media and prospects for the cultivation of marine algae. In: Hattori A (ed). Proceedings of the US-Japanese conference, cultures and collection of algae. Japanese Society of Plant Physiology, Hakone (Japan), pp 63-75

Rhodes L, Harwood T, Smith K, Argyle P, Munday R (2014). Production of ciguatoxin and maitotoxin by strains of *Gambierdiscus australes*, *G. pacificus* and *G. polynesiensis* (Dinophyceae) isolated from Rarotonga, Cook Islands. Harmful Algae 39:185-190.

Rhodes LL, Smith KF, Murray S, Harwood DT, Trnski T, Munday R (2017). The epiphytic genus *Gambierdiscus* (Dinophyceae) in the Kermadec Islands and Zealandia regions of the Southwestern Pacific and the associated risk of Ciguatera fish poisoning. Mar Drugs 15:219.

Rodríguez F, Fraga S, Ramilo I, Rial P, Figueroa RI, Riobó P, Bravo I (2017). Canary Islands (NE Atlantic) as a biodiversity 'hotspot'of *Gambierdiscus*: Implications for future trends of ciguatera in the area. Harmful Algae 67:131-143.

Rossignoli AE, Tudo A, Bravo I, Diaz PA, Diogène J, Riobo P (2020). Toxicity characterisation of *Gambierdiscus* species from the Canary Islands. Toxins (Basel) 12:134

Roué M, Darius HT, Picot S, Ung A, Viallon J, Gaertner-Mazouni N, Sibat M, Amzil Z, Chinain M (2016). Evidence of the bioaccumulation of ciguatoxins in giant clams (*Tridacna maxima*) exposed to *Gambierdiscus* spp. cells. Harmful Algae 57:78-87.

Roy S, Parenteau M, Casas-Monroy O, Rochon A (2012). Coastal ship traffic: a significant introduction vector for potentially harmful dinoflagellates in eastern Canada. Can J Fish Aquat Sci 69(4): 627-644.

Sambrook J, Fritsch EF, Maniatis T (1989). Molecular cloning: a laboratory manual. Cold spring harbor laboratory press.

Sánchez JLA, Henry OYF, Joda H, Solnestam BW, Kvastad L, Johansson E, Akan P, Lundeberg J, Lladach N, Ramakrishnan D, Riley I, O'Sullivan

CK (2016). Multiplex PCB-based electrochemical detection of cancer biomarkers using MLPA-barcode approach. Biosens Bioelectron 82: 224-232

Satake M, Murata M, Yasumoto T (1993). Gambierol: a new toxic polyether compound isolated from the marine dinoflagellate *Gambierdiscus toxicus*. J Am Chem Soc 115:361-362

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

Silva M, Rodriguez I, Barreiro A, Kaufmann M, Isabel Neto A, Hassouani M, Sabour B, Alfonso A, Botana LM, Vasconcelos V (2015). First report of ciguatoxins in two starfish species: *Ophidiaster ophidianus* and *Marthasterias glacialis*. Toxins 7: 3740-57.

Smith KF, Biessy L, Argyle PA, Trnski T, Halafihi T, Rhodes LL (2017). Molecular identification of *Gambierdiscus* and *Fukuyoa* (Dinophyceae) from environmental samples. Mar Drugs 15:243.

Toldrà A, Alcaraz C, Andree KB, Fernández-Tejedor M, Diogène J, Katakis I, O'Sullivan CK, Campàs M (2019a). Colorimetric DNA-based assay for the specific detection and quantification of *Ostreopsis* cf. *ovata* and *Ostreopsis* cf. *siamensis* in the marine environment. Harmful Algae 84:27-35.

Toldrà A, Alcaraz C, Diogène J, O'Sullivan CK, Campàs M (2019b). Detection of *Ostreopsis* cf. *ovata* in environmental samples using an electrochemical DNA-based biosensor. Sci Total Environ 689:655-661.

Toldrà A, Andree KB, Bertomeu E, Roque A, Carrasco N, Gairín I, Furones MD, Campàs M (2018a). Rapid capture and detection of ostreid herpesvirus-1 from Pacific oyster *Crassostrea gigas* and seawater using magnetic beads. PLoS One 13: e0205207.

Toldrà A, Andree KB, Fernández-Tejedor M, Diogène J, Campàs M (2018b). Dual quantitative PCR assay for identification and enumeration of *Karlodinium veneficum* and *Karlodinium armiger* combined with a simple and rapid DNA extraction method. J Appl Phycol 30: 2435-2445.

Tsumuraya T, Fujii I, Inoue M, Tatami A, Miyazaki K, Hirama M (2006). Production of monoclonal antibodies for sandwich immunoassay detection of ciguatoxin 51-hydroxyCTX3C. Toxicon 48: 287-94. DOI:

Tsumuraya T, Takeuchi K, Yamashita S, Fujii I, Hirama M (2012). Development of a monoclonal antibody against the left wing of ciguatoxin CTX1B: Thiol strategy and detection using a sandwich ELISA. Toxicon 60: 348-357.

Tsumuraya T and Hirama M (2019). Rationally designed synthetic haptens to generate anti-ciguatoxin monoclonal antibodies, and development of a practical sandwich ELISA to detect ciguatoxins. Toxins 11:533.

Tudó À, Toldrà A, Andree KB, Rey M, Fernández-Tejedor M, Campàs M, Diogène J (2018). First report of *Gambierdiscus* in the Western Mediterranean Sea (Balearic Islands). Harmful Algae News.

Tudó À, Toldrà A, Rey M, Todolí I, Andree KB, Fernández-Tejedor M, Campàs M, Sureda FX, Dogène J (2020a). *Gambierdiscus* and *Fukuyoa* as potential indicators of ciguatera risk in the Balearic Islands. Harmful Algae 99: 101913.

Tudó À, Gaiani G, Rey Varela M, Tsumuraya T, Andree KB, Fernández-Tejedor M, Campàs M, Diogène J (2020b). Further advance of *Gambierdiscus* species in the Canary Islands, with the first report of *Gambierdiscus belizeanus*. Toxins 12:692

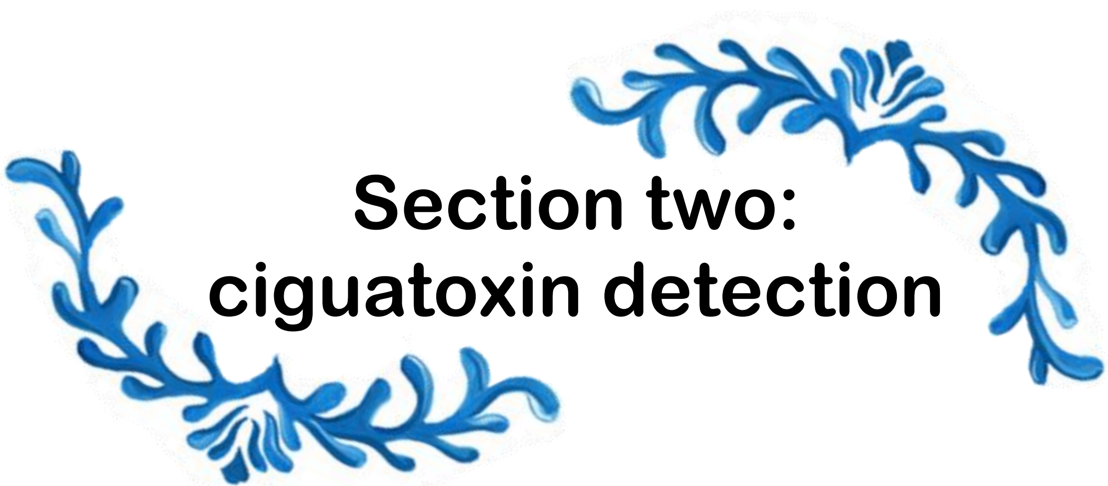
Utermöhl H (1958). Zur vervollkommnung der quantitativen phytoplankton-methodik: Mit 1 Tabelle und 15 abbildungen im Text und auf 1 Tafel. Internationale Vereinigung für theoretische und angewandte Limnologie: Mitteilungen 9: 1-38.

Vandersea MW, Kibler SR, Holland WC, Tester PA, Schultz TF, Faust MA, Holmes MJ, Chinain M, Litaker RW (2012). Development of semi-quantitative PCR assays for the detection and enumeration of *Gambierdiscus* species (gonyaulacales, Dynophyceae). J Phycol 48:902-915.

Watanabe R, Uchida H, Suzuki T, Matsushima R, Nagae M, Toyohara Y, Satake M, Oshima Y, Inoue A, Yasumoto T (2013). Gambieroxide, a novel epoxy polyether compound from the dinoflagellate Gambierdiscus toxicus GTP2 strain. Tetrahedron 69:10299-10303.

Withers NW (1983). Ciguatera research in the northwestern Hawaiian Islands: Laboratory and field studies on ciguatoxigenic dinoflagellates in the Hawaiian Archipelago. Second Symposium on Resource Investigations in the Northwestern Hawaiian Islands, Honolulu, HI, USA, pp 144-156.

Yasumoto T, Nakajima I, Oshima Y, Bagnis R (1979). A new toxic dinoflagellate found in association with ciguatera. In: Taylor L, Seliger HH (eds). Toxic Dinoflagellate Blooms. Elsevier New York, USA, pp 65-70.



Section two: ciguatoxin detection

Chapter 3

Advancing in the ciguatoxins detection challenge using a biosensor

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Abstract

The high relevance of ciguatoxins (CTXs) in seafood safety and their emerging occurrence far away from tropical areas highlight the need for simple and low-cost methods for the sensitive and rapid detection of these potent marine toxins in order to protect seafood consumers. Herein, an electrochemical immunosensor for the detection of CTXs is presented. A sandwich configuration is proposed using magnetic beads (MBs) as immobilisation supports for two capture antibodies that allow the detection of CTX1B, CTX3C, 54-deoxyCTX1B and 51-hydroxyCTX3C. PolyHRP-streptavidin is used for the detection of the biotinylated detector antibody and signal amplification. Experimental conditions are first optimised using colorimetry and subsequently used for electrochemical detection on electrode arrays. Limits of detection at the pg/mL level are achieved for CTX1B and 51-hydroxyCTX3C. The applicability of the immunosensor to the analysis of fish

samples is demonstrated, attaining detection of CTX1B at contents as low as 0.01 µg/kg and providing results that agree with those obtained using mouse bioassay (MBA) and cell-based assay (CBA). Liquid chromatography coupled to high-resolution mass spectrometry (LC-ESI-HRMS) is used to confirm the presence of CTXs in the fish. This user-friendly bioanalytical tool for the rapid detection of CTXs can mitigate ciguatera risk and contribute to the protection of consumer health.

1. Introduction

Ciguatera fish poisoning (CFP) is the most common and one of the most relevant seafood-borne diseases worldwide, affecting from 10,000 to 500,000 people per year, and probably even more due to underdiagnosis and underreporting (Friedman et al 2017) CFP is characterised by severe neurological, gastrointestinal and cardiovascular disorders that usually abate within a few days or weeks but can persist for months or years.(Lehane and Lewis 2000) CFP is caused by the ingestion of fish contaminated with ciguatoxins (CTXs), p otent lipophilic marine toxins with complex chemical structures(Murata et al. 1989; Satake et al. 1998; Yasumoto et al. 2000) produced by microalgae of the genus *Gambierdiscus* (Caillaud et al. 2011; Chinain et al. 2010; Litaker et al. 2017; Reverté et al. 2018) and *Fukuyoa* (Lewis et al. 2016; Litaker et al. 2017) that accumulate in fish through the food webs. There are several types of CTXs depending on their chemical structure. CTXs have been historically classified according to their geographical origin into Pacific (P), Caribbean (C) and Indic (I) CTXs. However, CTXs are emerging in places not previously expected according to their latitude, particularly in Europe. In recent years, several species of *Gambierdiscus* have been found in the Canary Islands (Bravo et al. 2019; Fraga and Rodríguez 2014; Fraga et al. 2011; Rodríguez et al. 2017) where several CFP outbreaks have also occurred (Boada et al. 2010; Bravo et al. 2015; Perez-Arellano et al. 2005). CTXs have also been detected in fish from other areas of the Macaronesia, *i.e.* Azores and Madeira archipelagos (Portugal) (Costa et al. 2018; Silva et al. 2015). *Gambierdiscus* sp. (Aligizaki and Nikolaidis 2008), *G. australes* (Tudó et al. 2018) and *F. paulensis* (Laza-Martínez et al. 2016) have been recorded in the Mediterranean Sea.

To protect consumer health, the United States Food and Drug Administration (US FDA) proposed guidance levels of ≤0.01 µg/kg of CTX1B equivalent toxicity in fish (US FDA, 2011). In Europe, although the legislation requires that no fish products containing CTXs are placed on the market (Regulation (EC) No. 853/2004), no regulatory limits have been established and no details about the analytical methodology to be used have been provided. Other parts of the world, such as Australia or New Zealand, provide guidelines on the susceptible fish species and the local areas where fish may be toxic (FSANZ) and, in Japan, the sale of barracuda and other fish species associated with CFP is banned, but no specific regulations for CTXs are provided.(MHWL 1953; 2001).

The mouse bioassay (MBA) has been the most widely used method to detect CTXs. Due to its insufficient detection capability and ethical concerns, other methods have been developed, including high-performance liquid chromatography coupled with mass spectrometry, cell-based assays (CBAs) and receptor binding assays (Reverté et al. 2014). The analysis of CTXs in fish is hampered by the fact that certified reference calibrants and materials are not readily available. This issue together with the chemical complexity of CTXs have hindered the production of specific antibodies. Hokama and co-workers produced anti-CTX polyclonal antibodies (pAbs) that were used for the development of some immunoassays (Hokama et al. 1977; 1983) and two immunostrip tests, marketed as Cigua-Check (Hokama et al. 1985, 1987) and Ciguatect kit (Park 1995). These antibodies showed high cross-reactivity with another marine toxin, okadaic acid, which raised high controversy regarding the performance of the tests, since false positive and false negative results were obtained (Dickey et al. 1994; Bienfang et al. 2011; Ebesu & Campora 2012). In fact, fish determined as positive in Israel,(Bentur and Spanier 2007) the first and only report on ciguateric fish in the Mediterranean, had been analysed using only the Cigua-Check kit. Therefore, confirmation of CTXs in fish from this area is still pending. On the other hand, synthetic haptens as an alternative to natural CTXs were exploited for the production of monoclonal antibodies (mAbs) that were subsequently used in immunoassays, and observed to have high enough specificity and sensitivity.(Nagumo et al. 2001, 2004; Oguri et al. 1999, 2003; Tsumuraya et al. 2006, 2010, 2012, 2014, 2018) Taking into account these successful results and with the aim to move towards compact and automated devices, the development of an electrochemical immunosensor for the detection of CTXs is undertaken for the first time.

In this work, three different mAbs (3G8, 10C9 and 8H4) that specifically bind to one of the wings of the four principal congeners of CTXs (CTX1B, CTX3C, 51-hydroxyCTX3C and 54-deoxyCTX1B) have been used to develop a sandwich immunosensor. Magnetic beads (MBs) are exploited as a support to provide an enlarged surface area for the immobilisation of mAbs, to shorten the analysis time and to minimise matrix effects. The applicability of the immunosensor to the analysis of fish is successfully demonstrated, being able to discriminate between contaminated and non-contaminated samples and allowing the detection of CTX1B contents at 0.01 μg/kg. Liquid chromatography coupled to electrospray ionisation high-resolution mass spectrometry (LC-ESI-HRMS) analysis has confirmed the presence of CTX1B in fish. The availability of this user-friendly bioanalytical tool for the rapid detection of CTXs can mitigate the ciguatera risk and contribute to protect consumer health.

2. Experimental section

2.1 Reagents and solutions

Dynabeads M-270 Carboxylic Acid (2 x 10^9 beads/mL) were supplied by Invitrogen (Life Technologies, S.A., Alcobendas, Spain). Potassium phosphate monobasic, potassium phosphate dibasic, potassium chloride, 4-morpholineethanesulfonic acid (MES) hydrate, N-(3-dimethylaminopropyl)-N′-ethylcarbodiimide hydrochloride (EDC), N-hydroxysuccinimide (NHS), Tween®-20, bovine serum album (BSA), anti-mouse IgG (whole molecule)-horseradish peroxidase antibody produced in rabbit (IgG-HRP), and 3,3',5,5'-tetramethylbenzidine (TMB) liquid substrate were purchased from Sigma-Aldrich (Tres Cantos, Spain). PolyHRP-streptavidin was obtained from Thermo Fisher (Barcelona, Spain). Milli-Q water (Millipore, Bedford, USA) was used to prepare all solutions. For the extractions, acetone and diethyl ether were obtained from Chem-lab (Zedelgem, Belgium), ethanol from J. T. Baker (Madrid, Spain), and methanol and *n*-hexane from Honeywell (Barcelona, Spain). For LC-ESI-HRMS, HPLC-MS grade acetonitrile and water were supplied by Chem-lab (Zedelgem, Belgium), and ammonium formate and formic acid by Sigma-Aldrich (Tres Cantos, Spain). CTX1B standard solution was obtained from Prof. Richard J. Lewis (The Queensland University, Australia) and calibrated (correction factor of 90%) in relation to the NMR-quantified CTX1B standard solution from Prof. Takeshi Yasumoto (Japan Food Research Laboratories, Japan). NMR-quantified 51-hydroxyCTX3C standard solution was kindly provided by Prof. Takeshi Yasumoto. 3G8, 10C9 and 8H4 mAbs had been prepared by immunising mice with keyhole limpet hemocyanine (KLH) conjugates of rationally designed synthetic haptens (Oguri et al., 1999, 2003; Nagumo et al., 2001, 2004; Tsumuraya et al., 2006, 2010, 2012, 2014). Biotin labelling of the 8H4 mAb was performed with the EZ-Link™ NHS-PEG4 Biotinylation Kit from Thermo Fisher (Barcelona, Spain) following the manufacturer's instructions. Unreacted NHS-PEG4-Biotin was removed using Zeba Spin Desalting Columns (7 kDa MWCO, 2mL) included in the kit.

2.2 Equipment

Magnetic separation was performed using a MagneSphere Technology Magnetic Separation Stand (for twelve 0.5-mL or 1.5-mL tubes) and a PolyATtract System 1000 Magnetic Separation Stand (for one 15-mL tube) from Promega Corporation (Madison, WI, USA). Colorimetric measurements were performed with a Microplate Reader KC4 from BIO-TEK Instruments, Inc. (Vermont, USA). Gen5 software was used to collect and evaluate data. Screen printed electrode arrays (DRP-8x110), a boxed connector (DRP-CAST8X) and a magnetic support (DRP-MAGNET8X) were purchased from Dropsens S.L. (Oviedo, Spain). The arrays consist of 8 carbon working electrodes of 2.5 mm in diameter, each with its carbon counter and silver reference electrodes. Amperometric measurements were performed with a

PalmSens potentiostat connected to an 8-channel multiplexer (MUX8) (Houte, The Netherlands). PalmSens PC software was used to collect and evaluate data.

2.3 Fish samples and extraction

Sampling was performed at various locations of the Indian Ocean close to Réunion and Maurice Islands. *Variola louti* (N=9), *Lutjanus bohar* (N=6) and *Thyrsitoides marleyi* (N=1) specimens were collected. Fishing dates and sites are summarised in Table 1. Fish samples were processed as previously described (Soliño et al. 2015). Briefly, 10 g of fish flesh homogenate were heated at 70 °C for 15 min in a water bath. Subsequently, 20 mL of acetone was added and the sample mixture was homogenised with an Ultra-turrax blender at 17500 *xg* for 5 min. The sample mixture was centrifuged at 3000 *xg* for 10 min to obtain the supernatant. The pellet was re-extracted with acetone, and supernatants were pooled, filtered with 0.45 μm nylon filters and evaporated at 55 °C. The dried extract was dissolved in 5 mL of methanol:water (9:1, *v:v*) and partitioned twice with 5 mL of *n*-hexane, and the *n*-hexane phases were discarded. The aqueous methanol solution was dried by rotary evaporation. The residue was dissolved in 5 mL of ethanol:water (1:3, *v:v*) and partitioned twice with 5 mL of diethyl ether. Diethyl ether fractions were pooled and dried. The residue was re-suspended in 4 mL of methanol and kept at -20 °C until analysis. For calculation purposes, 1 mL of extract contains 2.5 g equivalents of fish flesh.

2.4 Conjugation of the capture mAbs to MBs

3G8 and 10C9 mAb-MB conjugates were prepared as follows: (1) 8 μL of MB suspension were transferred to a tube and washed twice with 500 μL of MES (25 mM MES, pH 5.0) with vigorous mixing; for the washing steps, the tube was placed on the magnetic separation stand and the washing solution was removed; (2) 40 μL of 50 mg/mL EDC and 40 μL of 50 mg/mL NHS were added and incubated for 30 min; (3) the activated MBs were washed twice with MES; (4) 80 μL of 3G8 or 10C9 mAb in MES (from 1/50 to 1/3200 dilution for protocol optimisation and 1/50 dilution for the final assay) were added and incubated for 1 hour; (5) the mAb-MB conjugate was washed three times with PBS-Tween (0.1 M PBS, 0.05% *v/v* Tween®-20, pH 7.2) and re-suspended in 80 μL of the same buffer. All incubations were performed at room temperature and with slow tilt rotation. When amounts of MB varied, volumes were adjusted proportionally. To confirm the conjugation of the mAbs to the MBs and optimise the dilution to be used, 25 μL of mAb-MB conjugate were transferred to a new tube and incubated with 250 μL of anti-mouse IgG-HRP in PBS-Tween containing 1% *w/v* BSA (PBS-Tween-BSA) (1/1000 dilution) for 30 min; after two washing steps with PBS-Tween, 20 μL of immunoconjugate were transferred to a new tube, the supernatant was removed and 125 μL of TMB were incubated for 5 min; the tube was placed on the magnetic separation stand and 100 μL were taken for absorbance reading at 620 nm.

2.5 Colorimetric immunoassay and electrochemical immunosensor

Sandwich immunoassays were performed in 0.5-mL tubes following this protocol: (1) 150 µL of mAb-MB conjugates (75 µL of each mAb-MB conjugate) were exposed to 75 µL of CTX1B or 51-hydroxyCTX3C standard solution (congeners chosen as model CTXs) or fish extract (previously evaporated and re-suspended in PBS-Tween) for 30 min; (2) after three washing steps with PBS-Tween, a blocking step was performed with PBS-Tween-BSA (PBS-Tween containing 2% *w/v* BSA) for 30 min; (3) the conjugates were washed three times with PBS-Tween and incubated with 75 µL of biotin-8H4 mAb in PBS-Tween-BSA (from 1/50 to 1/4000 dilution for protocol optimisation and 1/2000 dilution for the final assay) for 30 min; (4) three washing steps were performed with PBS-Tween and 75 µL of polyHRP-streptavidin in PBS-Tween-BSA (from 1/500 to 1/5000 dilution for protocol optimisation and 1/1000 dilution for the final assay) were added and incubated for 30 min; (6) finally, three washing steps were performed in PBS-Tween and the content of each tube was re-suspended in 75 µL of the same buffer. All incubations were performed at room temperature and with slow tilt rotation. When amounts of MB varied, volumes were adjusted proportionally. Subsequent steps differed slightly between the immunoassay and the immunosensor and are described below. For the colorimetric immunoassay: (7) 10 µL of immunocomplexes were transferred to a new tube and the supernatant was removed; (8) 125 µL of TMB were incubated for 10 min; (9) tubes were placed on the magnetic separation stand and 100 µL of solution were taken for absorbance reading at 620 nm. Measurements were performed in duplicate or triplicate. For the electrochemical immunosensor: (7) 10 µL of immunocomplexes were placed on each working electrode of the 8-electrode array with a magnetic support on the back, the magnetic immunocomplex was captured and the supernatant was removed; (8) 10 µL of TMB were incubated for 2 min; (9) the TMB reduction current was measured using amperometry, applying −0.2 V (*vs.* Ag) for 5 s. Measurements were performed in triplicate or quadruplicate.

2.6 Mouse bioassay

The protocol was based on a standard method developed by ANSES. Fish extracts were solubilised in Tween-60 1-5% *v/v* saline solution, and then injected into three mice (male, OF1; 20 ± 2 g) by intraperitoneal (*i.p.*) route. The mice were observed continuously during the first 2 h, and then monitored regularly up to 24 h after injection. The interpretation of the results was based on the time-to-death and symptoms (profuse diarrhea, piloerection, respiratory disorders, dyspnoea and transient pre-erectional cyanosis of the penis, which can become priapism).

2.7 Cell-based assay

The CBA was performed as previously described (Diogène et al. 2017; Soliño et al. 2015). Briefly, neuro-2a (N2a) cells (ATCC, CCL131) were seeded in a

96-well microplate in 200 mL of RPMI medium containing 5% *v/v* fetal bovine serum (RPMI-FBS) at 42,500 cells per well, and incubated at 37 °C in a 5% CO_2 humid atmosphere for 24 h. Prior to exposure to CTX1B standard solution or fish extract, some N2a cells were pre-treated with ouabain and veratridine at 1 and 0.1 mM, respectively. CTX1B standard solution or fish extract were dried, reconstituted in 200 mL of RPMI-FBS medium, serially diluted, and 10 µL were added to the wells with and without ouabain/veratridine pre-treatment. After 24 h, cell viability was measured using the MTT assay (Manger et al., 1993). Measurements were performed in triplicate.

2.8 LC-ESI-HRMS analysis

One *V. louti* individual caught in March 2015, one *L. bohar* individual caught in February 2003 and the *T. marleyi* individual were analysed by LC-ESI-HRMS. An Orbitrap-Exactive HCD (Thermo Fisher Scientific, Bremen, Germany) mass spectrometer equipped with heated electrospray source (H-ESI II), a Surveyor MS Plus pump and an Accela Open AS auto-sampler kept at 15 °C (Thermo Fisher Scientific, San José, California) were used for the LC-ESI-HRMS analysis. The chromatographic separation was performed on a Kinetex XB-C18 reversed phase (100 mm × 2.1 mm, 2.6µm) (Phenomenex, Torrance,CA, USA) at a flow rate of 250 µL/min. Mobile phase A was water and B was acetonitrile/water (95:5), both containing 2 mM ammonium formate and 0.1% *v/v* formic acid. The gradient elution program 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 for re-equilibration (11 min 30% B). A 5-µL injection volume was used. The total duration of the method was 25 min. The analysis was carried out in electrospray positive ionisation (H-ESI+). CTX1B was used to optimize the source, transmission and HRMS conditions in positive mode. The final parameters were: spray voltage of 4.0 kV, capillary temperature of 275 °C, heater temperature of 300 °C, sheath gas flow rate of 35 psi and auxiliary gas flow rate of 10 (arbitrary units). Capillary voltage of 47.5 V, tube lens voltage of 186 V and skimmer voltage of 18 V were used. Nitrogen was employed as sheath, auxiliary and collision gas. The mass range was *m/z* 400-1500 in full scan acquisition mode. The resolution was 50,000 (*m/z* 200, FWHM) at a scan rate 2Hz, the automatic gain control was set as "balanced" (1e6) with a maximum injection time of 250 ms. Data were processed with Xcalibur 2.2 SP1 software (Thermo Fisher Scientific, Bremen, Germany). Automatic identification/quantification were performed. The peaks found were confirmed by the exact mass of $[M+H]^+$, $[M+NH_4]^+$ and $[M+Na]^+$ diagnostic ions with a mass accuracy of ± 3ppm (mass extraction window) and the retention time window. The following restrictive criteria were also used: 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 and the charge, the ring double bond equivalents and nitrogen rule were taken into account. Additionally, the monoisotopic pattern (M+1 ion) of these signals was used to assist in the further confirmation of the toxin identity. The relative ion intensities between

$[M+NH_4]^+$, $[M+Na]^+$ and their M+1 ions were calculated and matched taking into account a tolerance according to the EU Decision 2002/657/EC. An external standard calibration was carried out from 1 to 100 ng/mL of CTX1B with a limit of detection (LOD) of 0.3 ng/mL. The sum of the areas of $[M+H]^+$+$[M+NH_4]^+$+$[M+Na]^+$ signals was used for quantification purposes.

3. Results and discussion

The concept of the immunosensor is shown in Figure 1. On one side, two different mouse mAbs, 3G8 mAb able to bind to the left wing of CTX1B and 54-deoxyCTX1B (Tsumuraya et al. 2012) and 10C9 mAb able to bind to the left wing of CTX3C and 51-hydroxyCTX3C (Oguri et al. 2003), were immobilised separately on MBs and used as capture antibodies. On the other side, 8H4 mouse mAb, which binds to the right wing of CTX1B, CTX3C, 54-deoxyCTX1B and 51-hydroxyCTX3C (Tsumuraya et al. 2006), was biotinylated and used as a detector antibody. Following successive incubations of the magnetic immunocomplexes with the analyte and the biotinylated 8H4 mAb, polyHRP-streptavidin was used for signal reporting. Signal amplification was achieved by replacing the conventional HRP-streptavidin by polyHRP-streptavidin, a conjugate that contains a polymer with approximately 20 HRP molecules per streptavidin molecule. The experimental conditions to be used in the immunosensor were first optimised using colorimetric detection, and the immunoconjugates were transferred to electrode arrays to perform sequential electrochemical measurements.

3.1 Optimisation of the experimental conditions

Capture mAbs were conjugated to carboxylic acid-modified MBs through carbodiimide coupling using EDC-NHS. First, several 3G8 mAb dilutions were used to optimise the amount of antibody. Anti-mouse IgG-HRP was used to detect the immobilised antibody. As expected, absorbance values increased with increasing amounts of antibody (Figure S1). No saturation of the MBs was observed even with the highest antibody concentration tested (1/50 mAb dilution). This dilution was selected for further experiments as well as for 10C9 mAb.

The amount of biotinylated 8H4 mAb was optimised using 3G8 mAb at 1/50 dilution, CTX1B at 1000 and 0 pg/mL and polyHRP-streptavidin at 1/1000 dilution. The best signal-to-noise absorbance ratio was achieved with 1/2000 biotinylated 8H4 mAb dilution (Figure S2), which was selected for further experiments. These results demonstrated the correct performance of the system as well as the successful biotinylation of the 8H4 mAb, which maintains its affinity for the right wing of CTX1B once biotinylated. PolyHRP-streptavidin was selected to amplify the signals and its concentration was optimised to achieve the best signal-noise ratio. The 3G8 mAb-MB conjugates were exposed to CTX1B at 100 and 0 pg/mL and biotinylated 8H4 mAb at 1/2000 dilution, and subsequently incubated with a range of polyHRP-streptavidin dilutions

(Figure S3). Very low non-specific values were observed. In the presence of CTX1B, absorbance values achieved from 1/500 to 1/2000 dilutions did not show significant differences and the signal observed at a 1/5000 dilution was only slightly lower. Although saturation of the response was observed even with 1/2000 polyHRP-streptavidin dilution, a 1/1000 dilution was selected for further experiments to ensure polyHRP-streptavidin availability

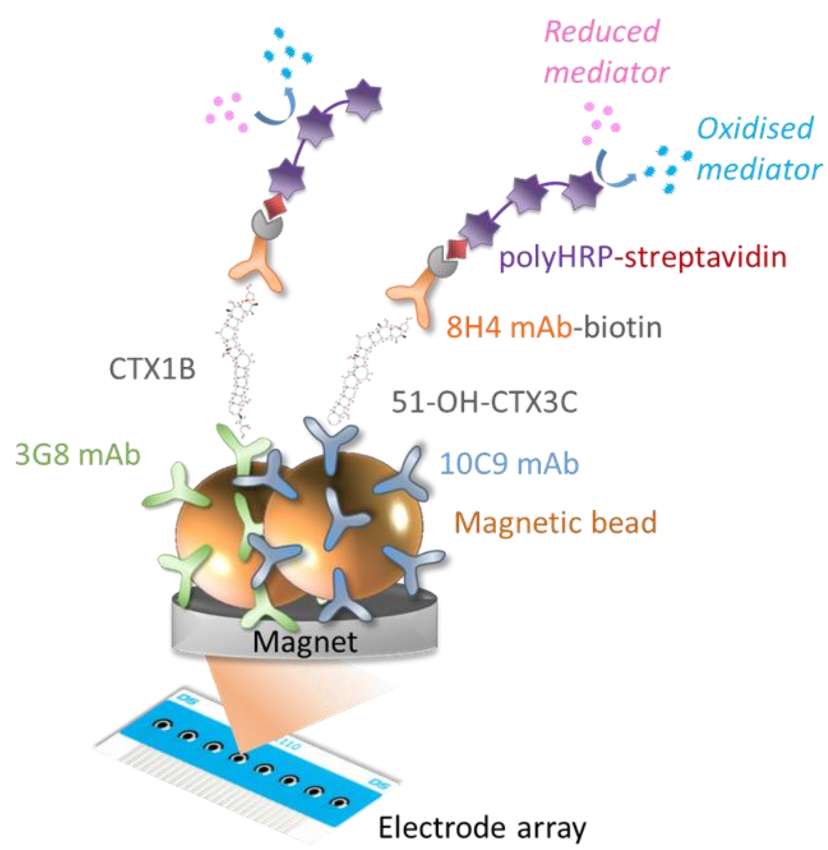

Figure 3. Representation of the electrochemical immunosensor for the detection of CTXs.

Table 1. Fish data and CTX1B equivalent contents (pg/g) (± standard deviation) obtained in their analysis using MBA, CBA, colorimetric immunoassay and electrochemical biosensor.

Species	Fishing date	Fishing site	MBA	CBA	Immunoassay	Immunosensor
Variola louti	January 2013	Saint-Gilles, Réunion	nd	nd	nd	nd
Variola louti	March 2013	Saint-Gilles, Réunion	nd	nd	nd	nd
Variola louti	March 2013	Saint-Gilles, Réunion	nd	nd	nd	nd
Variola louti	March 2013	Saint-Gilles, Réunion	nd	nd	nd	nd
Variola louti	April 2013	Saint-Gilles, Réunion	nd	nd	nd	nd
Variola louti	July 2003	La Pérouse Seamount, Réunion	++	9.74 ± 0.47	33.44 ± 2.04	26.14 ± 1.56
Variola louti	April 2004	La Pérouse Seamount, Réunion	++	81.66 ± 9.77	45.81 ± 13.99	44.40 ± 20.37
Variola louti	January 2003	La Pérouse Seamount, Réunion	++	580.06 ± 86.36	107.31 ± 5.18	97.41 ± 34.36
Variola louti*†	March 2015	Maurice	+++	2104.00 ± 224.43	279.77 ± 3.69	247.85 ± 35.56
Lutjanus bohar	September 2002	La Pérouse Seamount, Réunion	++	21.75 ± 0.30	9.19 ± 0.51	7.36 ± 0.64
Lutjanus bohar	August 2003	La Pérouse Seamount, Réunion	++	440.68 ± 20.94	9.02 ± 1.01	7.72 ± 0.90
Lutjanus bohar	August 2003	La Pérouse Seamount, Réunion	++	552.70 ± 83.91	22.76 ± 0.54	18.92 ± 5.41
Lutjanus bohar	December 2003	La Pérouse Seamount, Réunion	++	506.47 ± 86.36	27.12 ± 0.92	27.73 ± 8.79

Lutjanus bohar*	February 2003	La Pérouse Seamount, Réunion	++	1296.73 ± 181.60	149.46 ± 8.21	134.66 ± 32.8
Lutjanus bohar	January 2002	La Pérouse Seamount, Réunion	+++	2481.03 ± 727.47	142.46 ± 2.51	147.04 ± 28.44
Thyrsitoides marleyi*†	June 2015	Saint-Paul, Réunion	+++	600.48 ± 68.86	88.21 ± 9.50	78.12 ± 34.11

(Continues from previous page)
† Fish individuals involved in a poisoning case
* Fish individuals analysed by LC-ESI-HRMS
nd: not detected; ++: intermediate toxicity; +++: strong toxicity

3.2 Colorimetric characterisation

Using the optimised conditions, the calibration curve for CTX1B was constructed with 10 µL of 3G8 mAb-MB conjugate (equivalent to 1 µL of MBs). As expected, a dose-dependent response was observed (Figure 2A). The calibration curve was blank-subtracted and fitted to a sigmoidal logistic four-parameter equation. The LOD and limit of quantification (LOQ) were calculated using 3 and 10 times the standard deviation of the blank value (no CTX), and were 3.29 and 17.52 pg/mL, respectively. The calibration curve for 51-hydroxyCTX3C was then constructed using 10 µL of 10C9 mAb-MB conjugate. Again, a dose-dependent response was observed, with a slight saturation at high toxin concentrations (Figure 2B). LOD and LOQ values of 6.17 and 28.31 pg/mL were obtained, respectively. These values are lower than those attained with the colorimetric ELISA (LOD of 280 pg/mL for CTX1B)(Tsumuraya et al. 2012) but higher than those achieved with the fluorescence ELISA (LODs of 0.16 and 0.10 pg/mL for CTX1B and 51-hydroxyCTX3C, respectively) (Tsumuraya et al. 2018).

Since the purpose of the immunosensor is to detect as many CTX analogues as possible, both capture antibodies should be able to work together with no interferences from each other. To evaluate this issue, 10 µL of 3G8 mAb-MB conjugate and 10 µL of 10C9 mAb-MB conjugate were mixed together and calibration curves for CTX1B and 51-hydroxyCTX3C were constructed. The presence of twice the amount of MBs did not interfere in the recognition event and the measurement, as no significant differences were observed as compared to the calibration curves obtained using separated conjugates. Thus, in principle the immunosensor should be able to detect CTX1B and 51-hydroxyCTX3C simultaneously, as well as other analogues recognised by the mAbs, providing a global response. It is important to add that no cross-reactivity of 3G8 mAb-MB conjugates and 10C9 mAb-MB conjugates to 51-hydroxyCTX3C and CTX1B (at 500 pg/mL), respectively, was observed.

3.3 Electrochemical immunosensor

To develop the electrochemical biosensor, the magnetic immunocomplexes were transferred to 8-electrode arrays. Electrochemical calibration curves for CTX1B and 51-hydroxyCTX3C were first constructed using 10 µL of 3G8 mAb-MB or 10 µL of 10C9 mAb-MB conjugates, respectively (Figure 2C and 2D). No saturation of the amperometric response was observed at the highest CTX concentration tested, the dynamic ranges being well over two orders of magnitude for both CTXs. LOD and LOQ values of 1.96 and 2.94 pg/mL, respectively, were obtained for CTX1B, and 3.59 and 13.91 pg/mL for 51-hydroxyCTX3C. These values are lower than those obtained with the colorimetric approach, but still higher than the ones obtained with the fluorescence ELISA. Nevertheless, the electrochemical biosensor provides added advantages in terms of cost, possibility to be integrated into compact analysis devices and portability.

Subsequently, 10 µL of 3G8 mAb-MB conjugate and 10 µL of 10C9 mAb-MB were mixed together and immobilised on electrode arrays. Calibration curves for CTX1B and 51-hydroxyCTX3C were constructed, and results did not differ from those achieved with separated conjugates. The fact that the electrode surface was modified with twice the amount of magnetic immunocomplexes did not hamper the electrochemical measurement. Like in the colorimetric approach, CTX1B, 51-hydroxyCTX3C and the other CTXs analogues recognised by the mAbs should be detected together without a loss of sensitivity. Repeatability and reproducibility of the immunosensor for 100 pg/mL CTX1B were evaluated performing multiple measurements on the same (intra-day precision) and different days (inter-day precision), respectively. The relative standard deviation (RSD) value for the measurements performed on the same day with the same mAb-MB conjugate pool was 12% (N=3). The RSD value for the measurements performed on different days with different mAb-MB conjugate pools was 14% (N=6). These values show an appropriate reliability for the whole procedure including both immunosensor preparation and amperometric transduction

3.4 Fish matrix effects and recovery

Sample matrices contain compounds that may interfere in the assay. First, the effect of the fish matrix on the responses of the immunoassay and the immunosensor (using both mAb-MB conjugates together) was evaluated using a *V. louti* individual that had previously been determined as negative for CTXs by CBA. Absorbance and current intensity values at different extract dilutions were not significantly different from those obtained from the corresponding blanks (no fish extract), even at 2500 mg/mL. This experiment indicates that there is not non-specific adsorption of the capture antibody and/or the polyHRP-streptavidin on the system

In order to evaluate if the presence of fish matrix may interfere in the response of the immunoassay and the immunosensor towards CTXs, 100 pg/mL of CTX1B were spiked to several dilutions of the same *V. louti* extract. Absorbance and current intensity values were compared with those attained with the same amount of CTX1B in buffer. Recovery percentages are shown in Table 2. Although the highest matrix concentration tested had not shown any effect on the previous experiment, the CTX detection was affected. As the matrix was diluted, recovery percentages increased reaching nearly 90%. Despite the fact that matrix affects CTX quantification, these preliminary recovery values can be used as correction factors to be applied to the CTX quantifications provided by the immunoassay and the immunosensor in the analysis of naturally-contaminated fish in a first approach.

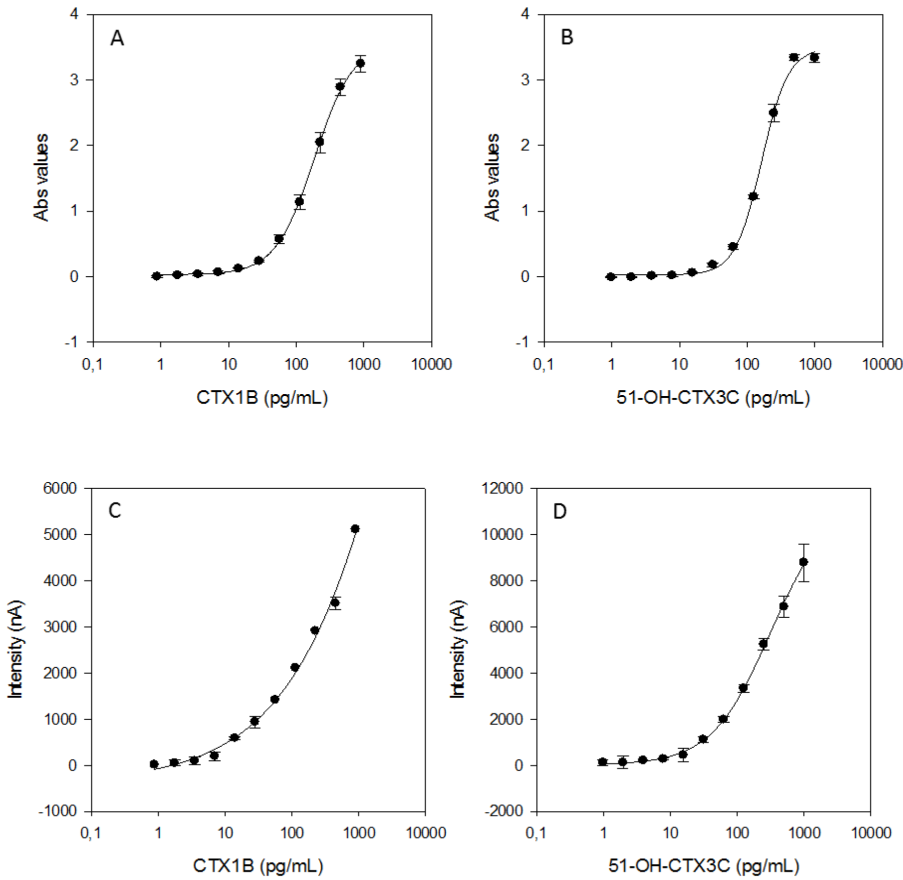

Figure 4. Calibration curves for CTX1B (A and C) and 51-hydroxyCTX3C (B and D) obtained using the colorimetric immunoassay (A and B) and the electrochemical immunosensor (C and D) (N=3). Curves are background-subtracted (Abs value = 0.089 ± 0.007; Intensity = 417 ± 121 nA).

To evaluate matrix effects between fish individuals, different CTX1B concentrations (from 3 to 100 pg/mL) were spiked to two non-contaminated *V. louti* extracts at 2500 mg/mL. According to the ANCOVA test, no significant differences were observed between individuals (P=0.65 for the immunoassay and P=0.38 for the immunosensor). Nonetheless, a more exhaustive analysis (including different fish species, of different size and from different geographical locations) would be required to refine the correction factors.

Table 2. CTX1B recovery values obtained in the analysis of a non-contaminated *V. louti* individual at different matrix concentrations using the colorimetric immunoassay and the electrochemical immunosensor. Values are expressed in percentages (%) and calculated with reference to the CTX1B spiking level of 100 pg/mL.

	2500 mg/mL	**1000 mg/mL**	**500 mg/mL**	**250 mg/mL**	**100 mg/mL**
Colorimetry	65 ± 3	77 ± 3	86 ± 6	88 ± 4	89 ± 3
Electrochemistry	58 ± 17	72 ± 2	76 ± 14	83 ± 17	89 ± 9

3.5 Analysis of fish samples spiked at 0.01 µg/kg CTX1B

Although regulatory limits for CTXs in fish have not yet been issued by official organisations, an important feature of the immunosensor presented herein should be the ability to detect at least CTX1B at 0.01 µg/kg. Effective LOQs were calculated from the calibration curves constructed from the CTX1B spiked *V. louti* extracts at 2500 mg/mL. The eLOQs achieved were 0.01 µg/kg and 0.002 µg/kg for the assay and biosensor, respectively, which are in agreement with the LOQs calculated from the calibration curve in buffer after applying the corresponding correction factors. The precision at the LOQ value was lower than 15% for both immunosensing tools. Thus, in principle, the developed tools should be able to detect CTX1B at 0.01 µg/kg.

To demonstrate this experimentally, the non-contaminated *V. louti* extract was spiked with 25 pg/mL of CTX1B and analysed at 2500 mg/mL (which corresponds to 0.01 µg/kg of CTX1B in the fish flesh). Once the absorbance and current intensity values were obtained, the previous recovery values achieved in the analysis of non-contaminated *V. louti* extract at 2500 mg/mL were used as correction factors and applied to the quantifications of the CTXs. Compared to the spiked level, the recovery values were 99% and 103% for the colorimetric immunoassay and the electrochemical biosensor, respectively.

It is important to take in mind that FDA guidance level is provided in CTX1B equivalent toxicity in fish. This equivalent toxicity is the composite toxicity in relation to CTX1B of the contaminated fish, in which several CTX congeners could be present. Thus, values achieved by the immunoapproaches can be straightforward compared with US FDA guidance levels when only CTX1B is present in the sample. The immunosensing tools are able to detect CTX3C, 51-hydroxyCTX3C and 54-deoxyCTX1B in addition to CTX1B, but in an extent not necessarily related to their toxicity. Otherwise, other non-structurally-related analogues are not detected by the immunoapproaches. Nevertheless, the detection of CTX1B at 0.01 µg/kg level highlights the good enough sensitivity of these bioanalytical tools.

3.6 Analysis of naturally-contaminated fish samples

Results for the MBA and CTXs contents determined by the immunoassay, the immunosensor and CBA are summarised in Table 1. (Table S1 shows crude results achieved by the immunosensing tools before applying the correction factors). Negative and positive individuals as determined by MBA and CBA were also negative and positive by the immunoassay and the immunosensor. An excellent correlation was obtained when comparing the CTX1B equivalent contents obtained using the immunosensor and the immunoassay ($r = 0.997$; $P < 0.001$) (Figure 3A). A good correlation was also observed between the immunosensor and the CBA ($r = 0.891$; $P < 0.001$) (Figure 3B). The usually higher CTXs contents obtained with the CBA as compared to the immunoassay and the immunosensor can be explained by the different recognition principles. Whereas the immunochemical tools detect analogues that possess specific wings in their structures (structural immunorecognition), CBA detects analogues that activate voltage-gated sodium channels (toxicological recognition). Thus, CBA could be detecting a higher number of CTXs and thus providing higher toxin contents. Since the cross-reactivity factors (CRFs) are not necessarily the same as the toxic equivalency factors (TEFs) and additionally many of them are unknown, quantifications cannot be always straightforwardly compared. Additionally, as the CBA is a toxicological assay, interferences from other compounds different from CTXs on the response cannot be discarded.

To confirm the presence of CTXs, LC-ESI-HRMS analysis of three naturally-contaminated individuals was performed. The analysis revealed the presence of CTX1B in the *V. louti* individual at 1609 pg/g (Figure 4, Figure S4). The presence of other CTX congeners was not confirmed. LC-ESI-HRMS provided around 6-fold higher CTXs content than that obtained using immunochemical tools. However, no CTXs were detected in the *L. bohar* and *T. marleyi* individuals analysed using LC-ESI-HRMS, despite the high toxicities or CTXs contents observed by MBA, CBA and the immunosensing tools. It is important to note that the LOQ of LC-ESI-HRMS is much higher than the LOQs attained with the immunochemical tools (*i.e.* ~400 pg/g *vs.* ~2-10 pg/g). Additionally, whereas LC-ESI-HRMS quantifies individual CTX analogues, the immunochemical tools provide a global response, being more useful for the detection of CTXs contents in multi-toxin samples where each analogue is at a low concentration.

A higher number of samples should be analysed to provide statistically sound comparisons with LC-ESI-HRMS analysis. Samples with higher CTXs contents and/or pre-concentration and clean-up steps for samples to be analysed by LC-ESI-HRMS would be necessary, this work being beyond the scope of this work. Nevertheless, the comparison of the results obtained with the immunological tools and CBA shows a good correlation. In the end, each methodology has advantages and limitations, and their application provides complementary information.

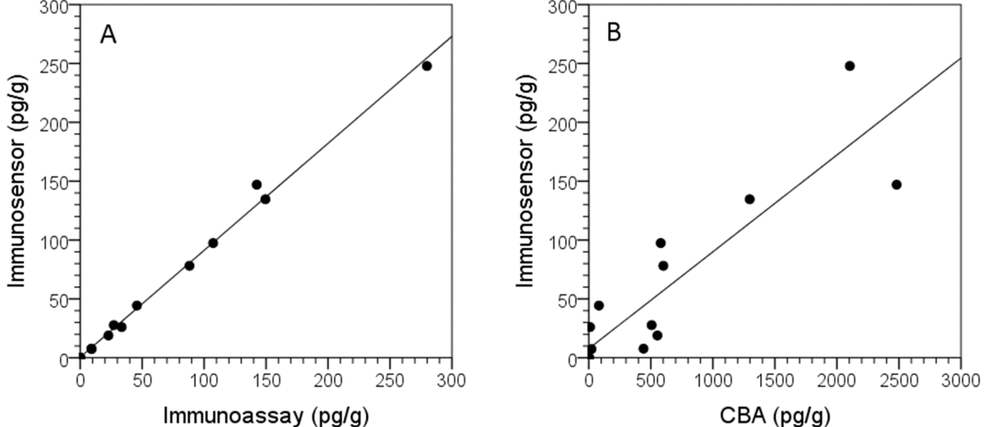

Figure 3. Correlations between CTX1B equivalent contents in fish provided by the electrochemical immunosensor and the colorimetric immunoassay (A) and the electrochemical immunosensor and CBA (B).

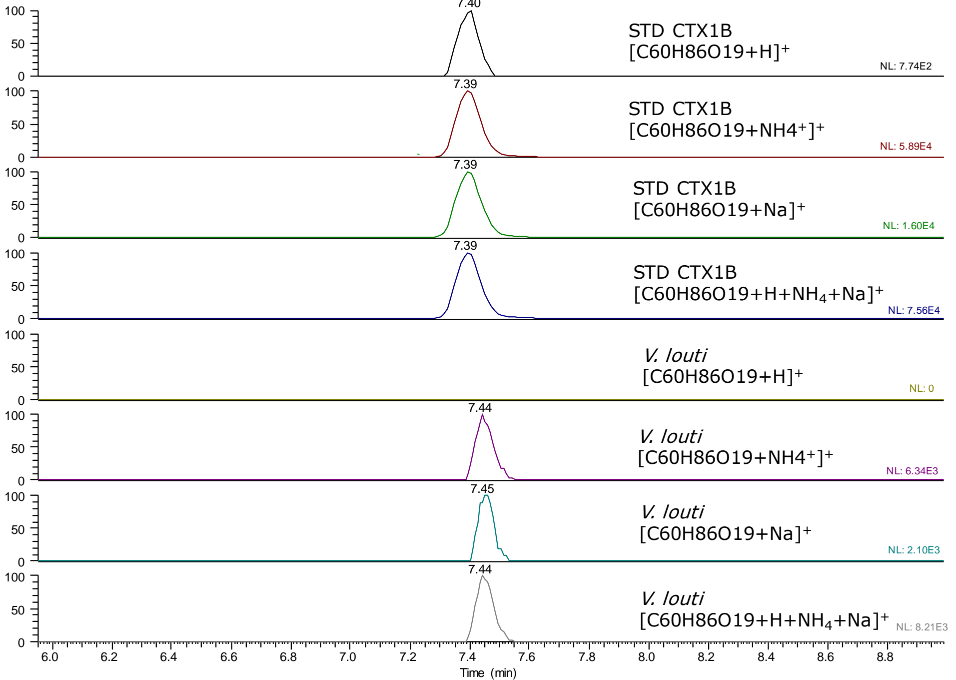

Figure 4. Extract ion chromatogram of CTX1B at *m/z* 1111.5836 $[M+H]^+$, 1128.6102 $[M+NH_4]^+$, 1133.5656 $[M+Na]^+$ and $[M+H+NH_4+Na]^+$ of CTX1B standard *and V. louti* extract.

4. Conclusion

An electrochemical immunosensor for the determination of CTXs in fish has been developed. Taking advantage of the sandwich configuration using magnetic beads as immobilisation supports and polyHRP-streptavidin for

signal amplification, together with the benefits provided by the electrochemical detection, the biosensor showed good analytical performance in terms of sensitivity and reproducibility and allowed the detection of CTX1B and 51-hydroxyCTX3C. The immunosensor was successfully applied to the analysis of fish samples, enabling the detection of CTX1B at 0.01 µg/kg and showing a good correlation with CTX levels determined by the CBA. Compared to CBA, the electrochemical immunosensor can tolerate higher matrix concentrations. Whilst the LOD for CTX1B achieved by the immunosensor is slightly higher than using CBA, the effective LOQ is similar. The magneto-immunosensor provides robustness, specificity, simplicity and rapidity in contrast with CBA, which requires working with "live" materials that need maintenance. On the other hand, the immunosensor attains an LOQ over two orders of magnitude lower than LC-ESI-HRMS, is much cheaper and does not require sophisticated instrumentation. Due to its lower cost, ease of operation, lack of need for maintenance and portability, there is no doubt that the electrochemical biosensor can be easily implemented in monitoring and research programs. Certainly, this work represents a successful and useful step forward the challenging CTXs detection.

Acknowledgments

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Supplementary material

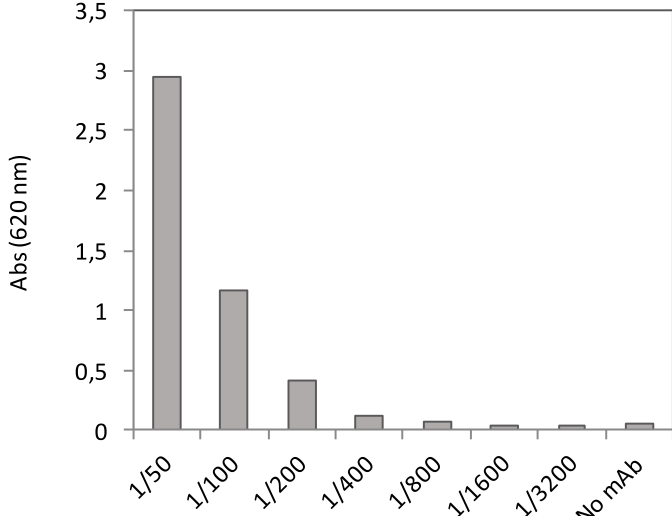

Figure S1. Conjugation of different 3G8 capture mAb dilutions to MBs. Signal is obtained after incubation of 3G8 mAb-MB conjugates with anti-mouse IgG-HRP and subsequent incubation with TMB.

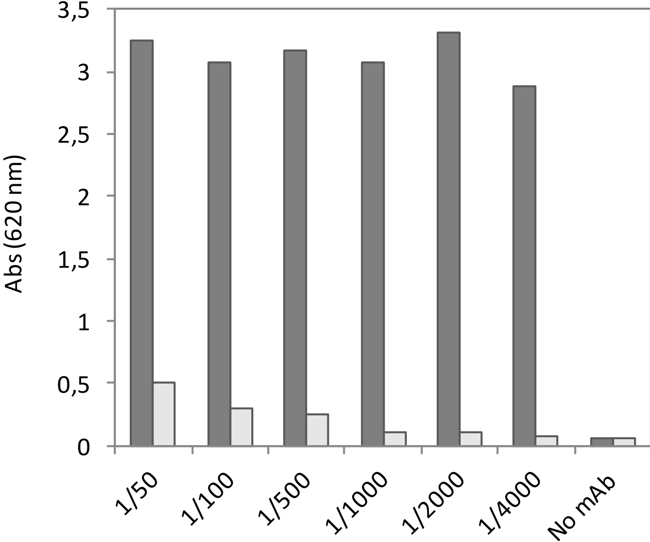

Figure S2. Optimisation of the biotinylated 8H4 detector mAb dilution. Dark grey bars show absorbance values after the incubation with different biotinylated 8H4 mAb dilutions in the presence of 1000 pg/mL CTX1B. Light grey bars show absorbance values in the absence of CTX1B.

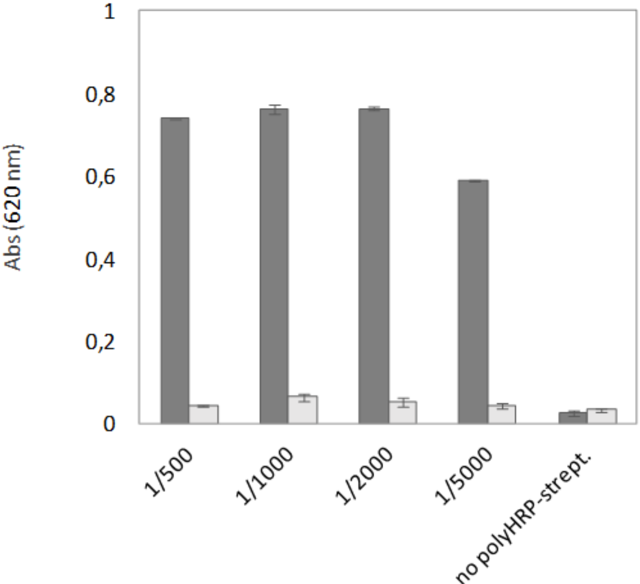

Figure S3. Optimisation of the polyHRP-streptavidin dilution. Dark grey bars show absorbance values after the incubation with different polyHRP-streptavidin dilutions in the presence of 100 pg/mL CTX1B. Light grey bars show absorbance values in the absence of CTX1B.

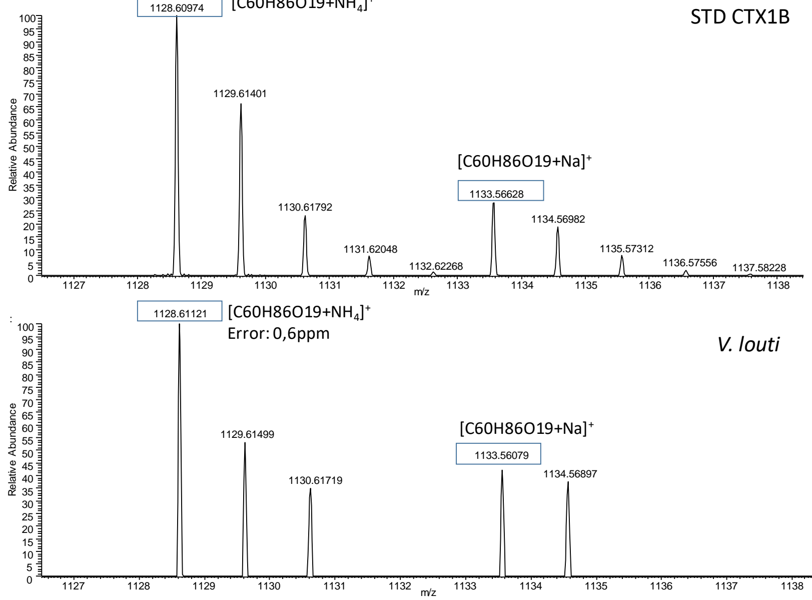

Figure S4. HRMS exact mass spectra of CTX1B standard and extract of *V. louti*.

Table S1. Fish data and CTX1B equivalent contents (pg/g) obtained in the analysis by colorimetric immunoassay and electrochemical biosensor without applying the correction factors

Species	Fishing date	Fishing site	Immunoassay	Immunosensor
Variola louti	January 2013	Saint-Gilles, Réunion	nd	nd
Variola louti	March 2013	Saint-Gilles, Réunion	nd	nd
Variola louti	March 2013	Saint-Gilles, Réunion	nd	nd
Variola louti	March 2013	Saint-Gilles, Réunion	nd	nd
Variola louti	April 2013	Saint-Gilles, Réunion	nd	nd
Variola louti	July 2003	La Pérouse Seamount,	21.57 ± 1.32	18.79 ± 1.12
Variola louti	April 2004	La Pérouse Seamount,	39.27 ± 12.00	33.57 ± 15.40
Variola louti	January 2003	La Pérouse Seamount,	94.73 ± 4.57	80.83 ± 28.51
Variola louti†*	March 2015	Maurice	246.98 ± 3.26	205.66 ± 29.50
Lutjanus bohar	September 2002	La Pérouse Seamount,	5.94 ± 0.33	4.24 ± 0.37
Lutjanus bohar	August 2003	La Pérouse Seamount,	5.83 ± 0.66	4.45 ± 0.52
Lutjanus bohar	August 2003	La Pérouse Seamount,	14.71 ± 0.35	10.91 ±3.12
Lutjanus bohar	December 2003	La Pérouse Seamount,	17.53 ± 0.59	15.99 ± 5.07
Lutjanus bohar	February 2003	La Pérouse Seamount,	131.94 ± 7.25	111.74 ± 27.21
Lutjanus bohar	January 2002	La Pérouse Seamount,	125.76 ± 2.22	122.01 ± 23.60
Thyrsitoides marleyi†	June 2015	Saint-Paul, Réunion	77.87 ± 8.39	64.82 ± 28.30

References

Aligizaki K, Nikolaidis G (2008). Morphological identification of two tropical dinoflagellates of the genera *Gambierdiscus* and *Sinophysis* in the Mediterranean Sea. J. Biol. Res -Thessalon 9:75-82.

Agence nationale de sécurité sanitaire de l'alimentation, de l'environnement et du travail (ANSES) (2015). Contamination des requins, notamment tigre et bouledogue, par des ciguatoxines: occurrence, méthodes analytiques, cas humains rapportés et éléments d'éthologie. Avis de l'Anses Rapport d'expertise collective. Avis de l'Anses Saisine n° 2013-SA-0198

Bentur Y, Spanier E (2007). Ciguatoxin-like substances in edible fish on the eastern Mediterranean, Clin Toxicol 45: 695-700.

Bienfang P, DeFelice S, Dowling A.(2011). Quantitative evaluation of commercially available test kit for ciguatera in fish. Food Sci Nutr 2:594-598

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

Bravo I, Rodriguez F, Ramilo I, Rial P, Fraga S (2019). Ciguatera-Causing Dinoflagellate *Gambierdiscus* spp.(Dinophyceae) in a subtropical region of North Atlantic Ocean (Canary Islands): morphological characterization and biogeography. Toxins 11:423.

Bravo J, Suárez F, Ramírez A, Acosta F (2015). Ciguatera, an emerging human poisoning in Europe. J Aquac Mar Biol 3:00053

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

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

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

Dickey R, Granade H, McClure F (1994). Evaluation of a solid-phase immunobead assay for detection of ciguatera-related biotoxins in Caribbean finfish. Mem Queensl Mus 34:481-488.

Diogène J, Reverte L, Rambla-Alegre M, Del Rio V, de la Iglesia P, Campàs M, Palacios O, Flores C, Caixach J, Ralijaona C, Razanajatovo I, Pirog A, Magalon H, Arnich N, Turquet J (2017). Identification of ciguatoxins

in a shark involved in a fatal food poisoning in the Indian Ocean. Sci Rep 7:8240.

Ebesu JSM, Campora CE (2012). Comment on "Quantitative Evaluation of Commercially Available Test Kit for Ciguatera in Fish". Food Nutr Sci 03:1233-1237.

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

Fraga S, Rodríguez F (2014). Genus *Gambierdiscus* in the Canary Islands (NE Atlantic Ocean) with description of *Gambierdiscus silvae* sp. nov., a new potentially toxic epiphytic benthic dinoflagellate. Protist 165:839-853.

Friedman MA, Fernandez M, Backer LC, Dickey RW, Bernstein J, Schrank K, Kibler S, Stephan W, Gribble MO, Bienfang P, Bowen RE, Degrasse S, Flores Quintana HA, Loeffler CR, Weisman R, Blythe D, Berdalet E, Ayyar R, Clarkson-Townsend D, Swajian K, Benner R, Brewer T, Fleming LE (2017). An Updated Review of Ciguatera Fish Poisoning: Clinical, Epidemiological, Environmental, and Public Health Management. Mar Drugs 15:72.

Food Standards Australia New Zealand (FZAN) (2006). A guide to the Australian Primary Production and Processing Standard for Seafood, Safe Seafood Australia. 2nd edn., Canberra.

Hokama Y, Abad MA, Kimura LH (1983). A rapid enzyme-immunoassay for the detection of ciguatoxin in contaminated fish tissues. Toxicon 21:817-824.

Hokama Y (1985). A rapid, simplified enzyme immunoassay stick test for the detection of ciguatoxin and related polyethers from fish tissues. Toxicon 23:939-946.

Hokama Y, Banner A, Boylan D (1977). A radioimmunoassay for the detection of ciguatoxin. Toxicon 15:317-325.

Hokama Y, Shirai L, Iwamoto L, Kobayashi M, Goto C, Nakagawa L (1987). Assessment of a rapid enzyme immunoassay stick test for the detection of ciguatoxin and related polyether toxins in fish tissues. Biol Bull 172:144-153.

Laza-Martínez A, David H, Riobó P, Miguel I, Orive E (2016). Characterization of a strain of *Fukuyoa paulensis* (Dinophyceae) from the Western Mediterranean Sea. J Eukaryot Microbiol 63:481-497

Lehane L, Lewis RJ (2000). Ciguatera: recent advances but the risk remains. Int J Food Microbiol 61:91-125.

Lewis RJ, Inserra M, Vetter I, Holland WC, Hardison DR, Tester PA, Litaker RW (2016). Rapid extraction and identification of maitotoxin and ciguatoxin-like toxins from Caribbean and Pacific *Gambierdiscus* using a new functional bioassay. PLoS One 11:e0160006

Litaker RW, Holland WC, Hardison DR, Pisapia F, Hess P, Kibler SR, Tester PA (2017). Ciguatoxicity of *Gambierdiscus* and *Fukuyoa* species from the Caribbean and Gulf of Mexico. PLoS One 12:e0185776

Ministry of Health W, and Labour (MHWL) (1953). A ban on domestic sales of barracuda. MHWL notification No. 20. vol 20.

Ministry of Health W, and Labour (MHWL) (2001). Handling of ciguatera fish, Office memorandum, by MHWL to heads of quarantine stations.

Murata M, Legrand AM, Ishibashi Y, Yasumoto T (1989). Structures of ciguatoxin and its congener. J Am Chem Soc 111:8929-8931.

Nagumo Y, Oguri H, Shindo Y, Sasaki S-y, Oishi T, Hirama M, Tomioka Y, Mizugaki M, Tsumuraya T (2001). Concise synthesis of ciguatoxin ABC-ring fragments and surface plasmon resonance study of the interaction of their BSA conjugates with monoclonal antibodies. Bioorg Med Chem Lett 11:2037-2040.

Nagumo Y, Oguri H, Tsumoto K, Shindo Y, Hirama M, Tsumuraya T, Fujii I, Tomioka Y, Mizugaki M, Kumagai I (2004) Phage-display selection of antibodies to the left end of CTX3C using synthetic fragments. J Immunol Methods 289:137-146.

Oguri H, Hirama M, Tsumuraya T, Fujii, I, Maruyama M, Uehara H, Nagumo Y (2003). Synthesis-based approach toward direct sandwich immunoassay for ciguatoxin CTX3C. J Am Chem Soc 125:7608-7612.

Oguri H, Tanaka S-i, Hishiyama S, Oishi T, Hirama M, Tumuraya T, Tomioka Y, Mizugaki M (1999). Designed hapten aimed at anti-ciguatoxin monoclonal antibody: synthesis, immunization and discrimination of the C2 configuration. Synthesis, pp 1431-1436.

Park D (1995). Detection of ciguatera and diarrheic shellfish toxins in finfish and shellfish with ciguatect kit. J AOAC Int(USA).

Perez-Arellano J-L, Luzardo OP, Brito AP, Cabrera MH, Zumbado M, Carranza C, Angel-Moreno A, Dickey RW, Boada LD (2005). Ciguatera fish poisoning, Canary Islands. Emerg Infect Dis 11:1981.

Reverté L, Soliño L, Carnicer O, Diogène J, Campàs M (2014). Alternative methods for the detection of emerging marine toxins: biosensors, biochemical assays and cell-based assays. Mar Drugs 12:5719-5763.

Reverté L, Toldrà A, Andree KB, Fraga S, de Falco G, Campàs M, Diogène J (2018). Assessment of cytotoxicity in ten strains of *Gambierdiscus* australes from Macaronesian Islands by neuro-2a cell-based assays. J Appl Phycol 30:2447-2461.

Rodríguez F, Fraga S, Ramilo I, Rial P, Figueroa RI, Riobó P, Bravo I (2017). Canary Islands (NE Atlantic) as a biodiversity ‘hotspot’of *Gambierdiscus*: implications for future trends of ciguatera in the area. Harmful algae 67:131-143.

Satake M, Fukui M, Legrand A-M, Cruchet P, Yasumoto T (1998). Isolation and structures of new ciguatoxin analogs, 2, 3-dihydroxyCTX3C and 51-hydroxyCTX3C, accumulated in tropical reef fish. Tetrahedron Lett 39:1197-1198.

Silva M, Rodriguez I, Barreiro A, Kaufmann M, Neto A, Hassouani M, Sabour B, Alfonso A, Botana L, Vasconcelos V (2015). First report of ciguatoxins in two starfish species: *Ophidiaster ophidianus* and *Marthasterias glacialis*. Toxins 7:3740-3757.

Soliño L, Widgy S, Pautonnier A, Turquet J, Loeffler CR, Quintana HAF, Diogène J (2015). Prevalence of ciguatoxins in lionfish (Pterois spp.) from Guadeloupe, Saint Martin, and Saint barthélmy islands (caribbean). Toxicon 102:62-68.

Tsumuraya T, Fujii I, Hirama M (2010). Production of monoclonal antibodies for sandwich immunoassay detection of Pacific ciguatoxins. Toxicon 56:797-803.

Tsumuraya T, Fujii I, Hirama M (2014). Preparation of anti-ciguatoxin monoclonal antibodies using synthetic haptens: sandwich ELISA detection of ciguatoxins. J AOAC Int 97:373-379.

Tsumuraya T, Fujii I, Inoue M, Tatami A, Miyazaki K, Hirama M (2006). Production of monoclonal antibodies for sandwich immunoassay detection of ciguatoxin 51-hydroxyCTX3C. Toxicon 48:287-294.

Tsumuraya T, Sato T, Hirama M, Fujii I (2018). Highly sensitive and practical fluorescent sandwich ELISA for ciguatoxins. Anal Chem 90:7318-7324.

Tsumuraya T, Takeuchi K, Yamashita S, Fujii I, Hirama M (2012). Development of a monoclonal antibody against the left wing of ciguatoxin CTX1B: Thiol strategy and detection using a sandwich ELISA. Toxicon 60:348-357.

Tudó À, Toldrà A, Andree KB, Rey M, Fernández-Tejedor M, Campàs M, Diogène J (2018). First report of *Gambierdiscus* in the Western Mediterranean Sea (Balearic Islands). Harmful Algae News.

US FDA (United States Food and Drug Administration) (2019). Fish and Fishery Products Hazards and Controls Guidance, 4th Ed.

Yasumoto T, Igarashi T, Legrand A-M, Cruchet P, Chinain M, Fujita T, Naoki H (2000) Structural elucidation of ciguatoxin congeners by fast-atom bombardment tandem mass spectroscopy. Journal of the American Chemical Society 122:4988-4989

Chapter 4

Multi-approached detection of a ciguateric fish in the Mediterranean Sea

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Abstract

Ciguatera fish poisoning occurs when seafood contaminated with ciguatoxins (CTXs) is consumed. This intoxication is an expanding phenomenon which repercussions can affect not only human health but also the fishery sector causing important economic losses. Since no antidote for this disease has been found yet, and the *Gambierdiscus/Fukuyoa* species, primary producers of ciguatoxin, are well established in tropical, sub-tropical and temperate areas (as the Mediterranean basin) the effort has to be focused on the prevention strategies. For this reason, in this work, 196 crude extracts of fishes from Mediterranean waters were screened with cell-based assay, and the unique positive individual identified (12.4 pg equiv. CTX1B/g flesh equiv.) was analyzed also with the immunosensor previously developed by our group, confirming the presence of CTXs (4.98 pg equiv. CTX1B/g flesh equiv.). Therefore, the crude extract of this particular individual was fractionated and subsequently re-analysed with the cell-based assay and the immunosensor to identify the exact fractions in which the CTX-like activity/CTXs were present. The interval of fractions in which the toxicological activity and the toxins were identified was comparable to the one previously obtained from the analysis of a shark individual naturally contaminated with CTXs. This study showed the

combination of two economical techniques can help in the pre-screening of naturally contaminated samples, reducing the use of instrumental techniques only to the analysis of particular individuals.

1. Introduction

Ciguatera fish poisoning (CFP) is a foodborne disease caused by the consumption of fish contaminated with ciguatoxins (CTXs) (Lewis 2001). These potent neurotoxins are produced by microalgae of the genera *Gambierdiscus* and *Fukuyoa*, which are epibenthic dinoflagellates that live attached to different substrates such as macroalgae, corals and rocks (Lewis and Holmes 1993). Herbivorous fishes graze those substrates and incorporate CTXs, which are accumulated in other organisms (mainly piscivorous fishes, but also crustaceans, echinoderms and bivalves) through the food web (Ledreux et al. 2014). CFP is known to affect at least from 10 000 to 500 000 people per year, and causes severe neurological, gastrointestinal and cardiovascular disorders that may persist for years (Friedman et al. 2017).

CFP was historically associated to tropical and subtropical areas, e.g. the Caribbean Sea, the Pacific, Atlantic and Indian Oceans. However, CFP has geographically expanded, due to international seafood trade and travel as well as changes in the aquatic environment, and emerged in places not previously expected according to their latitudes, particularly in Europe. In the Canary Islands (Spain), several CFP outbreaks have been reported (Gobierno de Canarias). CTXs have also been detected in fish from other areas of the Macaronesia, *i.e.*, Azores and Madeira archipelagos (Portugal) (Silva et al. 2015; Costa et al. 2018). Regarding *Gambierdiscus*, apart from their presence in the Canary Islands (Fraga et al. 2011; Fraga and Rodríguez 2014; Rodríguez et al. 2017; Bravo et al. 2019; Tudó et al., 2020a) and Madeira (Kaufmann and Böhm-Beck 2013), they have also been detected in the Mediterranean Sea, specifically in the Balearic Islands (Tudó et al. 2018; Tudó et al. 2020b; Gaiani et al. 2022), Greece (Aligizaki and Nikolaidis 2008; Aligizaki et al. 2018) and Cyprus (Aligizaki et al. 2018). *Fukuyoa* cells have also been found in the Mediterranean Sea (Laza-Martínez et al. 2016; Tudó et al. 2020b; Aligizaki et al. 2018), but not yet in the Macaronesia.

The purpose of this work has been the screening of CTXs in Mediterranean fish with the use of a cytotoxicity cell-based assay (CBA) and the further analysis of one positive individual fished in Cyprus waters.

2. Materials and methods

2.1. Reagents and materials

The CTX1B standard was obtained from Prof. Richard J. Lewis (The Queensland University, Australia) and calibrated (correction factor of 90%) in relation to the NMR-quantified CTX1B standard obtained from Prof. Takeshi Yasumoto (Japan Food Research Laboratories, Japan).

Methanol HPLC grade was obtained from Honeywell (Barcelona, Spain). Acetonitrile hypergrade for LC-MS was purchased from Merck (Darmstadt, Germany). Ultrapure water was obtained with a Milli-Q purification system (Millipore, Bedford, MA, USA).

Neuroblastoma murine cells (N2a) were purchased from ATCC LGC standards (USA). 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) was purchased from Chemlab (Spain).

Dynabeads M-270 Carboxylic Acid (2×10^9 beads/mL) were supplied by Invitrogen (Life Technologies, S.A., Alcobendas, Spain). 3G8, 10C9 and 8H4 mAbs had been prepared by immunizing mice with keyhole limpet hemocyanine (KLH) conjugates of rationally designed synthetic haptens (Oguri et al., 1999, 2003; Nagumo et al., 2001, 2004; Tsumuraya et al. 2006, 2010, 2012, 2014, 2018, 2019). Biotin labelling of the 8H4 mAb was performed with the EZ-Link™ NHS-PEG4 Biotinylation Kit from Thermo Fisher (Barcelona, Spain) following the manufacturer's instructions. Unreacted NHS-PEG4-Biotin was removed using Zeba Spin Desalting Columns (7 kDa MWCO, 2 mL) included in the kit. Potassium phosphate monobasic, potassium phosphate dibasic, potassium chloride, 4-morpholineethanesulfonic acid (MES) hydrate, N-(3-dimethylaminopropyl)-N′-ethylcarbodiimide hydrochloride (EDC), N-hydroxysuccinimide (NHS), Tween®-20, bovine serum album (BSA), and 3,3′,5,5′-tetramethylbenzidine (TMB) liquid substrate were purchased from Sigma-Aldrich (Tres Cantos, Spain). PolyHRP-streptavidin was obtained from Thermo Fisher (Barcelona, Spain).

2.2. Sampling

Fish samples were obtained from the Balearic Islands (n = 44; Palma/Majorca, Cabrera/Majorca, Maó/Menorca, Ciutadella/Menorca and Ibiza/Ibiza), Greece (Crete) (n = 70; Kissamos bay, Grambousa bay and Kolymbari bay) and Cyprus (n = 82; Zygi/Larnaca, Latsi/Paphos, Cavo Kiti/Larnaca, Paphos/Paphos, Cavo

Pyla/Larnaca, Larnaca/Larnaca and Zygi area/Limassol) during 2017, 2018, 2019 and 2020 (Figure 1). The sampled species were: *Chelon labrosus*, *Conger conger*, *Coriphaena hippurus*, *Dentex dentex*, *Diplodus sargus*, *Epinephelus aeneus*, *Epinephelus costae*, *Epinephelus marginatus*, *Muraena helena*, *Muraena* spp., *Pagrus pagrus*, *Seriola dumerili*, *Shyraena shyraena*, *Siganus luridus*, *Siganus rivulatus* and *Sphyraena viridensis*. For 3 of them, only the flesh was available; for 2 of them, only the liver was available; for the rest, both flesh and liver were available.

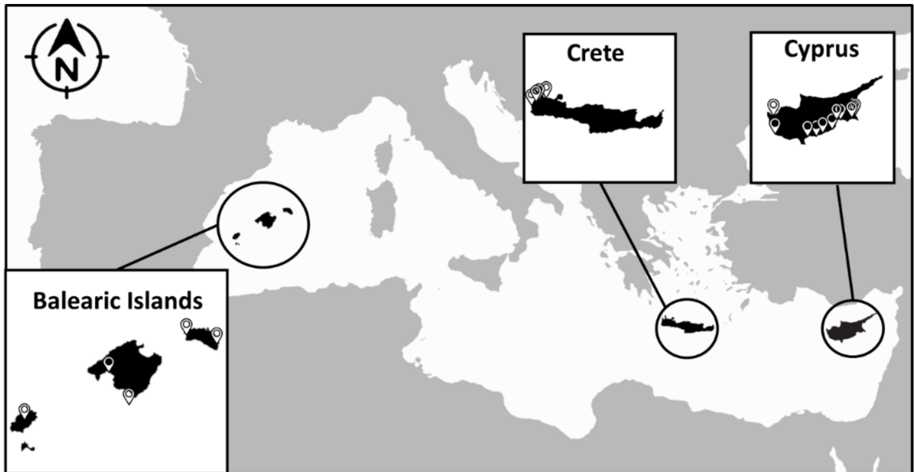

Figure 5. Fish sampling points.

2.3. Ciguatoxin extraction

Fish samples were extracted and purified as follows: 10 g of fish flesh or liver homogenate were heated at 70 °C for 15 min in a water bath. After cooling, 20 mL of acetone was added, and the sample mixture was homogenized with an Ultraturrax blender at 17,500 g for 5 min. The sample mixture was centrifuged at 3,000 g for 15 min to obtain the supernatant. The pellet was re-extracted with acetone, and supernatants were pooled, passed through 0.2-µm PTFE filters, rotary evaporated to a small volume, and adjusted to a volume of 4 mL with Milli-Q water. The sample was partitioned twice with 16 mL of diethyl ether. The water phases were discarded, and the diethyl ether phases were pooled and evaporated to dryness. The dried extract was resuspended in 2 mL of aqueous methanol (80%) and partitioned three times with 4 mL of n-hexane. The n-hexane phases were discarded, and the aqueous methanol phases were pooled and evaporated to dryness with N2. The dried extract was then resuspended in 4 mL of HPLC-grade methanol (100%), filtered with 0.2-µm PTFE membrane filters and stored at −20 °C until analysis by CBA and immunoassay, obtaining the "crude extract". For calculation purposes, 1 mL of crude extract contains 2.5 g equivalents of fish flesh. After the first screening with CBA, an additional fish flesh crude extract was prepared from the sample in which CTX-like activity was detected, using 130 g of flesh in a final volume of 52 mL (2.5 g

fish flesh/mL of methanol). This extract was used to study more deeply the toxins present in the fish, including fractionation steps through preparative chromatography.

2.4. Fish flesh extract fractionation

A total of 44 mL of fish flesh crude extract was evaporated to dryness with N_2 and resuspended in 1 mL of HPLC-grade methanol (110 g fish flesh/mL of extract). The analytical fractionation was performed on an Acquity UPLC® BEH C18, 1.7 µm (2.1 mm × 50 mm) column, protected with an Acquity UPLC® in-line filter and a VanGuard precolumn (2.1 mm × 5 mm), all of them from Waters Corporation (Milford, MA, USA), as previously described (Caillaud et al. 2011; Diogène et al. 2017). Binary gradient elution was achieved with mobile phase (A) water and (B) acetonitrile:water (95:5 *v/v*), both with 2 mM ammonium formate and 50 mM formic acid. The program started at 35% B, then linearly increased up to 100% B at 5.0 min. Such percentage was held to min 10.0 and returned to the initial conditions at min 10.1. Finally, the column was equilibrated to complete a total run-time cycle of 14 min. The auto sampler temperature, the oven temperature and the flow rate were set at 4 °C, 40 °C and 0.2 mL/min, respectively. The injection volume was 0.1 mL and, after loading, the syringe was washed for 4 seconds with 100% methanol at the flush port to avoid carry-over. The flow was diverted to waste by a 10-port Valco valve during the first 1.5 min of each run to keep the ion source clean. Fractions were collected every 0.5 min (n = 28). After fractionation, the content of each tube (1 mL) was evaporated to dryness, resuspended in 1 mL of HPLC-grade methanol, passed through 0.2-µm PTFE filters and stored at −20 °C until analysis by CBA and immunoassay.

2.5. Neuro-2a cell-based assay

The CBA for the detection of CTX-like activity was performed as previously described (Diogène et al., 2017). Briefly, N2a cells (ATCC, CCL131) were seeded in a 96-well microplate in 200 µL of RPMI medium containing 5% *v/v* fetal bovine serum (RPMI-FBS) at 34,000 cells/well, and incubated under a 5% CO_2 humid atmosphere for 24 h at 37 °C. Some N2a cells were treated with 100 µM ouabain and 10 µM veratridine. Then, 10 µL of CTX1B standard solution, fish crude extract or fraction (½ serially diluted from 12.5 to 0.1 pg/mL for CTX1B standard solution, from 200 to 25 mg/mL for fish crude extract, from 4.78 to 0.15 g equiv./mL for fraction), and from 17,000 to 10 cells/mL (depending on the strain) for microalgae extract, previously evaporated to dryness with N_2 and resuspended in 200 µL of RPMI-FBS medium, were added to the wells with and without the ouabain/veratridine

treatment (no treatment was used as a control to evaluate matrix effects). After 24 h, cell viability was measured using the MTT assay (Manger et al., 1993). Measurements were performed in triplicate.

The CBA for the detection of MTX-like activity was performed as previously described (Caillaud et al., 2010). The assay was very similar to the one for CTX-like activity, the only difference being the pre-treatment of some N2a cells with 30 µM SKF96365 for 30 min instead of ouabain and veratridine.

2.6. Magnetic bead-based immunoassay

Analyses were performed following our previous protocols (Leonardo et al. 2020; Gaiani et al. 2020). Briefly, 8 µL of magnetic bead suspension were transferred to a tube and activated by incubation with 40 µL of 50 mg/mL EDC and 40 µL of 50 mg/mL NHS (in 25 mM MES, pH 5.0) for 30 min. Subsequently, 80 µL of antibodies (3G8 or 10C9 mAb at 1/50 dilution in MES) were incubated for 1 h. The mAb-MB conjugates were washed, re-suspended in 80 µL of PBS-Tween (0.1 M PBS, 0.05% *v/v* Tween®-20, pH 7.2) and 75 µL of each conjugate were transferred into new tubes mixed together. After supernatant removal, 75 µL of CTX1B standard solution, fish crude extract or fraction (at 500, 100, 50 and 25 pg/mL for CTX1B standard solution, and ½ serially diluted from 10 to 2.5 g/mL for fish crude extract, and from 440 to 13.75 g equiv./mL for fraction), previously dried and resuspended in 250 µL of PBS-Tween, were added to the tube and incubated for 30 min. A blocking step was performed with PBS-Tween containing 2% *w/v* BSA. Then, the conjugates were incubated first with 75 µL of biotin-8H4 mAb and afterwards with 75 µL of polyHRP-streptavidin. All the incubations lasted for 30 min, were performed at room temperature with slow tilt rotation, and three washings with PBS-Tween were performed between each step. Finally, immunocomplexes were washed and re-suspended in 75 µL of PBS-Tween. Then, 10 µL of immunocomplexes were transferred to a new tube, the supernatant was removed and 125 µL of TMB were incubated for 10 min. Then, 100 µL of solution were taken for absorbance reading at 620 nm. Measurements were performed in triplicate.

3. Results

3.1. Analysis of fish crude extracts with the CBA and the immunoassay

Mediterranean fish flesh and liver crude extracts were screened by CBA for the presence of CTXs. No CTX-like activity was detected in fish flesh from the Balearic Islands and Crete. Only one CTX-like positive fish individual was detected out of 75 fish individuals from Cyprus, showing 12.4 pg equiv.

CTX1B/g flesh equiv. The positive fish was a *Seriola dumerili* of 6.9 kg caught the 8th August 2018 in Zygi/Larnaca district. No liver was available for that fish.

The flesh crude extract that resulted positive by CBA was analysed with the magnetic bead-based immunoassay, which revealed the presence of CTXs at a concentration of 4.98 pg CTX1B equiv./g flesh equiv.

3.2. Analysis of fish fractions with CBA and immunoassay

The analysis of the fractions at 13.77 g flesh equiv./mL by CBA showed cell mortality above 80% in fractions 2, 3 and 7-19 (Figure 2). Fractions 2 and 3 were neglected as cell mortality was thought to be due to the solvents used in the fractionation. Fractions 7 and 8 provided the same cell mortality as the control (no ouabain/veratridine) and thus, they were not considered either. Samples 17-19 showed cell mortalities higher than in the control, which may indicate CTX-like activity. Fractions 9 to 16, the ones where higher cell mortalities were observed (regardless of fractions 2 and 3), were further diluted and analysed again. The CBA of fractions at 1.72 g flesh equiv./mL showed CTX-like activity in fractions 15 (0.4 pg/g) and 16 (0.5 pg/g). The CBA fractions at 0.86 g flesh equiv./mL showed CTX-like activity in fractions 13 (3.7 pg/g) and 14 (1.9 pg/g). Figure 3 shows the results of the analysis by the CBA of the fractions at 0.86 g flesh equiv./mL, as an example. The sum of toxin contents in fractions where CTX-like activity was clearly observed is 6.5 pg equiv. CTX1B/g flesh equiv.

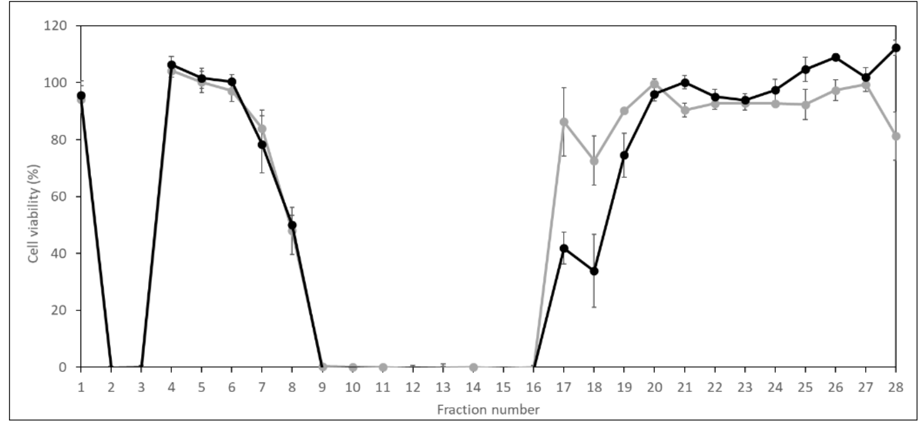

Figure 6. Toxicity of flesh fractions from 1 to 28 at 13.77 g flesh equiv./mL by CBA with ouabain/veratridine (black) and corresponding control without ouabain/veratridine (grey). Error bars represent standard deviation (SD) values for 3 replicates (n = 3).

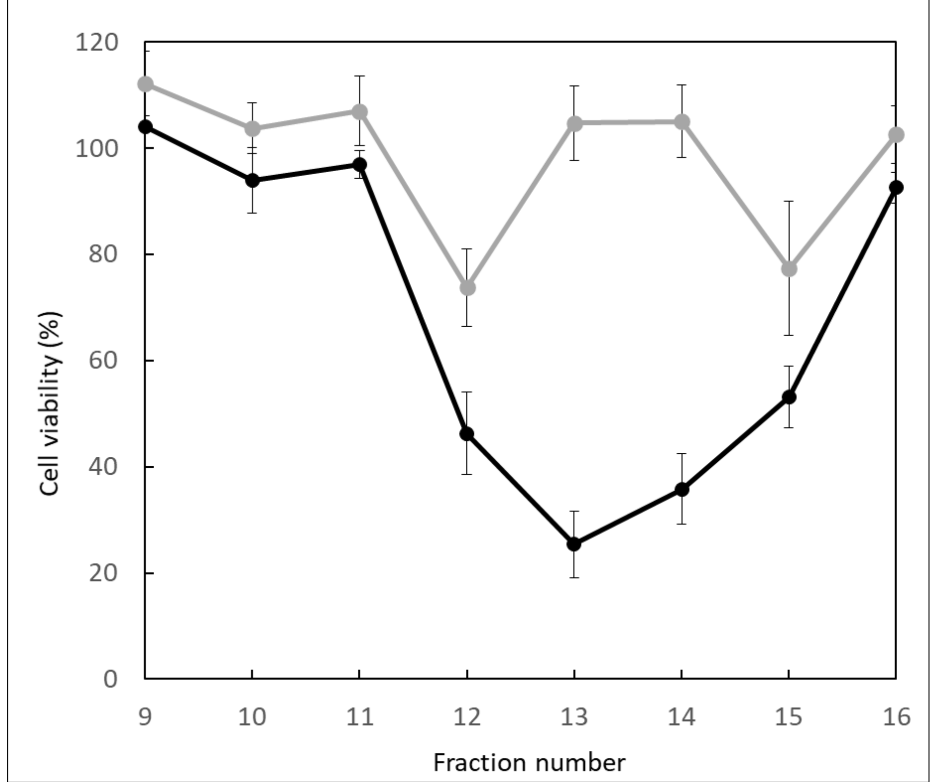

Figure 7. Toxicity of flesh fractions from 9 to 16 at 0.86 g flesh equiv./mL by CBA with ouabain/veratridine (black) and corresponding control without ouabain/veratridine (grey). Error bars represent standard deviation (SD) values for 3 replicates (n = 3).

The analysis of the fractions by the magnetic bead-based immunoassay revealed the presence of CTXs in fraction 15, when analysed at 110 g flesh equiv./mL, which contained 0.08 pg CTX1B equiv./g flesh equiv. (LOD = 0.01 pg CTX1B equiv./g flesh equiv.).

4. Discussion

Among all the fish studied in the Mediterranean (n = 196), only one fish from Cyprus presented CTX-like activity. Analysis of the fish crude extracts with CBA revealed CTX-like activity in a *Seriola dumerili* individual of 6.9 kg caught the 8th August 2018 in Zygi/Larnaca district. No liver was available for that fish. The toxin content was 12.4 pg equiv. CTX1B/g, just slightly over the guidance level of 0.01 ppb (10 pg/g) proposed by the United States Food and Drug Administration (US FDA, 2019).

Our recent work with anti-CTX antibodies has demonstrated that the MB-based immunoassay is very appropriate tool for the detection of CTXs in fish

(Leonardo et al. 2020) and microalgae (Gaiani et al. 2020; Tudó et al. 2020a). One of the advantages is the low limit of detection, comparable to that attained with CBA. Another important advantage is that matrix effects, if any, may cause a decrease of the signal and thus imply an underestimation of the CTX contents, but do not cause an enhancing effect. This robustness together with the high specificity of the antibodies used, make that no false positives are detected. When comparing with the results obtained with the CBA in the analysis of the flesh crude extract, much lower CTXs contents were found with the immunosensor (0.08 in front of 12.4 pg equiv. CTX1B/g flesh equiv.). It is necessary to keep in mind that the immunosensor recognises only some CTX congeners, *i.e.* CTX1B, 54-deoxyCTX1B, CTX3C and 51-hydroxyCTX3C, whereas the CBA is certainly responding to more CTX congeners. Nevertheless, the response detected by the MB-based immunoassay approach is another evidence of the presence of CTXs in this individual.

Regarding the fractionation experiment, a previous fractionation of a shark stomach extract with the same protocol showed CTX-like activity in fractions 8 to 22, with the highest toxicities in fractions 9 to 12 (Diogène et al. 2017). LC-ESI-HRMS revealed the presence of I-CTX-1&2 in these four fractions and I-CTX-3&4 in fractions 9 and 10. The fractionation of an extract from a solid-phase adsorption toxin tracking (SPATT) resin exposed to a *Gambierdiscus pacificus* culture also showed CTX-like activity in fractions 8, 10, 11 and 12 (retention time windows between 3.5–4.0 and 4.5–6.0 min). In that study, 2,3-dihydroxyCTX3C (5.17 and 5.82 min), 51-hydroxyCTX3C (5.82 min) and M-/L-seco-CTX3C (5.54 and 5.82 min) were detected by LC-MS/MS and could explain the reported toxicity (Caillaud et al. 2011). In our present work, fractionation of the fish flesh shows CTX-like activity in fractions 13, 14, 15 and 16. Therefore, the similarity of the chromatographic toxic profile with those observed in other works is another indication of the presence of CTXs in this individual, as well as the CTX-like compounds detected with the MB-based immunoassay in fraction 15. Since the antibodies only recognise CTX1B, 54-deoxyCTX1B, CTX3C and 51-hydroxyCTX3C, the lack of immunochemical signal from other fractions determined as positive by CBA could be due to the presence of other toxic CTX congeners. The different retention times of the different CTX congeners that may be present in the samples may justify the slight differences obtained in the toxic fractions among the studies. From a quantitative point of view, although the fractionation protocol reduces the matrix effects, it is also prone to toxin losses. This is reflected in the determination of CTXs contents, which was 6.5 in front of 12.4 pg equiv. CTX1B/g flesh equiv. with the CBA and 0.08 in front of 4.98 pg CTX1B equiv./g flesh equiv. with the magnetic bead-based immunoassay. Nevertheless,

the purpose of the fractionation experiment was the confirmation of CTXs presence rather than a precise quantification.

Previous studies indicate that *Gambierdiscus* and *Fukuyoa* are well established in the Mediterranean Sea. However, the toxicity of the strains from Greece and Cyprus is in general low. This, combined with low cell abundancies of ciguatoxin producing species may explain why that fish from Cyprus was only slightly toxic and why no other positive fish individuals (out of the 152 examined from Crete and Cyprus) have been detected so far. However, increases in abundance and toxicity due to climate change and the subsequent changes in several abiotic factors, and therefore spread of ciguateric fish and future CP cases cannot be ruled out.

5. Conclusions

The screening performed with CBA demonstrated the utility of this test to easily identify extracts in which a CTX-like activity is occurring. This feature combined with the robustness and specificity of the antibodies used in the immunosensor technique allowed to recognize CTXs in the fish individual positive for CTX-like activity, unique among the 196 crude extracts analysed. In this way, only this particular individual underwent a purification procedure, shortening considerably the time required for the analysis. Additionally, the use of these two tests identified the presence of CTX-like activity and CTXs in an interval of fractions similar to the one in which they were detected when the same protocol was performed for the analysis of a shark extract involved in a ciguatera outbreak. Therefore, even though these results are preliminary and require further analysis with instrumental techniques, they underline the need of routinely screening of fishes from the Mediterranean basin in order to prevent outbreaks. In this picture, the combined use of the CBA and the immunosensor would provide reliable results in a short period of time, reducing the use of instrumental analysis only to confirmation procedure for a reduced number of individuals. Thus, it would not take long to see this protocol integrated in monitoring systems.

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References

Aligizaki K., Iliadou M., Kappas I., Arsenakis M (2018). Is the eastern Mediterranean a "*Gambierdiscus* biodiversity hotspot"? New data from Greece and Cyprus. In: Hess P (ed). Abstract book of the 18th International Conference on Harmful Algae. IFREMER, Nantes, 493 pp.

Aligizaki K, Nikolaidis G (2008). Morphological identification of two tropical dinoflagellates of the genera *Gambierdiscus* and *Sinophysis* in the Mediterranean Sea. J. Biol. Res -Thessalon 9:75-82.

Bravo I, Rodriguez F, Ramilo I, Rial P, Fraga S (2019). Ciguatera-Causing Dinoflagellate *Gambierdiscus* spp. (Dinophyceae) in a subtropical region of North Atlantic Ocean (Canary Islands): morphological characterization and biogeography. Toxins 11:423.

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

Caillaud A, Yasumoto T, Diogène J (2010). Detection and quantification of maitotoxin-like compounds using a neuroblastoma (Neuro-2a) cell-based assay. Application to the screening of maitotoxin-like compounds in *Gambierdiscus* spp. Toxicon 56, 36-44.

Chinain M, Faust MA, Pauillac S (1999). Morphology and molecular analyses of three toxic species of *Gambierdiscus* (Dinophyceae): *G. pacificus*, sp. nov., *G. australes*, sp. nov., and *G. polynesiensis*, sp. nov. J Phycol 35:1282-1296.

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

Diogène J, Reverte L, Rambla-Alegre M, Del Rio V, de la Iglesia P, Campàs M, Palacios O, Flores C, Caixach J, Ralijaona C, Razanajatovo I, Pirog A, Magalon H, Arnich N, Turquet J (2017). Identification of ciguatoxins in a shark involved in a fatal food poisoning in the Indian Ocean. Sci Rep 7:8240.

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

Fraga S, Rodríguez F (2014) Genus *Gambierdiscus* in the Canary Islands (NE Atlantic Ocean) with description of *Gambierdiscus silvae* sp. nov., a new potentially toxic epiphytic benthic dinoflagellate. Protist 165:839-853.

Friedman MA, Fernandez M, Backer LC, Dickey RW, Bernstein J, Schrank K, Kibler S, Stephan W, Gribble MO, Bienfang P, Bowen RE, Degrasse S,

Flores Quintana HA, Loeffler CR, Weisman R, Blythe D, Berdalet E, Ayyar R, Clarkson-Townsend D, Swajian K, Benner R, Brewer T, Fleming LE (2017). An Updated Review of Ciguatera Fish Poisoning: Clinical, Epidemiological, Environmental, and Public Health Management. Mar Drugs 15:72.

Gaiani G, Cucchi F, Toldrà A, Andree KB, Rey M, Tsumuraya T, O'Sullivan CK, Diogène J, Campàs M (2022). Electrochemical biosensor for the dual detection of *Gambierdiscus australes* and *Gambierdiscus excentricus* in field samples. First report of *G. excentricus* in the Balearic Islands. Sci Total Environ 806:150915.

Gaiani G, Leonardo S, Tudó À, Toldrà A, Rey M, Andree KB, Tsumuraya T, Hirama M, Diogene J, O'Sullivan CK, Alcaraz C, Campàs M (2020). Rapid detection of ciguatoxins in *Gambierdiscus* and *Fukuyoa* with immunosensing tools. Ecotoxicol Environ Saf 204:111004.

Gobierno de Canarias. Sistema de Vigilancia Epidemiológica de la Intoxicación por Ciguatera en Canarias. https://www3.gobiernodecanarias.org/sanidad/scs/contenidoGenerico.jsp?idDocument=bb1799ed-b4c0-11de-ae50-15aa3b9230b7&idCarpeta=3ec36999-d4e1-11e2-8241-7543da9dbb8a

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

Laza-Martínez A, David H, Riobó P, Miguel I, Orive E (2016). Characterization of a strain of *Fukuyoa paulensis* (Dinophyceae) from the Western Mediterranean Sea. J Eukaryot Microbiol 63:481-497.

Ledreux A, Brand H, Chinain M, Bottein M-YD, Ramsdell JS (2014). Dynamics of ciguatoxins from *Gambierdiscus polynesiensis* in the benthic herbivore *Mugil cephalus*: Trophic transfer implications. Harmful Algae 39:165-174.

Leonardo S, Gaiani G, Tsumuraya T, Hirama M, Turquet J, Sagristà N, Rambla-Alegre M, Flores C, Caixach J, Diogène J (2020). Addressing the analytical challenges for the detection of ciguatoxins using an electrochemical biosensor. Anal Chem 92:4858-4865.

Lewis RJ (2001). The changing face of ciguatera. Toxicon 39, 97-106.

Lewis RJ, Holmes MJ (1993). Origin and transfer of toxins involved in ciguatera. Comp. Biochem. Physiol 106C:615-628.

Manger RL, Leja LS, Lee SY, Hungerford JM, Wekell MM (1993). Tetrazolium-based cell bioassay for neurotoxins active on voltage-sensitive sodium channels: semiautomated assay for saxitoxins, brevetoxins, and ciguatoxins. Anal Biochem 214:190-194.

Nagumo Y, Oguri H, Shindo Y, Sasaki S-y, Oishi T, Hirama M, Tomioka Y, Mizugaki M, and Tsumuraya T (2001). Concise synthesis of ciguatoxin ABC-ring fragments and surface plasmon resonance study of the interaction of their BSA conjugates with monoclonal antibodies. Bioorg Med Chem Lett 11:2037-2040.

Nagumo Y, Oguri H, Tsumoto K, Shindo Y, Hirama M, Tsumuraya T, Fujii I, Tomioka Y, Mizugaki M, Kumagai I (2004). Phage-display selection of antibodies to the left end of CTX3C using synthetic fragments. J Immunol Methods 289:137-146.

Oguri H, Hirama M, Tsumuraya T, Fujii, I, Maruyama M, Uehara H, Nagumo Y (2003). Synthesis-based approach toward direct sandwich immunoassay for ciguatoxin CTX3C. J Am Chem Soc 125:7608-7612.

Oguri H, Tanaka S-i, Hishiyama S, Oishi T, Hirama M, Tumuraya T, Tomioka Y, Mizugaki M (1999). Designed hapten aimed at anti-ciguatoxin monoclonal antibody: synthesis, immunization and discrimination of the C2 configuration. Synthesis, pp 1431-1436.

Rodríguez F, Fraga S, Ramilo I, Rial P, Figueroa RI, Riobó P, Bravo I (2017) Canary Islands (NE Atlantic) as a biodiversity 'hotspot'of *Gambierdiscus*: Implications for future trends of ciguatera in the area. Harmful algae 67:131-143

Silva M, Rodriguez I, Barreiro A, Kaufmann M, Isabel Neto A, Hassouani M, Sabour B, Alfonso A, Botana LM, Vasconcelos V (2015). First report of ciguatoxins in two starfish species: *Ophidiaster ophidianus* and *Marthasterias glacialis*. Toxins (Basel) 7:3740-3757.

Tsumuraya T, Fujii I, Inoue M, Tatami A, Miyazaki K, Hirama M (2006). Production of monoclonal antibodies for sandwich immunoassay detection of ciguatoxin 51-hydroxyCTX3C. Toxicon 48:287-294.

Tsumuraya T, Fujii I, Hirama M (2010). Production of monoclonal antibodies for sandwich immunoassay detection of Pacific ciguatoxins. Toxicon 56:797-803.

Tsumuraya T, Takeuchi K, Yamashita S, Fujii I, Hirama M (2012). Development of a monoclonal antibody against the left wing of ciguatoxin CTX1B: Thiol strategy and detection using a sandwich ELISA. Toxicon 60:348–357.

Tsumuraya T, Fujii I, Hirama M (2014). Preparation of anti-ciguatoxin monoclonal antibodies using synthetic haptens: Sandwich ELISA detection of ciguatoxins. J AOAC Int 97:373-379.

Tsumuraya T, Sato T, Hirama M, Fujii I (2018). Highly sensitive and practical fluorescent sandwich ELISA for ciguatoxins. Anal Chem 90:7318-7324.

Tsumuraya T, Hirama M (2019). Rationally designed synthetic haptens to generate anti-ciguatoxin monoclonal antibodies, and development of a practical sandwich ELISA to detect ciguatoxins. Toxins 11:533.

Tudó À, Toldrà A, Andree KB, Rey M, Fernández-Tejedor M, Campàs M, Diogène J (2018). First report of *Gambierdiscus* in the Western Mediterranean Sea (Balearic Islands). Harmful Algae News.

Tudó À, Gaiani G, Varela MR, Tsumuraya T, Andree KB, Fernández-Tejedor M, Campàs M, Diogène J (2020a). Further advance of *Gambierdiscus* species in the Canary Islands, with the first report of *Gambierdiscus belizeanus*. Toxins 12:692.

Tudó À, Toldrà A, Rey M, Todolí I, Andree KB, Fernández-Tejedor M, Campàs M, Sureda FX, Diogène J (2020b). *Gambierdiscus* and *Fukuyoa* as potential indicators of ciguatera risk in the Balearic Islands. Harmful Algae 99:101913.

US FDA (United States Food and Drug Administration) (2019). Fish and Fishery Products Hazards and Controls Guidance, 4th Ed.

Chapter 5

Rapid detection of ciguatoxin in *Gambierdiscus* and *Fukuyoa* with immunosensing tools

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Abstract

Consumption of seafood contaminated with ciguatoxins (CTXs) leads to a foodborne disease known as ciguatera. Primary producers of CTXs are epibenthic dinoflagellates of the genera *Gambierdiscus* and *Fukuyoa.* In this study, thirteen *Gambierdiscus* and *Fukuyoa* strains were cultured, harvested at exponential phase, and CTXs were extracted with an implemented rapid protocol. Microalgal extracts were obtained from pellets with a low cell abundance (20,000 cell/mL) and were then analyzed with magnetic bead (MB)-based immunosensing tools (colorimetric immunoassay and electrochemical immunosensor). It is the first time that these approaches are used to screen *Gambierdiscus* and *Fukuyoa* strains, providing not only a global indication of the presence of CTXs, but also the ability to discriminate between two series of congeners (CTX1B and CTX3C). Analysis of the microalgal extracts revealed the presence of CTXs in 11 out of 13 strains and provided new information about *Gambierdiscus* and *Fukuyoa* toxin profiles. The use of immunosensing tools in the analysis of microalgal extracts facilitates the elucidation of further knowledge regarding these dinoflagellate genera and can contribute to improved ciguatera risk assessment and management.

1. Introduction

Epibenthic dinoflagellates of the genera *Gambierdiscus* and *Fukuyoa* are known producers of ciguatoxins (CTXs), potent marine toxins responsible for a foodborne disease termed ciguatera. (Lewis 2001, Begier et al 2006, Litaker et al 2017, Larsson et al 2019) CTXs can accumulate in marine food webs, from herbivorous and detritivorous fishes that graze substrates colonized by *Gambierdiscus* and *Fukuyoa* (*e.g.* macroalgae, corals and rocks) to carnivorous fishes (Ledreux et al. 2014). In the process, other organisms like crustaceans, echinoderms and bivalves may also be implicated (Kelly et al. 1992, Silva et al. 2015, Roué et al. 2016).

Presently, eighteen species of *Gambierdiscus* are recognized worldwide: *G. toxicus, G. belizeanus, G. australes, G. pacificus, G. polynesiensis, G. caribaeus, G. carolinianus, G. carpenteri, G. excentricus, G. scabrosus, G. silvae, G. balechii, G. cheloniae, G. lapillus, G. honu, G. jejuensis, G. lewesii* and *G. holmesii* (Chinain et al. 1999, Litaker et al. 2009, Fraga and Rodriguez 2014, Nishimura et al. 2014, Rhodes et al. 2017, Jang et al. 2018, Kretzschmar et al. 2019). Regarding the genus *Fukuyoa*, only three species (*F. ruetzleri, F. yasumotoi* and *F. paulensis*) have been described (Holmes 1998, Litaker et al. 2009, Gómez et al. 2015). These species have been found mainly in tropical and subtropical areas, but also in temperate areas. Despite the wide distribution, there are zones where the diversity in terms of reported species is higher, such as the Canary Islands, coasts of the Caribbean and adjacent seas, and French Polynesia (Tester et al 2020). *Gambierdiscus* and *Fukuyoa* are all potential producers of bioactive compounds. In fact, in addition to CTXs (Yasumoto et al. 2000, Chinain et al. 2010) maitotoxins (MTXs) (Murata et al. 1993, Holmes and Lewis 1994, Pisapia et al. 2017b), gambieric acids (Nagai et al. 1992), gambierol (Satake et al. 1993), gambieroxide (Watanabe et al. 2013) and gambierone (Rodríguez et al. 2015, Murray et al. 2019) have also been detected in laboratory cultures of some species. Even if toxicity of these compounds on cell lines has been reported, it is not fully understood yet if they play a role in ciguatera intoxication (Kohli et al. 2015). During their accumulation through food webs, CTXs are often biotransformed and this may result in metabolites of higher toxicity than the algal parent compounds (Lehane and Lewis 2000, Ikehara et al. 2017). The CTXs profiles found in fish are determined by the *Gambierdiscus* and *Fukuyoa* species grazed by fishes, the congeners that these microalgae produce and the biotransformation processes occurring through the food web. Therefore, the oxidation of specific CTX algal precursors can lead

to species-specific and region-specific toxin profiles in fishes (Yogi et al. 2011, 2014). Thus, studies that aim to investigate toxic profiles of *Gambierdiscus* and *Fukuyoa* are extremely important not only to obtain fundamental knowledge about these genera, but also to understand and monitor the presence of CTXs in fishes and, more broadly, to better describe ciguatera intoxication and predict future outbreaks.

Several methods have been used to analyze *Gambierdiscus* or *Fukuyoa* species. The mouse bioassay (MBA) has been very useful during the first steps to identify CTXs and MTXs in microalgae but it has been demonstrated to lack sensitivity and specificity. As a consequence, other methods have been developed, including cell-based assays (CBAs), receptor binding assays (RBAs) and instrumental analysis techniques (*e.g.* liquid chromatography coupled to mass spectrometry, LC-MS/MS) (Reverté et al. 2014) CTXs have not been detected in all existing species of the genera *Gambierdiscus* and *Fukuyoa*, and even when the presence is confirmed, the contents are very low (few fg/cell) (Kohli et al. 2015). Nevertheless, the species *G. polynesiensis* and *G. excentricus* have shown, consistently over the years, a CTX-like toxicity significantly higher than other species, producing up to several pg/cell of CTX compounds (Chinain et al. 2010, Fraga et al. 2011, Rhodes et al. 2014, Litaker et al. 2017, Pisapia et al. 2017a, Sibat et al. 2018). Therefore, these two species are viewed as the most important CTXs producers in the Pacific and Atlantic Oceans, respectively.

Gambierdiscus and *Fukuyoa* cells are armored dinoflagellates with cellulose thecae difficult to disrupt. Therefore, a key point for the correct determination of the toxin content is the extraction procedure, which usually involves several purification steps to obtain a clean extract (Caillaud et al. 2010). The first step of this procedure is the intrinsic pellet extraction, which is performed in absolute methanol (Caillaud et al. 2011, Kretzschmar et al. 2017, Litaker et al. 2017, Munday et al. 2017, Pisapia et al. 2017a, Rhodes et al. 2017, Larsson et al. 2018), aqueous methanol (Roeder et al. 2010), or a combination of both (Chinain et al. 2010, Pawlowiez et al. 2013, Clausing et al. 2018, Reverté et al. 2018, Longo et al. 2019, Rossignoli et al. 2020), whilst Lewis et al (2016) extracted pellets with a methanol:water:hexane solution. To facilitate cell disruption, sonication is usually involved in the extraction process, through sonicator probes (Chinain et al. 2010, Lewis et al. 2016, Litaker et al. 2017, Pisapia et al. 2017a, Clausing et al. 2018, Larsson et al. 2018, Reverté et al. 2018, Rossignoli et al. 2020), or ultrasonic baths (Roeder et al. 2010, Rhodes et al. 2017, Longo et al. 2019), or, alternatively, the use of a bead beater (Pisapia et al. 2017a). According to the grade of purity needed, crude extracts have to

undergo a first purification step that usually involves liquid/liquid solvent partitioning to separate CTXs from MTXs (Chinain et al. 2010, Lewis et al. 2016, Litaker et al. 2017, Pisapia et al. 2017a, Pisapia et al. 2017b, Clausing et al. 2018). If the extracts are highly concentrated in biomass, further purification steps are needed prior to the analysis with LC-MS/MS. These steps include the use of chromatography, either Solid Phase Extraction (SPE) (Darius et al. 2007, Chinain et al. 2010, Rhodes et al. 2010) or High Performance Liquid Chromatography (HPLC) (Chinain et al. 2010). Evidently, this procedure is time consuming, involves the use of several reagents and instrumentation, and requires skilled personnel. Therefore, the development of more rapid, simpler and equally efficient techniques is desirable.

Recently, our group has developed an immunosensor for the detection of CTXs in fish samples (Leonardo et al. 2020). This technique involves the use of monoclonal antibodies (mAbs) showing high specificity and sensitivity for their CTX targets (Oguri et al. 1999, 2003 Nagumo et al. 2001, 2004, Tsumuraya et al. 2006, 2010, 2012, 2014, 2018), and their exploitation in a sandwich colorimetric immunoassay and electrochemical immunosensor on magnetic beads (MBs). Specifically, the 3G8 mAb has affinity for the left wing of CTX1B and 54-deoxyCTX1B (Tsumuraya et al. 2012), the 10C9 mAb for the left wing of CTX3C and 51-hydroxyCTX3C (Oguri et al. 2003), and the 8H4 mAb for the right wing of the four congeners(Tsumuraya et al. 2006) (Figure 1).

CTX1B : R = OH
54-deoxCTX1B : R = H

51-hydroxyCTX3C : R = OH
CTX3C : R = H

Figure 1. Schematic representation of the four CTXs congeners recognized by the antibodies used in this work.

In this work, the developed MB-based immunoassay and immunosensor have been exploited to investigate the CTXs production of nine *Gambierdiscus* strains belonging to three species (*G. australes, G. excentricus* and *G. caribaeus)* and four *Fukuyoa paulensis* strains (Figure 2). A rapid CTXs extraction protocol using a bead beater has been evaluated with the intent to accelerate the analytical process. Results have been compared to the CBA. The immunosensing tools provided a qualitative estimation and discrimination of two series of congeners (CTX1B and CTX3C) of these microalgal strains.

2. Experimental Section

2.1. Reagents and solutions

Dynabeads M-270 Carboxylic Acid (2×10^9 beads/mL) were supplied by Invitrogen (Life Technologies, S.A., Alcobendas, Spain). Potassium phosphate monobasic, potassium phosphate dibasic, potassium chloride, 4-morpholineethanesulfonic acid (MES) hydrate, N-(3-dimethylaminopropyl)-N′-ethylcarbodiimide hydrochloride (EDC), N-hydroxysuccinimide (NHS), Tween®-20, bovine serum albumin (BSA), and 3,3’,5,5’-tetramethylbenzidine (TMB) liquid substrate were purchased from Sigma-Aldrich (Tres Cantos, Spain). PolyHRP-streptavidin was obtained from Thermo Fisher (Barcelona, Spain). Milli-Q water (Millipore, Bedford, USA) was used to prepare solutions.

For the extractions, methanol was obtained from Honeywell (Barcelona, Spain). CTX1B standard solution was obtained from Prof. Richard J. Lewis (The Queensland University, Australia) 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. 3G8, 10C9 and 8H4 mAbs had been prepared by immunizing mice with keyhole limpet hemocyanine (KLH) conjugates of rationally designed synthetic haptens[53-61] Biotin labelling of the 8H4 mAb was performed with the EZ-Link™ NHS-PEG4 Biotinylation Kit from Thermo Fisher (Barcelona, Spain) following the manufacturer's instructions. Unreacted NHS-PEG4-Biotin was removed using Zeba Spin Desalting Columns (7 kDa MWCO, 2mL) included in the kit.

2.2. Equipment

A Bead Beater (BioSpec, Bartlesville, USA) was used for the extraction of CTXs. An Allegra X-15R (Beckman Coulter, Brea, USA) centrifuge was used to obtain the microalgal pellets and in the CTXs extraction after using the sonicator. An Eppendorf 5415D (Hamburg, Germany) centrifuge was used in the CTXs extraction after using the bead beater. Magnetic separation was performed using a MagneSphere Technology Magnetic Separation Stand (for 12 0.5-mL or 1.5-mL tubes) and a PolyATtract System 1000 Magnetic Separation Stand (for one 15-mL tube) from Promega Corporation (Madison, USA). Colorimetric measurements were performed with a Microplate Reader KC4 from BIO-TEK Instruments, Inc. (Vermont, USA). Gen5 software was used to collect and evaluate data. Arrays of eight screen printed carbon electrodes (DRP-8x110), a boxed connector (DRP-CAST8X) and a magnetic support (DRP-MAGNET8X) were purchased from Dropsens S.L. (Oviedo, Spain). The arrays consist of 8 carbon working electrodes of 2.5 mm in diameter, each with its own carbon counter electrode and silver reference electrode. Amperometric measurements were performed with a PalmSens potentiostat connected to an 8-channel multiplexer (MUX8) (Houte, The Netherlands). Data were collected and evaluated with the PalmSens PC software.

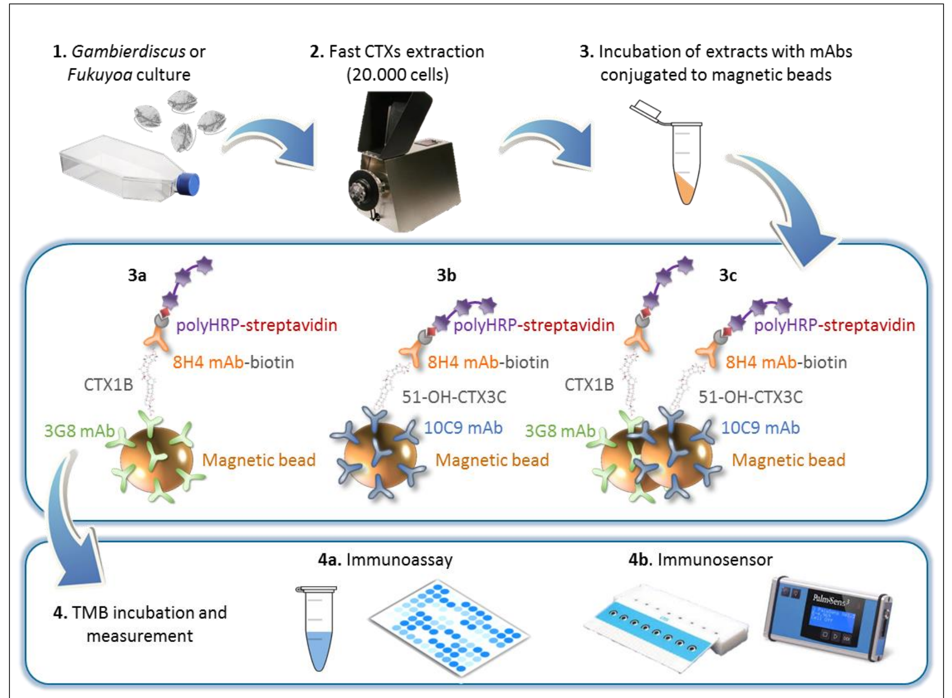

Figure 2. Schematic representation of the *Gambierdiscus* and *Fukuyoa* cultures, rapid CTXs extraction and subsequent analysis of the extracts with the MB-based colorimetric immunoassay and electrochemical immunosensor.

2.3. Microalgal cultures

Several *Gambierdiscus* ($N = 9$) and *Fukuyoa* ($N = 4$) strains were used: 1) from IRTA collection (*G. australes* IRTA-SMM-13_07; *F. paulensis* IRTA-SMM-17_206, IRTA-SMM-17_211 and IRTA-SMM-17_220); 2) from Culture Collection of Microalgae (CCVIEO) of the Instituto Español de Oceanografía in Vigo, Spain (*G. excentricus* VGO791; *F. paulensis* VGO1185); and 3) from a sampling performed in the Canary Islands, Spain, in September 2016 and 2017, and recently incorporated to IRTA collection (*G. australes* IRTA-SMM-16_286; *G. excentricus* IRTA-SMM-17_01, IRTA-SMM-17_126, IRTA-SMM-17_407, IRTA-SMM-17_428 and IRTA-SMM-17_432; *G. caribaeus* IRTA-SMM-17_03). In total, 2 *G. australes* strains, 6 *G. excentricus* strains, 1 *G. caribaeus* strain, and 4 *F. paulensis* strains were evaluated. For the sampling, macroalgae were collected, mixed with 1 L of seawater, vigorously shaken and filtered through a 200 µm mesh. Microalgal cells were isolated with a glass pipette following the capillary method (ikehara and Rosowski 1973) and cultivated, first in 24-well microplates and then in tissue culture polystyrene flasks.

All the clonal cultures were grown in ES medium (Provasoli 1968) containing filtered and autoclaved seawater from L'Ametlla de Mar, Spain, and adjusted to a practical salinity of 36. Cultures were maintained at 24 ± 0.5 °C under a photon flux rate of 100 µmol m^{-2} s^{-1} with a 12:12 h light:dark regime. Culture aliquots were fixed with 3% Lugol's iodine and counted following the Sedgwick-Rafter method (Greeson 1977) using a Kolkwitz chamber (Hydro-Bios, Altenholz, Germany) under an inverted light microscope (Leica DMIL, Spain). All the cultures were collected at the exponential phase (*ca.* 21 days). Pellets containing 10^4 cells were prepared by centrifugation (3200 g, 20 min) and stored at −20 °C until CTXs extraction. Additionally, pellets of strains from the sampling were prepared and stored at −20 °C for subsequent DNA extraction.

Extraction of genomic DNA was performed using a bead beating system and the phenol/chloroform/isoamylalcohol method (Toldrà et al 2018). Extracted DNA samples (50 µL) were quantified and checked for their purity using a NanoDrop 2000 spectrophotometer (Thermo Scientific). The D8-D10 domain of the 28S rDNA gene was amplified using PCR and the pair of primers FD8/RB (5-GGATTGGCTCTGAGGGTTGGG-3/5 GATAGGAAGAGCCGACATCGA-3) (Chinain et al 1999). 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 2 µL of template DNA (10–50 ng). Amplifications were carried out in a Nexus Gradient Thermal Cycler (Eppendorf, Spain) and included 45 cycles of amplification following a three-step protocol (95 °C for 30 s, 60 °C for 45 s and 72 °C for 30 s). Each PCR reaction was checked by agarose gel electrophoresis. PCR products of ~950 bp were purified with QIAquick PCR Purification Kit and bidirectionally sequenced (Sistemas Genómicos, LLC, Valencia, Spain). Forward and reverse sequence reads were edited using BioEdit v7.0.5.2 (Hall 1999) to create consensus sequences for each strain. Sequences were aligned using MAFFT v.7 (Rozewicki et al. 2019). The phylogenetic relationships were inferred by Maximum Likelihood (ML) using RaxML v.8 (Stamatakis 2014) and Bayesian Inference (BI) using Mr. Bayes v.3.2.2 (Huelsenbeck and Ronquist 2001). Sequences were deposited in GenBank (Table S1).

2.4. Ciguatoxins (CTXs) extraction

Toxin extraction was performed comparing the use of a sonicator (Reverté et al., 2018) and a bead beater. The sonicator protocol was as follows: 1) two pellets of 10^4 cells were pooled together in a 15-mL tube using 5 mL of MeOH; 2) sonication was conducted for 15 min at 38% of amplitude 3 sec on/2 sec off using a 3 mm diameter sonicator probe (Watt ultrasonic processor VCX 750

(Newton, USA); 3) the extract was centrifuged (3200 g, 10 minutes), transferred to a new tube and dry-blown under N_2 gas at 40 °C; 4) 5 mL of MeOH were added to the first tube and steps 2 and 3 were repeated twice (transferring the supernatants to the tube with the already evaporated extract). The bead beater protocol was as follows: (1) two pellets of 10^4 cells were pooled together into a 2-mL screw-cap cryotube containing ~50 µg of 0.5 mm diameter zirconium glass beads using 1 mL of MeOH; (2) bead beating was conducted for 3 or 6 runs of 40 s each; (3) the extract was centrifuged (3700 g, 1 min), transferred to a glass vial and dry-blown under N_2 gas at 40 °C. Dried extracts were stored at -20 °C until analysis.

2.5. Cell-based assay (CBA)

The CBA was performed as previously described (Diogène et al. 2017). Briefly, neuro-2a (N2a) cells (ATCC, CCL131) were seeded in a 96-well microplate in 200 µL of RPMI medium containing 5% *v/v* fetal bovine serum (RPMI-FBS) at 42,500 cells per well, and incubated under a 5% CO_2 humid atmosphere for 24 h at 37 °C. Prior to exposure to CTX1B standard solution or microalgal extract, some N2a cells were pre-treated with ouabain and veratridine at 1 and 0.1 mM, respectively. CTX1B standard solution or microalgal extract were dried, reconstituted in 200 µL of RPMI-FBS medium, 1/2 serially diluted (from 575.0 to 4.5 pg/mL for CTX1B standard solution and from 90.000 to 11.250 cells/mL for microalgal extract), and 10 µL were added to the wells with and without ouabain/veratridine pre-treatment (no pre-treatment used as a control to evaluate matrix effects). After 24 h, cell viability was measured using the MTT assay (Manger et al. 1993). Measurements were performed in triplicate.

2.6. Colorimetric immunoassay and electrochemical immunosensor

Analyses were performed following our previous protocol (Leonardo et al. 2020) with some modifications. Briefly, 8 µL of MB suspension were transferred to a tube and activated by incubation with 40 µL of 50 mg/mL EDC and 40 µL of 50 mg/mL NHS (in 25 mM MES, pH 5.0) for 30 min. Subsequently, 80 µL of antibodies (3G8 or 10C9 mAb at 1/50 dilution in MES) were incubated for 1 h. The mAb-MB conjugates were washed, re-suspended in 80 µL of PBS-Tween (0.1 M PBS, 0.05% *v/v* Tween®-20, pH 7.2) and transferred into new tubes either separately (75 µL of conjugate) or mixed together (150 µL containing 75 µL of each conjugate). After supernatant removal, 75 µL of microalgal extract (evaporated extract resuspended in 250 µL of PBS-Tween), CTX standard (CTX1B or 51-OH-CTX3C) or both (for the spiking experiment) were added to the tube and incubated for 30 min. From this step on, the protocol of our previous work was followed without any change.

At first, a blocking step was performed with PBS-Tween-BSA. Then, the conjugates were incubated first with 75 µL of biotin-8H4 mAb and afterwards with 75 µL of polyHRP-streptavidin. All the incubations lasted for 30 min, were performed at room temperature with slow tilt rotation, and three washings with PBS-Tween were performed between each step. Finally, immunocomplexes were washed and re-suspended in 75 µL of PBS-Tween. For the analysis two different procedure were followed, for the colorimetric immunoassay: 10 µL of immunocomplexes were transferred to a new tube, the supernatant was removed and 125 µL of TMB were incubated for 10 min. Then, 100 µL of solution were taken for absorbance reading at 620 nm. Measurements were performed in triplicate. Instead, for the electrochemical immunosensor: 10 µL of immunocomplexes were placed on each working electrode of the 8-electrode array, the supernatant was removed and 10 µL of TMB were incubated for 2 min; the TMB reduction current was measured using amperometry (−0.2 V (*vs.* Ag) for 5 s). Measurements were performed in quadruplicate.

2.7. Statistical analysis

Multivariate analysis of variance (two-way MANOVA) was first used to analyze differences in CTXs quantifications between the immunoassay and the immunosensor and among strains of different species. MANOVA is used when several dependent variables are measured on each sampling unit instead of only one variable (for more details, see Suarez-Serrano et al. 2010 and Rovira et al.2012). Significances were further explored with two-way analysis of variance (ANOVA). In addition to *P* values, the partial eta squared (η_p^2) was used as a measure of effect size (*i.e.* importance of factors). Similar to regression coefficient (r^2), η_p^2 is the proportion of variation explained for a certain effect, and has the advantage over eta squared of not depending on the number of sources of variation used in the ANOVA, thus it could be compared among different designs (Tabachnick et al. 2007). In contrast to *P* value, η_p^2 has the advantage that allows the proper comparison of treatments (*e.g.* a lower *P* value does not necessarily mean that a factor has stronger effect; Alcaraz et al. 2008). Adjusted (or marginal) means of a dependent variable are the means for each level of the factor, and were used to describe the differences among strains and quantification tools. All statistical analyses were performed with SPSS 25.0.

3. Results

3.1. Ciguatoxins (CTXs) extraction

To demonstrate the efficiency of the bead beater protocol to extract CTXs, pellets from a culture of the strain *G. excentricus* IRTA-SMM-17_428 were

extracted with two different bead beater settings and also with sonication as a reference method. CTXs extraction was evaluated using CBA (IC_{50} = 0.90 pg/mL, IC_{80} (limit of detection, LOD) = 0.40 pg/mL). Each extraction was performed in duplicate, and each extract was also analyzed in duplicate with the CBA. Observation of the cells under the light microscope indicated that all the protocols caused cell lysis (results not shown). As can be seen in Figure 3, the different techniques resulted in similar CTXs extraction yields, and no differences were observed between performing 3 or 6 bead beater runs. The bead beater protocol can be considered the most suitable for CTXs extraction because it is more rapid and simpler. Indeed, the time required for the toxin extraction from microalgal pellets is as low as 2 min in comparison to the 60 min used by Pisapia and coworkers (2017a) or the 45 min required with sonication. Additionally, evaporation of the 15 mL of MeOH required for the sonication protocol takes longer than the evaporation of the 1 mL used with the bead beater. Furthermore, using a bead beater it is possible to extract up to eight samples at the same time, whereas the sonicator can extract only one sample at a time. Therefore, for subsequent experiments, samples were extracted using 3 bead beater runs.

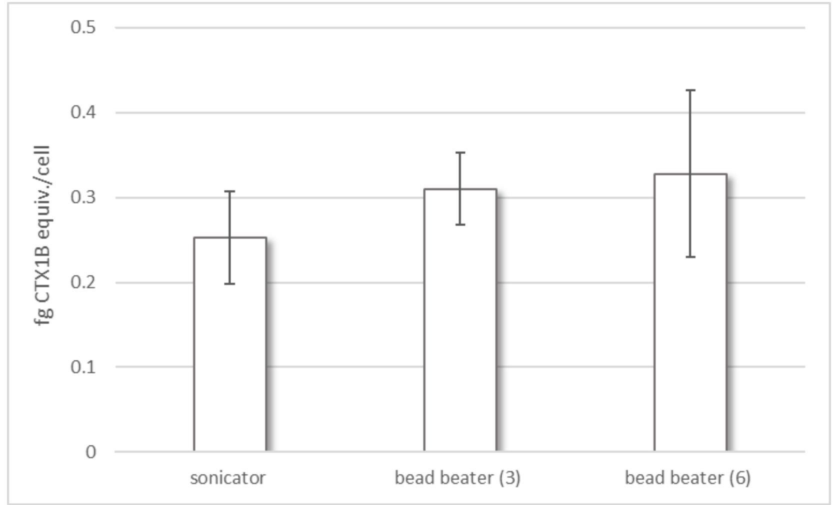

Figure 3. CTXs extracted (fg CTX1B equiv./cell) from *G. excentricus* IRTA-SMM-17_428 using sonicator, bead beater 3 times and bead beater 6 times, and evaluated with CBA.

3.2. Colorimetric immunoassay and electrochemical immunosensor

Microalgal extracts from *Gambierdiscus* and *Fukuyoa* cultures were analyzed using the colorimetric immunoassay, the electrochemical immunosensor and the CBA (Figure 4 and Table S2). Regarding the analysis with the immunoassay and the immunosensor, both approaches should be able to detect at least four congeners among CTXs: CTX1B, 54-deoxyCTX1B, CTX3C and 51-

hydroxyCTX3C. This is due to the ability of 3G8 mAb to bind to the left wing of CTX1B and 54-deoxyCTX1B (Tsumuraya et al. 2012), of 10C9 mAb to bind to the left wing of CTX3C and 51-hydroxyCTX3C (Oguri et al. 2003), and of 8H4 mAb to bind to the right wing of the four congeners (Tsumuraya et al. 2006). Whereas in our previous work both capture antibodies (3G8 and 10C9) were used together to analyze fish extracts (Leonardo et al. 2020), thus providing a global response, in this study they have also been used separately to obtain an estimation of the amount of CTX1B or CTX3C series of congeners of several *Gambierdiscus* and *Fukuyoa* strains. Therefore, quantifications are expressed in fg/cell of CTX1B equiv. when the 3G8 mAb was used alone, in fg/cell of 51-OH-CTX3C equiv. when the 10C9 mAb was used alone, and in fg/cell of CTX1B equiv. and 51-OH-CTX3C equiv. when the two antibodies were used together. Regarding the CBA, where the CTXs recognition principle is based on a toxicological effect instead of a structural affinity, quantifications are expressed in fg/cell of CTX1B equiv.

Analyses with the immunoassay and the immunosensor revealed the presence of CTXs in 11 out of 13 extracts (all except for IRTA-SMM-13_07 and IRTA-SMM-17_211). In general terms, as expected, the CTXs contents determined when using two capture antibodies were higher than when using only one. This is certainly explained by the presence of the two different series of congeners, even if one of them was not detected separately because of the LOD of the method. It is also important to note that although in some cases the immunoassay showed higher CTXs contents, the immunosensor was able to detect the presence of CTXs in samples where the immunoassay was not capable. This can be attributed to the lower LODs of the immunosensor (1.96 and 3.59 pg/mL compared to 3.29 and 6.17 pg/mL, for CTX1B and 51-OH-CTX3C respectively (Leonardo et al. 2020).

In order to evaluate the matrix effect, an experiment was performed, where CTX1B and 51-OH-CTX3C standard solutions (at 100 pg/mL) were spiked into the extracts that were negative by the immunoassay, the immunosensor and CBA (IRTA-SMM-13_07 and IRTA-SMM-17_211). Results showed practically no matrix effects in both the immunoassay (87% and 86% CTX1B recovery and 100% and 87% 51-OH-CTX3C recovery for IRTA-SMM-13_07 and IRTA-SMM-17_211, respectively) and the immunosensor (97% and 89% CTX1B recovery and 87% and 102% 51-OH-CTX3C recovery for IRTA-SMM-13_07 and IRTA-SMM-17_211, respectively).

The results obtained show the predominance of CTX1B congeners in 4 out of 6 *G. excentricus* strains (IRTA-SMM-17_126, IRTA-SMM-17_407, IRTA-SMM-17_428 and IRTA-SMM-17_432), ranging from 0.06 to 0.77 fg/cell of

CTX1B equiv., and 1 out of 4 *F. paulensis* strains (VGO1185) (0.27-0.33 fg/cell of CTX1B equiv.). In these strains, CTX3C congeners were not detected, or only at very small amounts (0.01-0.04 fg/cell of 51-OH-CTX3C equiv.). Interestingly, 2 out of 4 *F. paulensis* strains (IRTA-SMM-17_206 and IRTA-SMM-17_220) revealed the presence of CTXs only when both capture antibodies were used together in the immunoassay (although due to the lower LODs obtained with the immunosensor, it was able to detect very low amounts in one of them when using the antibodies separately) and 1 out of 4 (IRTA-SMM-17_211) did not show any presence of CTXs at all. On the contrary, CTX3C congeners were the unique or most abundant in 1 out of 2 *G. australes* strains (IRTA-SMM-16_286, 0.16-0.37 fg/cell of 51-OH-CTX3C equiv. in front of 0.04 fg/cell of CTX1B). In the other *G. australes* strain (IRTA-SMM-13_07), no CTXs were detected. CTX3C congeners were also predominant in 2 out of 6 *G. excentricus* strains (IRTA-SMM-17_01 and VGO791), ranging from 0.16 to 0.54 fg/cell of 51-OH-CTX3C equiv. Regarding the *G. caribaeus* strain (IRTA-SMM-17_03), equal amounts of both CTX congeners were detected (although slightly different depending on the immunosensing tool that was used).

Microalgal extracts were also screened with CBA, in order to compare the presence of CTXs detected with the immunosensing tools with the toxicity. As mentioned above, it must be considered that, even if all the tests have the objective to assess the presence of CTX congeners, their detection principle is different, and so results can differ between them. CTX-like activity was only detected in 4 out of 13 strains (IRTA-SMM-17_407, IRTA-SMM-17_428, IRTA-SMM-17_432 and VGO791) with the CBA, all belonging to the species *G. excentricus*.

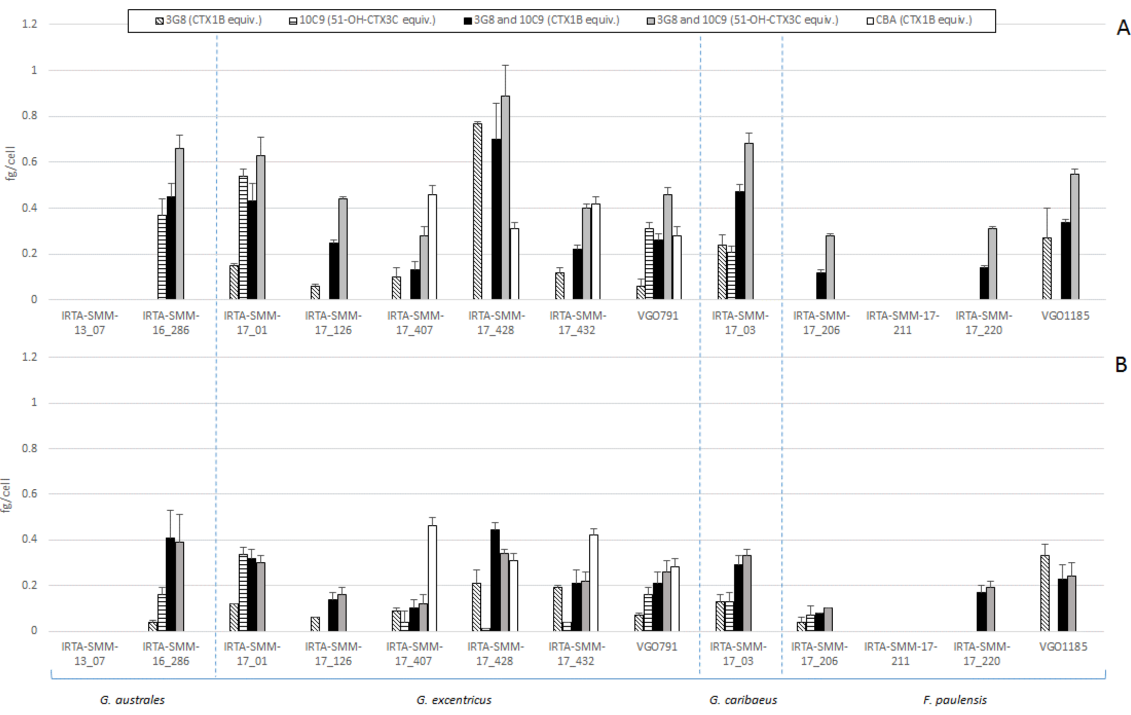

Figure 4. CTXs (fg/cell) extracted from different *Gambierdiscus* and *Fukuyoa* strains using the bead beater protocol, and evaluated with the colorimetric immunoassay (A) and the electrochemical immunosensor (B). CBA results are in both A and B for comparison purposes. Dashed lines separate genera and species.

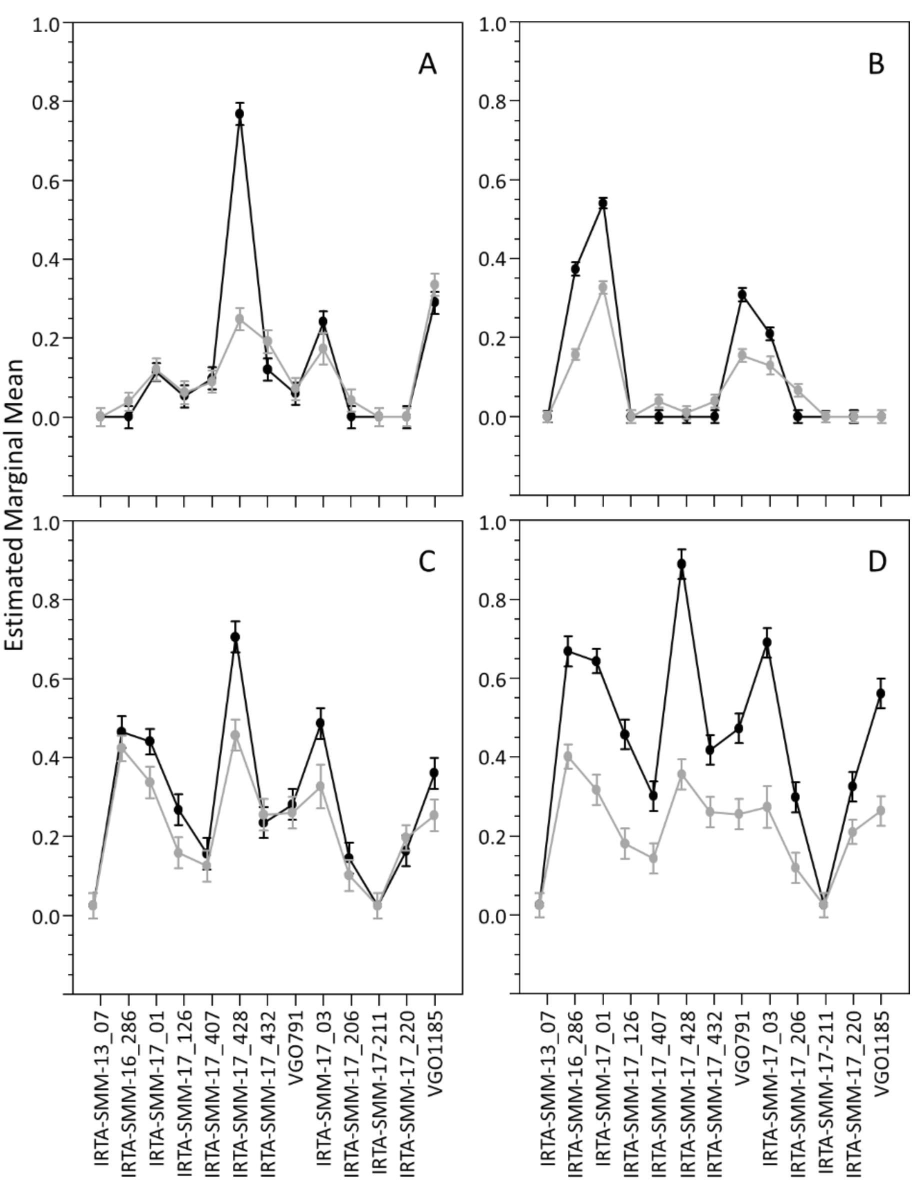

Figure 5. ANOVA adjusted means of CTXs quantifications for *Gambierdiscus* and *Fukuyoa* strains when using the 3G8 mAb and providing the results in CTX1B equiv. (A), when using the 10C9 mAb and providing the results in 51-OH-CTX3C equiv. (B), when using both mAbs and providing the results CTX1B equiv. (C), and when using both mAbs and providing the results 51-OH-CTX3C equiv. (D). Black dots refer to the results obtained with the immunoassay. Grey dots refer to the results obtained with the immunosensor.

4. Discussion

There is a general lack of studies about toxic profiles of *Gambierdiscus* and *Fukuyoa*, probably due to the complexity of the compounds produced by these genera. Furthermore, cultivating microalgae at a scale large enough to obtain a high cell abundance requires time and space. The subsequent pellet extraction is not straightforward either. In addition, there is a lack of CTXs standards, and the available ones are extremely costly. Regardless, in the past decade, some studies have focused on the identification of CTXs in different strains of *Gambierdiscus* and *Fukuyoa*.

Several *G. australes* strains have been identified as CTXs producers using RBA (17 to 30 fg/cell of CTX3C equiv.) and LC-MS/MS (Roeder et al. 2010). Lewis and coworkers (Lewis et al. 2016) used a CBA with human neuroblastoma cells to assess the CTX-like activity of a *G. australes* strain without finding any toxicity. Subsequently, Pisapia and coworkers (Pisapia et al. 2017a) identified other *G. australes* strains as CTX3C equiv. producers (from 0.6 to 2.7 fg/cell) using CBA. CBA was again used to screen other *G. australes* strains, in which the presence of CTX1B equiv. ranging from 200 up to 679 fg/cell (Reverté et al. 2018) and from 31.1 to 107.16 fg/cell (Rossignoli et al. 2020) was demonstrated. In the present study, CTXs contents are lower and one strain did not show toxicity at all. Discrepancies can arise due to different reasons including, for instance, the age of the culture. In fact, laboratory cultures seem to decrease their ability to produce toxins with time, as recorded for one of the *G. australes* strains (IRTA-SMM-13_07), which is the same as that used in Reverté et al. (2018).

Owing to its high toxicity, of *ca.* 1000 fg/cell of CTX1B equiv., as observed using a CBA, *G. excentricus* has also attracted attention of researchers worldwide (Fraga et al. 2011). CBA was also used to determine the CTX-like activity in the study of Pisapia and coworkers (Pisapia et al. 2017a), providing similar results (*ca.* 1400 fg/cell of CTX3C equiv.). Other works have reported quantifications of 469 fg/cell of CTX3C equiv. (Litaker et al. 2017) and from 128.2 up to 510.6 fg/cell of CTX1B equiv. (Rossignoli et al. 2020) in *G. excentricus* strains, also with CBA. It must be underlined that in all these studies, *G. excentricus* strains were identified as the most toxic among other *Gambierdiscus* species. In the current work, the *G. excentricus* strain VGO 791 showed lower CTXs contents than in previous works (Fraga et al. 2011, Pisapia et al. 2017a), again probably due to the age of the culture and the growth conditions. Nevertheless, even if not all the *G. excentricus* strains registered the highest CTXs contents, all of them showed the presence of CTXs (unlike *G. australes* and *F. paulensis*, for which some of them did not), and the strain

that showed the highest CTXs contents belongs to the species *G. excentricus*. Additionally, CBA also only showed CTX-like activity in some of the *G. excentricus* strains. These results again place this species among the most toxic known to date, and one of the most important to monitor.

Gambierdiscus caribaeus strains have also been screened for toxicity with CBA, obtaining no CTX-like activity (Lewis et al. 2016), 1.6 fg/cell of CTX3C equiv. (Pisapia et al. 2017a), 0.66 fg/cell of CTX13C equiv. (Litaker et al. 2017) and 2.59 fg/cell of CTX1B equiv. (Rossignoli et al. 2020). CTXs quantifications obtained in the current work are close to these values. Here, the laboratory culture for this species is much younger compared to that of *G. australes*, and probably its ability for toxin production has not yet changed in response to the artificial growth conditions.

When considering the genus *Fukuyoa*, the lack of studies is even more evident. The genus *Fukuyoa* was split from *Gambierdiscus* in 2015, when molecular and morphologic criteria from two *Gambierdiscus* species *(G. yasumotoi* and *G. ruetzleri*) were used to define this new genus (Gómez et al. 2015). Therefore, the *G.* cf *yasumotoi* identified as non-toxic in Rhodes et al. (2014) is a *Fukuyoa* species. Subsequent studies on *F. paulensis* did not present any CTX-like activity either (Gómez et al. 2015, Munday et al. 2017, Larsson et al. 2019), with the exception of the work of Laza-Martínez and coworkers (Laza-Martínez et al. 2016) where one *F. paulensis* strain (Dn135EHU) was identified as a 54-deoxy-CTX1B producer by LC-HRMS. *Fukuyoa paulensis* cultures analyzed in our work showed the presence of CTX congeners in the majority of the strains (even in the VGO1185 strain, reported as negative in Gomez et al. 2015), confirming the potential hazard of the genus *Fukuyoa*.

The CTXs production of Gambierdiscus and Fukuyoa is quite complex to evaluate. As it emerges from the literature, CBA has been the most commonly used tool to analyze microalgal extracts. Our results with CBA showed that CTX quantification has been possible only in 4 strains, whereas the immunosensing tools have detected CTXs in 11 out of 13. This can be attributed to the different recognition principles and interfering compounds. Whereas in the immunosensing tools, the detection is based on a structural affinity between CTXs and antibodies, in the CBA, CTXs bind to the voltage-gated sodium channels (VGSCs) of cells and block them, in an open state, causing a toxic effect. Even if in this work the CBA did not show any matrix effects and its LOD was as low as 0.4 pg/mL of CTX1B equiv. (lower than the LODs of the immunosensing tools), the detection of CTXs could be hidden by the presence of other *Gambierdiscus* or *Fukuyoa* toxic compounds. In fact, MTX is known to interfere in the CBA if no additional treatments are performed, but it is not

recognized by the antibodies. Specificity of antibodies has always been a crucial issue for their applicability. Whereas the first immunoassays for CTXs showed cross-reactivity towards other marine toxins such as okadaic acid (Hokama et al. 1992, Campora et al. 2006), this problem was overcome by the production of more specific antibodies (Campora et al. 2008a). The use of these new antibodies in the analysis of fish extracts significantly improved the correlations with other techniques (Campora et al. 2008b, 2010). The antibodies used in the current work are highly specific and do not cross-react with the marine toxins brevetoxin A, brevetoxin B, okadaic acid and MTX (Tsumuraya et al. 2014). Additionally, two different capture antibodies are used, thus the system is able to detect a higher number of CTXs congeners than in other works where only one antibody is used. It is also important to note that this is the first time that immunochemical approaches have been applied to the analysis of microalgae.

Interestingly, for the 4 strains where CTX-like activity was detected, quantifications obtained using the CBA and immunosensing tools were in the same order of magnitude. Instead, in our previous work (Leonardo et al. 2020), quantifications obtained for fish extracts using CBA were around one order of magnitude higher. These results suggest that whereas the CTXs congeners that are found in microalgal extracts may mostly belong to the two series of CTX congeners detectable by immunosensing tools, the CTXs congeners in fish may have undergone biotransformation processes.

It is necessary to be aware that we cannot rule out the possibility that the microalgal extracts contain other CTX congeners different from the four targets of this study (*i.e.* with different wings) that may go unnoticed by the immunosensing tools. Instead, LC-MS/MS technique is able to discriminate among all the CTX congeners, provided a standard is available. However, instrumental analysis techniques are strongly affected by the matrix effect caused by other compounds produced by *Gambierdiscus* and *Fukuyoa* (especially MTX), and so, as mentioned above, the extract has to undergo several purifications steps prior to the analysis. These steps usually cause toxin losses during the process. Consequently, pellets with high cell abundances are required to perform this analysis, and obtaining such cultures is costly in terms of time and space. Another important issue here is that whereas the immunosensing tools, like the CBA, provide a global response, LC-MS/MS detects individual CTX congeners. Therefore, the tools of this work may be more useful for the analysis of multi-toxin samples where each CTX congener is at a low concentration.

One particularity of our study is the low concentration of cells that have been used for toxin extraction and analysis (20,000 cell/mL), compared to the concentration in the order of 1,000,000 cell/mL used in the majority of other studies. In fact, during a bloom in the Canary Islands, the concentration of *Gambierdiscus* spp. was estimated to reach 10^4 cells g^{-1} wet weight (Soler-Onís et al. 2016). Therefore, the fact that the immunosensing tools are able to detect CTXs at such low cell abundances makes them suitable for the analysis of field samples. Additionally, the protocol used herein for the rapid CTXs extraction requires very simple instrumentation, which can be portable and thus appropriate for *in situ* analysis. The results demonstrate that the bead beater protocol is suitable for CTXs extraction, considerably reducing time and costs, since it is possible to extract up to eight samples in 2 min.

More and more *Gambierdiscus* species are being found in non-endemic regions, such as *G. australes* that has been recently identified in the Balearic waters (Tudó et al. 2018). Whether this is due to an actual increase in their worldwide expansion, or because there are better tools to detect them, global warming will certainly act in favor of their proliferation. Therefore, there is a clear necessity for tools that can detect toxins from extracts with low abundance of microalgal cells, and they must be reliable, rapid, inexpensive and easy to use. The analysis obtained with the immunosensing tools will not only provide information regarding the ecology of *Gambierdiscus* and *Fukuyoa* genera, but will also be important for the socioeconomy and human health, with the ability to predict an intoxication outbreak, facilitating the avoidance of long-term neurological diseases and human fatalities related to ciguatera.

5. Conclusions

This study examined nine *Gambierdiscus* strains belonging to three species (*G. australes, G. excentricus* and *G. caribaeus)* and four *Fukuyoa paulensis* strains. Microalgal extracts were obtained using a rapid and efficient CTXs extraction protocol and analyzed with an immunoassay and an immunosensor, which used MBs for the immobilization of antibodies. The unique features of this study are the ability to discriminate between two series of CTX congeners, giving more information on the toxic profile of *Gambierdiscus* and *Fukuyoa* species, the absence of interferences from non-structurally related compounds, and the high sensitivity of the immunosensing tools used for CTXs detection, which has avoided the requirement of large-scale cultures.

The approach presented in this work can be included in the group of methods ready to be used for ciguatera management (such as CBA, LC-MS/MS and RBA), providing a better understanding of CTXs production in the genera

Gambierdiscus and *Fukuyoa*. The use of the immunosensing tools can open the way for regional and international comparative studies on the CTXs production of those genera and, consequently, on ciguatera as an expanding phenomenon.

Acknowledgments

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Supplementary material

Table S1. *Gambierdiscus* and *Fukuyoa* strains used in this study.

Strain	Species	Sampling location and year	Sampling point	Source	GenBank accession number	Sequenced region
IRTA-SMM-13_07	*G. australes*	SGI, Portugal, 2013	30°8’18.00” N, 15°52’4.20” W	Reverté et al. 2018	KY564320	D1-D3
IRTA-SMM-16_286	*G. australes*	Lanzarote, Spain, 2016	28°54’56.48” N, 13°42’38.20” W	This study	MT119197	D8-D10
IRTA-SMM-17_01	*G. excentricus*	Gran Canaria, Spain, 2017	28°9’14.52” N, 15°41’58.78” W	This study	MT119198	D8-D10
IRTA-SMM-17_126	*G. excentricus*	Gran Canaria, Spain, 2017	28°6’24.12” N, 15°42’40.14” W	This study	MT119199	D8-D10
IRTA-SMM-17_407	*G. excentricus*	La Gomera, Spain, 2017	28°4’57.99” N, 17°19’56.00” W	This study	MT119200	D8-D10
IRTA-SMM-17_428	*G. excentricus*	La Gomera, Spain, 2017	28°4’57.99” N, 17°19’56.00” W	This study	MT119201	D8-D10
IRTA-SMM-17_432	*G. excentricus*	La Gomera, Spain, 2017	28°4’57.99” N, 17°19’56.00” W	This study	MT119202	D8-D10
VGO 791	*G. excentricus*	Tenerife, Spain, 2004	28°50’2.40” N, 16°49’8.34” W	Fraga et al. 2011	JF303066; JF303075	D1-D3; D8-D10

IRTA-SMM-17_03	*G. caribaeus*	El Hierro, 2017	27°49’26.48” N, 17°53’42.70” W	This study	MT119203	D8-D10
IRTA-SMM-17_206	*F. paulensis*	Mallorca, 2017	39°25’6.43” N, 3°16’15.55” E	Submitted work	MT119204	D8-D10
IRTA-SMM-17_211	*F. paulensis*	Menorca, 2017	39°58’54.18” N, 3°50’3.47” E	Submitted work	MT119205	D8-D10
IRTA-SMM-17_220	*F. paulensis*	Menorca, 2017	39°55’3.13” N, 4°1’51.18” E	Submitted work	MT119206	D8-D10
VGO 1185	*F. paulensis*	Ubatuba, Brazil	23°30’3.09” S, 45°7’7.32” W	Gómez et al., 2015	KM886379	18S; D1-D4; ITS

(Continues from previous page)

SGI: Selvagem Grande Island

Table S2. CTXs (fg/cell) extracted from different *Gambierdiscus* and *Fukuyoa* strains using the bead beater protocol, and evaluated with the colorimetric immunoassay, the electrochemical immunosensor and the CBA.

Species	Strain	3G8 (CTX1B equiv.)		10C9 (51-OH-CTX3C equiv.)		3G8 and 10C9 (CTX1B equiv.)		3G8 and 10C9 (51-OH-CTX3C equiv.)		CBA (CTX1B equiv.)
		Immunoassay	Immunosensor	Immunoassay	Immunosensor	Immunoassay	Immunosensor	Immunoassay	Immunosensor	
G. australes	IRTA-SMM-13_07	nd	nd	nd	nd	nd	nd	nd	nd	nd
	IRTA-SMM-16_286	nd	0.04 ± 0.01	0.37 ± 0.07	0.16 ± 0.03	0.45 ± 0.06	0.41 ± 0.12	0.66 ± 0.06	0.39 ± 0.12	nd
G. excentricus	IRTA-SMM-17_01	0.15 ± 0.01	0.12 ± 0.00	0.54 ± 0.03	0.34 ± 0.03	0.43 ± 0.08	0.32 ± 0.04	0.63 ± 0.08	0.30 ± 0.03	nd
	IRTA-SMM-17_126	0.06 ± 0.01	0.06 ± 0.00	nd	nd	0.25 ± 0.01	0.14 ± 0.03	0.44 ± 0.01	0.16 ± 0.03	nd
	IRTA-SMM-17_407	0.10 ± 0.04	0.09 ± 0.01	nd	0.04 ± 0.05	0.13 ± 0.04	0.10 ± 0.04	0.28 ± 0.04	0.12 ± 0.04	0.46 ± 0.04
	IRTA-SMM-17_428	0.77 ± 0.01	0.21 ± 0.06	nd	0.01 ± 0.00	0.70 ± 0.16	0.44 ± 0.03	0.89 ± 0.14	0.34 ± 0.02	0.31 ± 0.03
	IRTA-SMM-17_432	0.12 ± 0.02	0.19 ± 0.01	nd	0.04 ± 0.00	0.22 ± 0.02	0.21 ± 0.06	0.40 ± 0.02	0.22 ± 0.04	0.42 ± 0.03
	VGO791	0.06 ± 0.03	0.07 ± 0.01	0.31 ± 0.03	0.16 ± 0.03	0.26 ± 0.03	0.21 ± 0.05	0.46 ± 0.03	0.26 ± 0.05	0.28 ± 0.04
G. caribaeus	IRTA-SMM-17_03	0.24 ± 0.04	0.13 ± 0.03	0.21 ± 0.02	0.13 ± 0.04	0.47 ± 0.03	0.29 ± 0.04	0.68 ± 0.05	0.33 ± 0.03	nd
F. paulensis	IRTA-SMM-17_206	nd	0.04 ± 0.02	nd	0.07 ± 0.04	0.12 ± 0.01	0.08 ± 0.00	0.28 ± 0.01	0.10 ± 0.00	nd
	IRTA-SMM-17_211	nd	nd	nd	nd	nd	nd	nd	nd	nd
	IRTA-SMM-17_220	nd	nd	nd	nd	0.14 ± 0.01	0.17 ± 0.03	0.31 ± 0.01	0.19 ± 0.03	nd
	VGO1185	0.27 ± 0.13	0.33 ± 0.05	nd	nd	0.34 ± 0.01	0.23 ± 0.06	0.55 ± 0.02	0.24 ± 0.06	nd

nd: not detected

References

Alcaraz C, Pou-Rovira Q, and García-Berthou E (2008). Use of a flooded salt marsh habitat by an endangered cyprinodontid fish (*Aphanius iberus*). Hydrobiologia 600:177-185.

Begier EM, Backer LC, Weisman RS, Hammond RM, Fleming LE, Blythe D (2006). Outbreak bias in illness reporting and case confirmation in ciguatera fish poisoning surveillance in south Florida. Public Health Rep 121:658-665.

Caillaud A, de la Iglesia P, Darius HT, Pauillac S, Aligizaki K, Fraga S, Chinain M, Diogène J (2010). Update on methodologies available for ciguatoxin determination: perspectives to confront the onset of ciguatera fish poisoning in Europe. Mar Drugs 8:1838-1907.

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

Campora CE, Hokama Y, Ebesu JS. (2006). Comparative analysis of purified Pacific and Caribbean ciguatoxin congeners and related marine toxins using a modified ELISA technique. J Clin Lab Anal 20:121-125

Campora CE, Hokama Y, Yabusaki K, Isobe M (2008a). Development of an enzyme-linked immunosorbent assay for the detection of ciguatoxin in fish tissue using chicken immunoglobulin Y. J Clin Lab Anal 22:239-245.

Campora CE, Dierking J, Tamaru CS, Hokama Y, Vincent D (2008b). Detection of ciguatoxin in fish tissue using sandwich ELISA and neuroblastoma cell bioassay. . J Clin Lab Anal 22:246-253.

Campora CE, Dierking J, Tamaru CS, Hokama Y, Anderson B, Vincent D (2010). Evaluating the risk of ciguatera fish poisoning from reef fish grown at marine aquaculture facilities in Hawai'i. J World Aquac Soc 41:61-70.

Chinain M, Faust MA, Pauillac S (1999). Morphology and molecular analyses of three toxic species of *Gambierdiscus* (Dinophyceae): *G. pacificus*, sp. nov., *G. australes*, sp. nov., and *G. polynesiensis*, sp. nov. J Phycol 35:1282-1296.

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

Clausing RJ, Losen B, Oberhaensli FR, Darius HT, Sibat M, Hess P, Swarzenski PW, Chinain M, Dechraoui-Bottein MY (2018). Experimental evidence of dietary ciguatoxin accumulation in an herbivorous coral reef fish. Aquat Toxicol 200:257-265.

Darius H, Ponton D, Revel T, Cruchet P, Ung A, Fouc MT, Chinain M (2007). Ciguatera risk assessment in two toxic sites of French Polynesia using the receptor-binding assay. Toxicon 50:612-626.

Diogène J, Reverte L, Rambla-Alegre M, Del Rio V, de la Iglesia P, Campàs M, Palacios O, Flores C, Caixach J, Ralijaona C, Razanajatovo I, Pirog A, Magalon H, Arnich N, Turquet J (2017). Identification of ciguatoxins in a shark involved in a fatal food poisoning in the Indian Ocean. Sci Rep 7:8240.

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

Fraga S, Rodriguez F (2014). Genus *Gambierdiscus* in the Canary Islands (NE Atlantic Ocean) with description of *Gambierdiscus silvae* sp. nov., a new potentially toxic epiphytic benthic dinoflagellate. Protist 165:839-853

Gómez F, Qiu D, Lopes RM, Lin S (2015). *Fukuyoa paulensis* gen. et sp. nov., a new genus for the globular species of the dinoflagellate *Gambierdiscus* (Dinophyceae). PLoS One 10.

Greeson PE (1977). Methods for collection and analysis of aquatic biological and microbiological samples. In: Techniques of water-resources investigations of the United States Geological Survey, vol 5. Laboratory analysis. US Department of the Interior, Geological Survey

Hall TA (1999). BioEdit: a user-friendly biological sequence alignment editor and analysis program for Windows 95/98/NT. Nucleic Acids Symp Ser 41: 95–98.

Hokama Y, Hong T, Isobe M, Ichikawa Y, Yasumoto T (1992). Cross-reactivity of highly purified okadaic acid (OA), synthetic, spiroketal east sphere of OA and ciguatoxin. J Clin Lab Anal 6:54-58.

Holmes MJ, Lewis RJ (1994). Purification and characterisation of large and small maitotoxins from cultured *Gambierdiscus toxicus*. Natural Toxins 2:64-72.

Holmes MJ (1998). *Gambierdiscus yasumotoi* sp. nov. (Dinophyceae), a toxic benthic dinoflagellate from Southeastern Asia. J Phycol 34:661-668.

Hoshaw RW, Rosowski JR (1973). Methods for microscopic algae. In: Stein JR (ed). Handbook of phycological methods. Cambridge University Press, New York (USA), pp 53-67.

Huelsenbeck JP, Ronquist F (2001). MRBAYES: Bayesian inference of phylogenetic trees. Bioinformatics 17:754-755.

Ikehara T, Kuniyoshi K, Oshiro N, Yasumoto T (2017). Biooxidation of ciguatoxins leads to species-specific toxin profiles. Toxins (Basel) 9:205.

Jang SH, Jeong HJ, Yoo YD (2018). *Gambierdiscus jejuensis* sp. nov., an epiphytic dinoflagellate from the waters of Jeju Island, Korea, effect of temperature on the growth, and its global distribution. Harmful Algae 80:149-157.

Kelly AM., Kohler CC, Tindall DR (1992). Are crustaceans linked to the ciguatera food chain? Environ Biol Fishes 33:275-286. DOI: 10.1007/BF00005871

Kohli GS, Farrell H, Murray SA (2015). *Gambierdiscus*, the cause of ciguatera fish poisoning: An increased human health threat influenced by climate change. In: Botana LM, Louzao C, Vilariño N (eds). Climate change and marine and freshwater toxins. De Gruyter, Berlin (Germany), pp 273-312.

Kretzschmar AL, Verma A, Harwood T, Hoppenrath M, Murray S (2017). Characterization of *Gambierdiscus lapillus* sp. nov.(Gonyaulacales, Dinophyceae): a new toxic dinoflagellate from the Great Barrier Reef (Australia). J Phycol 53:283-297.

Kretzschmar AL, Larsson ME, Hoppenrath M, Doblin MA, Murray SA (2019). Characterisation of two toxic *Gambierdiscus* spp. (Gonyaulacales, Dinophyceae) from the Great Barrier Reef (Australia): *G. lewisii* sp. nov. and *G. holmesii* sp. nov. Protist 170.

Larsson ME, Laczka OF, Harwood DT, Lewis RJ, Himaya SWA, Murray SA, Doblin MA (2018). Toxicology of *Gambierdiscus* spp. (Dinophyceae) from tropical and temperate Australian waters. Mar Drugs 16.

Larsson ME, Harwood TD, Lewis RJ, Swa H, Doblin MA (2019). Toxicological characterization of *Fukuyoa paulensis* (Dinophyceae) from temperate Australia. Phycological Res 67:65-71.

Laza-Martínez A, David H, Riobó P, Miguel I, Orive E (2016). Characterization of a strain of *Fukuyoa paulensis* (Dinophyceae) from the Western Mediterranean Sea. J Eukaryot Microbiol 63:481-497.

Ledreux A, Brand H, Chinain M, Bottein M-YD, Ramsdell JS (2014). Dynamics of ciguatoxins from *Gambierdiscus polynesiensis* in the benthic herbivore *Mugil cephalus*: Trophic transfer implications. Harmful Algae 39:165-174.

Lehane L, Lewis RJ (2000). Ciguatera: recent advances but the risk remains. Int. J Food Microbiol 61:91-125.

Leonardo S, Gaiani G, Tsumuraya T, Hirama M, Turquet J, Sagristà N, Rambla-Alegre M, Flores C, Caixach J, Diogène J (2020). Addressing the analytical challenges for the detection of ciguatoxins using an electrochemical biosensor. Anal Chem

Lewis RJ (2001). The changing face of ciguatera. Toxicon 39:97-106.

Lewis RJ, Inserra M, Vetter I, Holland WC, Hardison DR, Tester PA, Litaker RW (2016). Rapid extraction and identification of maitotoxin and ciguatoxin-like toxins from Caribbean and Pacific *Gambierdiscus* using a new functional bioassay. PLoS One 11:e0160006

Litaker RW, Vandersea MW, Faust MA, Kibler SR, Chinain M, Holmes MJ, Holland WC, Tester PA (2009). Taxonomy of *Gambierdiscus* including four new species, *Gambierdiscus caribaeus*, *Gambierdiscus carolinianus*, *Gambierdiscus carpenteri* and *Gambierdiscus ruetzleri* (Gonyaulacales, Dinophyceae). Phycologia 48:344-390.

Litaker RW, Holland WC, Hardison DR, Pisapia F, Hess P, Kibler SR, Tester PA (2017). Ciguatoxicity of *Gambierdiscus* and *Fukuyoa* species from the Caribbean and Gulf of Mexico. PLoS One 12:e0185776.

Longo S, Sibat M, Viallon J, Darius HT, Hess P, Chinain M (2019). Intraspecific variability in the toxin production and toxin profiles of in vitro cultures of *Gambierdiscus polynesiensis* (Dinophyceae) from French Polynesia. Toxins (Basel) 11:735.

Manger RL, Leja LS, Lee SY, Hungerford JM, Wekell MM (1993). Tetrazolium-based cell bioassay for neurotoxins active on voltage-sensitive sodium channels: semiautomated assay for saxitoxins, brevetoxins, and ciguatoxins. Anal Biochem 214:190-194.

Munday R, Murray S, Rhodes LL, Larsson EM, Harwood DT (2017). Ciguatoxins and maitotoxins in extracts of sixteen *Gambierdiscus* isolates and one *Fukuyoa* isolate from the South Pacific and their toxicity to mice by intraperitoneal and oral administration. Marine Drugs 15.

Murata M, Naoki H, Iwashita T, Matsunaga S, Sasaki M, Yokoyama A, Yasumoto T (1993). Structure of maitotoxin. J Am Chem Soc 115:2060-2062

Murray JS, Selwood AI, Harwood DT, van Ginkel R, Puddick J, Rhodes LL, Rise F, Wilkins AL (2019). 44-Methylgambierone, a new gambierone analogue isolated from *Gambierdiscus australes*. Tetrahedron Lett 60:621-625.

Nagai H, Murata M, Torigoe K, Satake M, Yasumoto Y (1992). Gambieric acids, new potent antifungal substances with unprecedented polyether structures from a marine dinoflagellate *Gambierdiscus toxicus*. J Org Chem 57:5448-5453.

Nagumo Y, Oguri H, Shindo Y, Sasaki S-y, Oishi T, Hirama M, Tomioka Y, Mizugaki M, and Tsumuraya T (2001). Concise synthesis of ciguatoxin ABC-ring fragments and surface plasmon resonance study of the interaction of their BSA conjugates with monoclonal antibodies. Bioorg Med Chem Lett 11:2037-2040.

Nagumo Y, Oguri H, Tsumoto K, Shindo Y, Hirama M, Tsumuraya T, Fujii I, Tomioka Y, Mizugaki M, Kumagai I (2004). Phage-display selection of antibodies to the left end of CTX3C using synthetic fragments. J Immunol Methods 289:137-146.

Nishimura T, Sato S, Tawong W, Sakanari H, Yamaguchi H, Adachi M (2014). Morphology of *Gambierdiscus scabrosus* sp. nov.(Gonyaulacales): a new epiphytic toxic dinoflagellate from coastal areas of Japan. J Phycol 50:506-514.

Oguri H, Tanaka S-i, Hishiyama S, Oishi T, Hirama M, Tumuraya T, Tomioka Y, Mizugaki M (1999). Designed hapten aimed at anti-ciguatoxin monoclonal antibody: synthesis, immunization and discrimination of the C2 configuration. Synthesis, pp 1431-1436.

Oguri H, Hirama M, Tsumuraya T, Fujii I, Maruyama M, Uehara H, Nagumo Y (2003). Synthesis-based approach toward direct sandwich immunoassay for ciguatoxin CTX3C. J Am Chem Soc 125:7608-7612.

Pawlowiez R, Darius HT, Cruchet P, Rossi F, Caillaud A, Laurent D, Chinain M (2013). Evaluation of seafood toxicity in the Australes archipelago (French Polynesia) using the neuroblastoma cell-based assay. Food Addit Contam Part A Chem Anal Control Expo Risk Assess 30:567-586.

Pisapia F, Holland WC, Hardison DR, Litaker RW, Fraga S, Nishimura T, Adachi M, Nguyen-Ngoc L, Sechet V, Amzil Z, Herrenknecht C, Hess P (2017a). Toxicity screening of 13 *Gambierdiscus* strains using neuro-2a and erythrocyte lysis bioassays. Harmful Algae 63:173-183.

Pisapia F, M. Sibat M, Herrenknecht C, Lhaute K, Gaiani G, Ferron PJ, Fessard V, Fraga S, Nascimento SM, Litaker RW, Holland WC, Roullier C, Hess P (2017b). Maitotoxin-4, a novel MTX analog produced by *Gambierdiscus excentricus*. Mar Drugs 15:220.

Provasoli L (1968). Media and prospects for the cultivation of marine algae. In: Hattori A (ed). Proceedings of the US-Japanese conference, cultures and collection of algae. Japanese Society of Plant Physiology, Hakone (Japan), pp 63-75.

Reverté L, Soliño L, Carnicer O, Diogène J, Campàs M (2014). Alternative methods for the detection of emerging marine toxins: biosensors, biochemical assays and cell-based assays. Mar Drugs 12:5719-5763.

Reverté L, Toldrà A, Andree KB, Fraga S, de Falco G, Campàs M, Diogène J (2018). Assessment of cytotoxicity in ten strains of *Gambierdiscus australes* from Macaronesian Islands by neuro-2a cell-based assays. J Appl Phycol 30:2447-2461.

Rhodes LL., K. F. Smith KF, R. Munday R, A. I. Selwood AI, P. S. McNabb PS, P. T. Holland PT, and M.-Y. Bottein M-Y (2010). Toxic dinoflagellates (Dinophyceae) from Rarotonga, Cook Islands. Toxicon 56:751-758.

Rhodes L, Harwood T, Smith K, Argyle P, Munday R (2014). Production of ciguatoxin and maitotoxin by strains of *Gambierdiscus australes, G. pacificus* and *G. polynesiensis* (Dinophyceae) isolated from Rarotonga, Cook Islands. Harmful Algae 39:185-190.

Rhodes LL, Smith KF, Murray S, Harwood DT, Trnski T, Munday R (2017). The epiphytic genus *Gambierdiscus* (Dinophyceae) in the Kermadec Islands and Zealandia regions of the Southwestern Pacific and the associated risk of ciguatera fish poisoning. Mar Drugs 15:219.

Rodríguez I, Genta-Jouve G, Alfonso C, Calabro K, Alonso E, Sánchez JA, Alfonso A, Thomas OP, Botana LM (2015). Gambierone, a ladder-shaped polyether from the dinoflagellate *Gambierdiscus belizeanus*. Org Lett 17:2392-2395.

Roeder K, K. Erler K, Kibler S, Tester P, Van The H, Nguyen-Ngoc L, Gerdts G, Luckas B (2010). Characteristic profiles of ciguatera toxins in different strains of *Gambierdiscus* spp. Toxicon 56:731-738.

Rossignoli AE, Tudo A, Bravo I, Diaz PA, Diogène J, Riobo P (2020). Toxicity characterisation of *Gambierdiscus* species from the Canary Islands. Toxins (Basel) 12:134.

Roué M, Darius HT, Picot S, Ung A, Viallon J, Gaertner-Mazouni N, Sibat M, Amzil Z, Chinain M (2016). Evidence of the bioaccumulation of ciguatoxins in giant clams (*Tridacna maxima*) exposed to *Gambierdiscus* spp. cells. Harmful Algae 57:78-87.

Rovira A, Alcaraz C, Ibáñez C (2012). Spatial and temporal dynamics of suspended load at-a-cross-section: the lowermost Ebro River (Catalonia, Spain). Water Res 46:3671-3681. DOI: 10.1016/j.watres.2012.04.014

Rozewicki J, Li S, Amada KM, Standley DM, Katoh K (2019). MAFFT-DASH: integrated protein sequence and structural alignment. Nucleic Acids Res 47:W5-W10.

Satake M, Murata M, Yasumoto T (1993). Gambierol: a new toxic polyether compound isolated from the marine dinoflagellate *Gambierdiscus toxicus*. J Am Chem Soc 115:361-362.

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

Silva M, Rodriguez I, Barreiro A, Kaufmann M, Isabel Neto A, Hassouani M, Sabour B, Alfonso A, Botana LM, Vasconcelos V (2015). First Report of Ciguatoxins in Two Starfish Species: *Ophidiaster ophidianus* and *Marthasterias glacialis*. Toxins (Basel) 7:3740-3757.

Soler-Onís E, Fernandez-Zabala J, Ojeda-Rodriguez A, Amorin A (2016). Bloom of *Gambierdicus caribaeus* in the temperate-subtropical waters of El Hierro, Canary Islands (North East Atlantic). Harmful Algae News 55:14-17.

Stamatakis A (2014). RAxML version 8: a tool for phylogenetic analysis and post-analysis of large phylogenies. Bioinformatics 30:1312-1313.

Suárez-Serrano A, Alcaraz C, Ibanez C, Trobajo R, Barata C (2010). *Procambarus clarkii* as a bioindicator of heavy metal pollution sources in the lower Ebro River and Delta. Ecotoxicol Environ Saf 73:280-286.

Tabachnick BG, and Fidell LS (2007). Using multivariate statistics. Pearson, Boston (USA).

Tester PA, Berdalet E, Litaker RW (2020). Climate change and benthic harmful microalgae. Harmful Algae 91:101655.

Toldrà A, Andree KB, Fernández-Tejedor M, Diogène J, Campàs M (2018). Dual quantitative PCR assay for identification and enumeration of *Karlodinium veneficum* and *Karlodinium armiger* combined with a simple and rapid DNA extraction method. J Appl Phycol 30:2435-2445.

Tsumuraya T, Fujii I, Inoue M, Tatami A, Miyazaki K, Hirama M (2006). Production of monoclonal antibodies for sandwich immunoassay detection of ciguatoxin 51-hydroxyCTX3C. Toxicon 48:287-294.

Tsumuraya T, Fujii I, Hirama M (2010). Production of monoclonal antibodies for sandwich immunoassay detection of Pacific ciguatoxins. Toxicon 56:797-803.

Tsumuraya T, Takeuchi K, Yamashita S, Fujii I, Hirama M (2012). Development of a monoclonal antibody against the left wing of ciguatoxin CTX1B: thiol strategy and detection using a sandwich ELISA. Toxicon 60:348-357.

Tsumuraya T, Fujii I, Hirama M (2014). Preparation of anti-ciguatoxin monoclonal antibodies using synthetic haptens: sandwich ELISA detection of ciguatoxins. J AOAC Int 97: 373-379.

Tsumuraya T, Sato T, Hirama M, Fujii I (2018). Highly Sensitive and Practical Fluorescent Sandwich ELISA for Ciguatoxins. Anal Chem 90:7318-7324.

Tudó À, Toldrà A, Andree KB, Rey M, Fernández-Tejedor M, Campàs M, Diogène J (2018). First report of *Gambierdiscus* in the Western Mediterranean Sea (Balearic Islands). Harmful Algae News

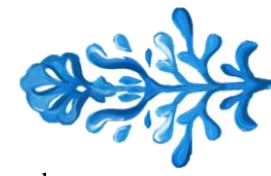
Watanabe R,Uchida H, Suzuki T, Matsushima R, Nagae M, Toyohara Y, Satake M, Oshima Y, Inoue A, Yasumoto T (2013). Gambieroxide, a novel epoxy polyether compound from the dinoflagellate *Gambierdiscus toxicus* GTP2 strain. Tetrahedron 69:10299-10303.

Yasumoto T, Igarashi I, Legrand A-M, Cruchet P, Chinain M, Fujita T, Naoki H (2000). Structural elucidation of ciguatoxin congeners by fast-atom bombardment tandem mass spectroscopy. J Am Chem Soc 122:4988-4989.

Yogi K, Oshiro N, Inafuku Y, Hirama M, Yasumoto T (2011). Detailed LC-MS/MS analysis of ciguatoxins revealing distinct regional and species characteristics in fish and causative alga from the Pacific. Anall Chem 83:8886-8891.

Yogi K, Sakugawa S, Oshiro N, Ikehara T, Sugiyama K, Yasumoto T (2014). Determination of toxins involved in ciguatera fish poisoning in the Pacific by LC/MS. J AOAC Int 97:398-402.

General discussion



Ciguatera fish poisoning (CFP) has threatened human heath for centuries, and the inexistence of an antidote makes this disease still an issue. In fact, the occurrence of a CFP event can have direct effect not only on human health but also on the economy via fishery closure and on the tourism via labelling certain regions as "ciguatera-at risk destination". The only actual action that can be taken to manage ciguatera is the prevention of it, by improving the detection methods of ciguatoxins (CTXs) and DNA of the CTX-producing species. In fact, despite the undeniable utility of traditional methods for *Gambierdiscus/Fukuyoa* and CTXs detection, they need an update because they are expensive, time consuming and personnel need to be trained. Thus, the development of cheap, rapid, reliable and easy-to-use tools for *in situ* analysis can act as a solution for CFP anticipation.

The work developed in this thesis presents solutions for the rapid DNA detection of the CTXs producing genera *Gambierdiscus* and *Fukuyoa* and for CTXs. At first, for DNA detection, rapid isothermal amplification was coupled to a colorimetric assay, and then multiplex PCR approach was integrated in lateral flow assays and in a dual biosensor for the simultaneous detection of two toxin producing species, *G. australes* and *G. excentricus*. Furthermore, for CTXs detection, an immunosensor has been developed, allowing the detection of the four main congeners of CTXs in fish and algal samples. The application of these strategies has been deeply studied in this thesis, by constructing calibration curves with natural material, by evaluating the specificity, by testing them with samples artificially created and finally by applying them directly to the analysis of natural samples.

Detection of DNA from *Gambierdiscus*

The current increase in the detection of *Gambierdiscus* species (Aligizaki and Nikolaidis 2008; Tudó et al. 2018, 2020a, 2020b) in European waters has increased the warning of a possible ciguatera outbreak. The first effort performed by our group in the direction of the development of quick and fast technique to detect those species, was the exploitation of an isothermal technique that, with the implementation of modified primers, allowed obtaining, in 30 min and at a constant temperature of 37 °C, an amplified product flanked with oligonucleotides tails. Then, a sandwich hybridization assay was performed, and thanks to the use of capture probes and a horseradish peroxidase (HRP)-labelled reporter probe, it was possible to differentiate between *Gambierdiscus/Fukuyoa* and other microalgae genera and among two

toxin-producing species (*G. australes* and *G. excentricus*) and their congeneric species, as described in Chapter 1. Despite its advantageous features, this technique is not commonly used, mostly because the kit needed to perform the test is expensive and its efficiency gets lower and lower once opened, compromising the inter-day reproducibility. Additionally, a purification step was required after the amplification, otherwise the background signal showed too high absorbance values. Even with this additional step, it was still possible to slightly detect this background noise. Nevertheless, the comparison between absorbance values obtained from the analysis of target amplified products and controls left no doubt about the discrimination ability of the system, but all template DNAs used for the evaluation of the assay performance were highly concentrated (1 ng/µL) and extracted from laboratory monoclonal cultures, which made easier obtaining good quality DNA. It is necessary to mention that, even with equally concentrated template DNAs, it was possible to observe differences in the assay yields, depending on the primer set used. In fact, the highest absorbance values were obtained in the detection of *Gambierdiscus/Fukuyoa* genera. This is probably due to a better efficiency of those primers during the isothermal amplification. Additionally, not all the strains belonging to the same species provided the same absorbance value in the assay, as it is possible to observe especially in the detection of *G. excentricus* strains. The most probable reason behind this non-conformity is the difference that may exist in the rDNA copy number of the samples analyzed. In fact, the rDNA copy number can vary between species, strains, geographical origins and even cell growth phases (Galluzzi et al. 2010; Vanderesea et al. 2012). The extraction and analysis of DNA from natural samples is a complicated task to perform, therefore, to test if the system would be able to detect few amounts of DNA, extraction was been performed from a single cell of various *Gambierdiscus/Fukuyoa* species. The extract obtained was then tested with the primer sets for the genera and the two species, demonstrating that the system was efficient even with the DNA template equivalent to 1/3 of a cell. The same was done with genera different than the targeted ones and no signal was obtained. To explore even more the discrimination ability of the system, mixes of target DNAs at a final concentration of 1 ng/µL were tested. The results obtained showed absorbance values only in presence of target species, although much lower than the ones obtained with just one target species. It is clear that DNA concentration and rDNA copy number are not the only crucial parameters, and the presence of non-target DNA in a sample may cause a steric hindrance and inhibit the efficiency of the isothermal amplification. Therefore, even though the achieved results were promising, this strategy was not chosen for the screening of field samples due to the limitations previously discussed about the DNA isothermal amplification kit.

Therefore, the effort was focused on the development of PCR-based techniques that could be implemented into portable devices for the *in situ* detection of microalgae. With this purpose in mind (i.e. the analysis of field samples), this thesis focused on the development of an electrochemical biosensor for the simultaneous detection of the two CTXs producing species, *G. australes* and *G. excentricus,* in natural samples. At first, a fast extraction technique was developed. The DNA extraction is a key step for the analysis of environmental samples and there exists a huge variety of protocols and commercially available kits to do so. However, they usually require long times and are laboratory dependent, and so other types of techniques should be explored for the *in situ* extraction. It must be underlined that the long laboratory-based extraction techniques usually result in highly purified DNA that makes the amplification easier, thing that is more difficult to achieve with shorter protocols. Therefore, a compromise has to be found. One of the first attempts was performed by Toldrà et al. (2018), who applied a fast DNA extraction method to the analysis of field samples containing the target species *Karlodinium veneficum* and *Karlodinium armiger.* Thus, this technique was also used here. Despite the application of the bead beating step, which resulted in yields comparable to the conventional method in the *Karlodinium* work, the reaction yield in our work was nearly the half when compared to the Phenol/Chloroform/Isoamyl alcohol method used as a reference. Hence, another technique was tried, involving a MBs-based protocol. The technique was tested with and without the bead beating step, but it resulted in low absorbance values in both cases. Clearly, the bead beating step cannot be taken out from the protocol since it is necessary to break the armored *Gambierdiscus* cells, especially the *G. australes* ones. Therefore, another trial was performed using a portable bead beater in order to make a step forward in the *in situ* extraction of field samples. With this particular method, the obtained results were comparable to the ones obtained with the reference method, therefore it was chosen as the extraction procedure prior the analysis. One of the reasons behind the better yield obtained with portable bead beater in comparison with the desk one could be the duration of the bead beating step. In fact, the run of the desk one lasts 45 s, otherwise it would heat too much the samples, and instead the run of the portable one lasts 1 minute straight. As it possible to deduce from the obtained results, the system (i.e. extraction and dual amplification) seems to work better for *G. australes* than for *G. excentricus*, in accordance to what we observed in the performance of the isothermal amplification (Chapter 1). Again, this can be due either to a difference in the rDNA copy number between species or even strains or to a better efficiency of the primers for *G. australes* DNA. Anyhow, the chosen rapid DNA extraction technique enables to obtain DNA good enough for the amplification in less than 30 minutes. The major disadvantage of this technique is the fact that the battery charge of the portable bead beater allows the extraction of five samples. Nevertheless, if used during sampling campaigns, it

can be recharged between sites, allowing performing the bead beating step immediately after the collection of samples.

The method used for the DNA detection was based on a sandwich hybridization assay with the use of maleimide-coated MBs and species-specific capture probes together with an HRP-labelled reporter probe. The strategy developed is similar to the one of Medlin et al. (2020), although with a few differences. In our work, the species-specific primers for *G. australes* and *G. excentricus* designed for RPA (Chapter 2), were used together to perform PCR. These primers included tails that, after PCR, gave amplified products flanked with single-stranded oligonucleotides at each end. Afterwards, the amplified products were incubated with the MBs modified with the specific capture probes, and then an HRP-labelled reporter probe was added. For the electrochemical detection, the *G. australes* oligocomplexes were immobilized on one of the working electrodes of a dual electrode array, and the *G. excentricus* oligocomplexes were immobilized on the other one. The use of 3,3′,5,5′-tetramethylbenzidine (TMB) Enhanced One Component HRP Membrane Substrate allowed the simultaneous detection of amplified DNA on both electrodes because, when oxidized by the HRP, it precipitates on the electrode on which the chemical reaction is happening, without interference with the other one. The reduction current intensity was proportional to the amount of amplified product and consequently to the number of microalgal cells present in the samples. In our work, the calibration curve of the system was constructed starting from a 10^4 cell pellet, performing 1/10 serial dilutions and extracting each pellet dilution with the newly developed fast DNA extraction technique. Since the DNA extraction part is crucial, as mentioned above, in order to assess its efficiency, DNA dilutions (i.e. extraction of DNA from 10^4 cells, and subsequent 1/10 serial dilutions of the extracted DNA) were also tested and compared. Results showed higher reduction current intensities with increasing concentrations of DNA for both DNA extracted from cells as well as dilutions of genomic DNA, as expected. The analysis of dilutions of *G. australes* genomic DNA resulted in higher absorbance values in comparison to the corresponding extracted cells. Instead, the analysis of dilutions of *G. excentricus* genomic DNA and extracted cells resulted in similar absorbance values. Probably, the cells of this strain and culture are easier to disrupt in comparison to the *G. australes* ones, and thus this thought is reinforced by the results obtained from the performance of the lateral flow dipstick assay, in which the obtained black line is darker for *G. excentricus* than for *G. australes* (Annex 10). It is important to consider that in the calibration curve obtained from the cell dilutions, there is one extraction step for each cell dilution and the efficiency of the extraction can change depending on the number of cells, being lower when cells are more dispersed in the lysis buffer. In both cases, an LOD of 10 cells was reached for the target species. The LOD achieved by Medlin

and coworkers (2020) was be similar or even lower than ours (it corresponds to 1pM of RNA, which in their work ranged from 10 to 444 cultivated cells), they targeted more *Gambierdiscus* species (*G. australes, G. excentricus* and *G. silvae*), and no PCR was performed. However, their technique was tested only with synthetic DNA, and it has not been applied yet to the analysis of genomic DNA/RNA or the screening of field samples, for which the PCR step would be necessary. Instead, our strategy resulted efficient in the extraction and amplification of genomic DNA, hence is more suitable for the analysis of field samples. Additionally, the simultaneous detection of these two species at different cell concentrations and ratios was tried. The combinations to test are infinite and, of course, it is impossible to test them all. Therefore, we decided to select two cell concentrations (10^3 and 10^2) that can be easily found in natural samples and that provided different absorbance values when tested in the calibration curves. The nine possible combination of cells were analyzed, and detection and discrimination were successfully achieved. Again, as observed in previous experiments, the presence of the non-target species affects the detection of the target one when they are at different concentrations. Nevertheless, at equal concentration of cells the intensity values are similar, indicating that even if the detection of one species is affected by the presence of the other, the system recognizes and discriminates between them simultaneously. Driven by this results, we decided to analyze field samples. So, epiphytic microalgae samples collected in Majorca (Balearic Islands, Spain) were screened. The test performed with pure extract resulted in no amplification, indicating that the sample matrix affected the performance of the PCR. Before adding long and tedious purification steps to our protocol to reduce the matrix effect, we decided to dilute the extracted samples, since PCR is known to work also at low concentrations of template DNA. With the application of this simple stratagem, it was possible to obtain signals and use the calibration curves to estimate the number of microalgal cells, which were close to the ones obtained with light microscopy. Sure, the strategy presented by our group is longer and less species are targeted, if compared to the one proposed by Medlin et al (2020). Nevertheless, not only it allowed the detection of genomic DNA extracted from laboratory cultures, but also the screening of environmental samples. It is important to mention that, during the analysis of field samples, we registered absorbance values that indicated the presence of DNA from *G. excentricus*, which was surprising, since it had not been previously reported in Balearic Island waters. Therefore, we isolated several single cells from Majorca, we extracted the DNA, amplified it, and sent to sequencing, and thus obtaining the identification of 5 *G. excentricus* among the analyzed cells. The detection strategy proposed combined with the fast extraction technique represent an important step forward in the practical application of *in situ* detection of toxin producing species, providing punctual warnings of *Gambierdiscus* presence in an area and so facilitating quick

management decisions. Moreover, this system can be easily modified for the simultaneous detection of other microalgae species (pending the design of tailed primers that do not cross-react with each other), and so it could be useful for the monitoring of their presence in an area.

Detection of CTXs

One of the major problems when trying to avoid and prevent CFP is that fishes contaminated with CTXs do not look, smell and taste differently than the non-contaminated ones. Additionally, since CTXs are thermostable toxins, neither cooking nor freezing the fish would deactivate them. Moreover, since the symptoms can appear between 1 and 48 hours after the ingestion by the consumer, the ingestion and the symptoms can occur in different places, making difficult spotting ciguatera outbreaks. Therefore, the development of fast, reliable and easy-to-use tools for the screening of fish extracts are of extreme interest, not only for scientific purposes but also for fisheries and public health.

The detection of CTXs in fish samples is a challenging task, and the most used technique (i.e. LC-MS/MS) requires highly trained personnel, it is expensive, and requires a laboratory to be performed. Therefore, researchers are trying to achieve the same goal by developing strategies that would avoid the use of sophisticated analytical instrumentation and focusing more on the simpler, faster and cheaper portable systems, like biosensors. In my thesis, the first one was developed (Chapter 3), targeting the detection of four congeners of CTXs belonging to the CTX1B and the CTX3C groups. Three different mAbs, two capture ones, which specifically bind to the right wing of the CTX1B and 54-deoxyCTX1B (3G8) and of the CTX3C and 51-hydroxyCTX3C (10C9), and a detector one, which has either a specific bind or a cross-reactivity with the left wing (8H4) of all the four congeners, were used. MBs were used for the immobilization of the capture mAbs. Then, the mAb-functionalized MBs were exposed to CTX standards (CTX1B or 51-OH-CTX3C) or extracts of fish naturally contaminated with CTXs, followed by the addition of the detector antibody previously biotinylated. Subsequently, polyHRP-streptavidin was incubated and, finally, the immunocomplexes were placed on the working electrodes of an eight-electrode array. Subsequently, a plastic support with magnets underneath each working electrode was used to block MBs in the right position. Then, TMB liquid substrate was incubated, and the reduction current intensity was measured with amperometry. We observed that the fish matrix affected the detection of CTXs, and so recovery values were calculated. In this way, by applying them to the results obtained following our protocol, there is no need to dilute or further purify the samples. In addition, an evaluation of matrix effects between fish individuals was performed, in which different CTX1B concentrations were spiked into two *Variola louti* individuals negative

for CTXs, and the experiment yields obtained were similar among those individuals. Effective limit of quantifications (eLOQs) were calculated from the calibration curves constructed from the CTX1B spiked at 2500 mg/mL of *V. louti* extracts. The achieved eLOQs were 0.01 and 0.002 μg/kg for the colorimetric immunoassay and electrochemical biosensor. Sure, this last experiment should be performed with fishes of different species, size and origin. Even though there are no regulatory limits for CTXs in fish, we decided to test if, at least, our system was able to detect CTX1B at 0.01 μg/kg, the United States Food and Drug Administration (US FDA) guidance level). The non-contaminated *V. louti* was spiked at the FDA threshold with CTX1B, which was successfully detected. Finally, fishes naturally contaminated from la Réunion were analysed and the amount of CTX congeners detected correlated well with the results obtained by the colorimetric immunoassay, mouse bioassay (MBA) and cell-based assay (CBA). In fact, all the assays performed indicated the same individuals as negative or positive for CTXs. In terms of CTXs content, the CBA detected higher levels compared to the immunosensor. This can be explained by the different recognition principles. In the immunochemical tools, the recognition is structural (they detect specific structures, as the wings of CTXs). Instead, in the CBA is toxicological (it detects the analogues that activates the voltage-gated sodium channels). Thus, the CBA would respond to the activity of several CTXs at the same time, giving a composite response of the CTXs content in a sample. However, it would also respond to other toxic compounds that would have as target the same one of CTXs, without being able to discriminate. Additionally, a complete correspondence may be not obtained since the fish samples were from the Indian Ocean and could more probably contain I-CTXs than P-CTXs (although, as it is mentioned below, CTX1B was found in one of the fishes). Even if this strategy allows detecting only few of the many CTXs existing congeners, the detection is not affected by the presence of marine toxins other than CTXs. Furthermore, due to the high robustness of the Abs, samples do not require many purification steps, shortening the assay time. It must be underlined that the performance of additional purification steps could have helped in increasing the toxin recovery values, since matrix effects would have been removed. However, it is also true that it could have provided lower CTXs contents, due to losses during the purification steps or even elimination of some congeners. In order to confirm the presence of CTXs in the fish extracts, 3 individuals positive for CTXs were screened with LC-ESI-HRMS. It was possible to obtain results just from one individual, which presented a CTX1B content of 1069 pg/g (six-fold higher than the value obtained with immunosensing tools), and no other congeners were detected. Therefore, despite its limitations, the strategy is fast, easy to perform and reliable, and it detected CTXs at low contents at which the LC-ESI-HRMS technique could not work. It definitely represents a step forward into the development of portable devices for the *in situ* detection

of CTXs, since it is easy to use, fast and reliable, so, it could be implemented in monitoring systems.

Chapter 4 stands as another example of the applicability of the system in natural samples with low concentration of CTXs. The strategy was applied to the screening of one fish from Cyprus, which had presented CTX-like activity in CBA. It was a *Seriola dumerili* individual of 6.9 kg, and the toxin content was 12.4 pg of CTX1B equiv./g with CBA, just slightly over the guidance level of 0.01 µg/kg (10 pg/g). Again, the crude extract was tested with LC-MS/MS and no CTXs congeners were detected. Therefore, since something similar happened in the analysis of the fish extracts from La Reunion, the crude extract was also screened with the immunosensing technique, resulting in 4.98 pg of CTX1B equiv./g. It is not surprising the fact that a lower content of CTXs was detected compared to CBA, as observed in the previous analyses (Chapter 3). In fact, as mentioned above, our system is designed for the recognition of four CTXs congeners, whereas the CBA can detect the CTXs-like activity of any of them. Nevertheless, it is important to mention again that our strategy is less affected by the presence of non-target toxic compounds compared to the CBA, making the detection of false positives most unlikely to happen. However, since this fish was the only one that showed CTXs activity, among many others analysed, further investigation on the CTXs present in the individual were considered as necessary. Hence, in this particular case, a purification process was applied. The crude extract was fractionated and the obtained fractions were tested with both CBA and immunoassay. The results showed CTX-like activity with CBA in 8 of the 28 fractions and the sum of toxin contents in these fractions was 6.5 pg of CTX1B equiv./g fish flesh. Instead, the analysis performed with the immunoassay revealed the presence of CTXs only in fraction 15 (0.08 pg of CTX1B equiv./g fish flesh). As mentioned above, the fact that CBA detected a higher CTX-like activity could mean that in these fractions there would be either other congeners that our system does not target or other toxic compounds with an activity similar to CTXs. Nevertheless, the combined response of our system and the CBA evidenced the presence of CTXs in this individual, even though the absence of LC-MS/MS analysis makes difficult to assert the actual presence of CTXs congeners in this particular fish. Anyhow, our strategy can be seen as an optimal tool for pre-screening, since the analysis of several samples can be performed at the same time. In this way, the samples that present CTXs content can be quickly identified and then undergo further purification and extra analyses, also including with other techniques, as it was done with the fish individual from Cyprus.

The challenge of identifying new ciguatera-at-risk regions must not rely completely on detecting CTXs in fish samples. In fact, the managing of ciguatera is biased by the fact that fishes move, and so the intoxication episode

and the caught of the intoxicated fish, if possible, can be separated not only in time but also in space. Thus, scientists started studying CTXs production ability of *Gambierdiscus* species. However, this task is not as easy as it might appear. Indeed, after the success in detecting CTXs in fish samples, our group decided to tackle the challenge of the detection of CTXs in microalgal producers. Therefore, several strains of *Gambierdiscus* and *Fukuyoa* were tested in order to investigate the differences in toxin production among species (Chapter 5). In this work, 20.000 cells from 9 *Gambierdiscus* and 4 *Fukuyoa* strains were cultured, extracted and analyzed with the strategy previously developed (Chapter 5). Nevertheless, since the capture Abs (3G8 and 10C9) targeted two different groups of CTX congeners, they were used combined together, but also separately in order to discriminate between them. Little is known about the CTXs production of these species, probably because they are quite difficult to manage and generally huge amounts of biomass are required to perform CTXs production studies and *Gambierdiscus* species are known to grow slowly in laboratories. The results obtained with the immunosensor showed the presence of CTX congeners in 11 out of the 13 strains analyzed. A higher CTXs content was obtained when the two capture Abs were combined together, in comparison to the detection achieved with just one. This is due to the presence of both series of congeners which simultaneously detection results in a higher signal that trespass the LOD of the system. Clearly, the presence of just one congener may not be detected because of the mentioned LOD of the system. In the analysis performed with the Abs separately, a predominance of CTX1B equiv. was observed in 4 out of 6 *G. excentricus* strains (0.06 to 0.21 fg/cell), and 1 out of 4 *F. paulensis* strains (0.33 fg/cell). On the other hand, *G. australes* and the other 2 *G. excentricus* strains showed a higher abundance of CTX3C equiv. (0.16 fg/cell and 0.04-3.54 fg/cell, respectively). The unique strain of *G. caribaeus* tested showed an equal amount of both the congeners (0.13 fg/cell). Additionally, the same microalgal extracts were screened with CBA, which identified CTX-like activity only in 4 out of the 6 *G. excentricus* strains. This finding was not surprising since *G. excentricus* has been identified as one of the most toxin producing species in previous studies, where CTXs-like activity ranged from 128.2 up to 1000 fg of CTX1B equiv./cell and from 469 up to 1400 fg of CTX3C equiv./cell (Fraga et al. 2011; Litaker et al. 2017; Pisapia et al. 2017; Rossignoli et al. 2020). Even if it did not show CTXs-like activity in this study, also *G. australes* is a well-known toxin producer. In fact, several data are available that testify its CTXs-like activity, which ranged from 31.1 up to 679 fg of CTX1B equiv./cell and of 0.6 up to 2.7 fg of CTX3C equiv./cell (Rhoeder et al. 2010; Lewis et al. 2016; Pisapia et al. 2017; Reverté et al. 2018; Rossignoli et al. 2020). Similarly, *G. caribaeus* did not show CTXs-like activity in this study, although in previous work did. Its CTX-like activity ranged from 0.66 up to 2.59 fg of CTX1B equiv./cell and from none up to 1.6 fg of CTX3C equiv./cell (Lewis et al. 2016; Litaker et al. 2017; Pisapia et al. 2017; Rossignoli

et al. 2020). The first observation that catches the eye is the much lower CTXs content detected in our study for all these *Gambierdiscus* species compared to other works. Instead, regarding *F. paulensis,* the situation is slightly different, as our study is one of the few that confirmed its potential hazard. Indeed, this genus has been labelled as non-toxic in several studies (Rhodes et al. 2014; Munday et al. 2017; Larsson et al 2019), and only in the study of Laza-Martínez et al. (2016) it was identified as a producer of 54-deoxy-CTX1B. Another important observation that has to be noticed is that that *Gambierdiscus* species can lose their production ability within time if maintained for long periods at laboratory conditions, as happened with the *G. australes* IRTA-SMM-13_17 strain analyzed in Reverté et al. (2018) and in Chapter 5, making its study even more complicated. Additionally, also the contrary can occur, as it happened for the *F. paulensis* strain VGO1185, which did not show CTXs-like activity in the study of Gómez et al. (2015), but it did in ours. It has not to be forgotten that, even if the strategy allows to detect only four CTX congeners, it is not affected by the presence of the other toxic compounds produced by *Gambierdiscus* and *Fukuyoa*, as mentioned above, and so providing reliable results. In fact, maitotoxins, toxins commonly found in these genera, are known to affect in the execution of CBA, if no pretreatment is performed. In order to reduce even more the assay time, a trial without evaporation procedure, and so with the extracts in 100% methanol was performed. This test did not work, probably because of the high methanol concentration. In fact, in a previous work by our group although with other Abs (Leonardo et al. 2017), samples containing 10-20 % methanol were exposed to the Abs, and it was possible to perform the analysis. Therefore, reducing the amount of MeOH could be a solution for a further reduction of the experimental time. Moreover, in my thesis, a new fast CTXs extraction technique was developed, allowing operating with as low as 20.000 cells, an amount that can be easily found in natural environments, making it suitable for the screening of field samples. The number of cells chosen for this experiment is a compromise between the possibility to detect CTXs in algal pellets and a low cell concentration. It would be of extreme interest to optimize even more the fast extraction technique to be able to work with an even lower number of cells in order to reduce the algal culturing procedure, which is time consuming, requires specific material and conditions, and needs experienced personnel. Another important feature of this study is that the use of the biosensor permitted the identification for the first time of two different groups of CTXs congeners from the same extract, giving new information about the *Gambierdiscus* and *Fukuyoa* toxin profiles. For this reason, this strategy was chosen to characterize a *G. belizeanus* strain firstly reported in the Canary Islands, detecting the production of congeners of both the CTX1B (0.13 fg/cell) and CTX3C (0.17 fg/cell) series (Annex 8). The analysis performed with both antibodies combined showed again a higher toxin content (0.35 fg of CTX1B equiv./cell). In the same work, the CTXs-like

activity detected with the CBA for this species resulted in 5.6 fg of CTX1B equiv./cell, a much higher content than the one obtained with the immunosensor, similarly to previously observations. It must be underlined that the all the algal extract tested came from either the Mediterranean Sea or the Canary Islands, therefore, as for the fish extracts tested in Chapter 3, they are most likely to contain CTXs different than the P-CTXs. Thus, it would be of interest and useful testing this technique on other *Gambierdiscus* species from all over the world, especially *G. polynesiensis*, which is considered one of the most toxic. Additionally, in order to make further advances in the direction of the actual *in situ* detection of CTXs congeners, this strategy was also applied directly to the analysis of field samples (Chapter 2). In this work, macroalgae substrates were collected in Majorca, extracted, and then exposed to both capture mAbs simultaneously, to maximize the probabilities to detect CTXs. Results showed CTX contents in one sample (13.35 ± 0.5 pg of CTX1B equiv./cell) and traces of CTX (below the LOQ) in 3 out of 9 analyzed samples, demonstrating that the system is suitable for the screening of field samples. Even if these are preliminary results and certainly further studies and optimizations are needed, they underline the efficiency and the applicability of biosensing tools for monitoring programs, contributing to the prevention of ciguatera outbreaks. It would be useful to apply the strategy to samples from areas in which the *Gambierdiscus* species are well established, such as the Canary Island, the Great Caribbean Region or the French Polynesia, in order to assess the actual efficacy of the system in the screening of natural samples of regions endemic for ciguatera. The current techniques for ciguatera managing rely on long and expensive laboratory-based techniques, and thus the intoxication episode, the symptoms appearance and the CTXs detection can be separated by a long interval of time, making difficult spotting the outbreaks. Therefore, some scientists focused on the development of easier, cheaper, faster and highly sensitive techniques to detect CTXs, exploiting the specificity of the antigen-antibody reaction. Some of these efforts resulted in the manufacturing of the commercially available kits Cigua-Check (Hokama 1985; Hokama et al. 1987) and Ciguatect (Park 1995). Despite the undoubtable advance represented by these tests, since no extraction of fish samples was needed, they showed cross-reactivity with other marine toxins (Hokama et al. 1989).

In conclusion, it has been mentioned several times in the discussion above that the strategies presented need optimization to provide better and more reliable results. As true as it is, the work presented in this thesis is undoubtedly paving the way for the development of biotechnological devices for the *in situ* detection of CTXs. In fact, the investigation performed allowed to successfully develop several techniques for the detection of the *Gambierdiscus* DNA at low concentrations and in field samples. Additionally, the applicability of the biosensor for CTXs detection has been demonstrated not only in fish samples from La Réunion, but also in a fish from the Mediterranean, a possible future area of ciguatera expansion. This last belief is reinforced by the successful application of the biosensor in the identification of CTXs congeners in Mediterranean algal samples from both laboratory *Gambierdiscus* cultures and the environment. Sure, inter-laboratory studies would help in proving the usefulness of these tools in monitoring programs, complementing the current methods. Overall, this thesis demonstrates the versatility and robustness of molecular and immunochemical tools for the analysis of complex matrixes such as fish extracts and environmental algal samples, without the need of multiple purification steps. I do believe that the strategies presented in my thesis could be easily extrapolated for the development of tools that would target other marine toxins or DNA of microalgal producers, helping in the prevention of several other seafood borne diseases.

References:

Aligizaki K, Nikolaidis G (2008). Morphological identification of two tropical dinoflagellates of the genera *Gambierdiscus* and Sinophysis in the Mediterranean Sea. J Biol Res 9:75-82

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

Galluzzi L, Bertozzini E, Penna A, Perini F, Garcés E, Magnani M (2010). Analysis of rRNA gene content in the Mediterranean dinoflagellate *Alexandrium catenella* and *Alexandrium taylori*: implications for the quantitative real-time PCR-based monitoring methods. J Appl Phycol 22:1–9.

Gómez F, Qiu D, Lopes RM, Lin S (2015) *Fukuyoa paulensis* gen. et sp. nov., a new genus for the globular species of the dinoflagellate *Gambierdiscus* (Dinophyceae). PLoS One 10.

Hokama Y (1985). A rapid, simplified enzyme immunoassay stick test for the detection of ciguatoxin and related polyethers from fish tissues. Toxicon 23:939-946.

Hokama Y, Shirai LK, Iwamoto LM, Kobayashi MN, Goto CS, Nakagawa LK (1987). Assessment of a rapid enzyme immunoassay stick test for the detection of ciguatoxin and related polyether toxins in fish tissues. Biol Bull 172:144-153.

Hokama Y, Honda SAA, Asahina AY, Fong JML, Matsumoto CM, Gallacher TS (1989). Cross-reactivity of ciguatoxin, okadaic acid, and polyethers with monoclonal antibodies. Food Agr Immunol 1:29-35.

Laza-Martínez A, David H, Riobó P, Miguel I, Orive E (2016). Characterization of a strain of *Fukuyoa paulensis* (Dinophyceae) from the Western Mediterranean Sea. J of Eukaryot Microbiol 63:481-497.

Leonardo S, Rambla-Alegre M, Samdal IA, Miles CO, Kilcoyne J, Diogène J, O'Sullivan CK, Campàs M (2017). Immunorecognition magnetic supports for the development of an electrochemical immunoassay for azaspiracid detection in mussels. Biosens Bioelectron 92:200-206.

Lewis RJ, Inserra M, Vetter I, Holland WC, Hardison DR, Tester PA, Litaker RW (2016). Rapid extraction and identification of maitotoxin and ciguatoxin-like toxins from Caribbean and Pacific *Gambierdiscus* using a new functional bioassay. PLoS One 11.

Litaker RW, Holland WC, Hardison DR, Pisapia F, Hess P, Kibler SR, Tester PA (2017). Ciguatoxicity of *Gambierdiscus* and *Fukuyoa* species from the Caribbean and Gulf of Mexico. PLoS One 12:e0185776.

Medlin LK, Gamella M, Mengs G, Serafín V, Campuzano S, Pingarrón JM (2020). Advances in the detection of toxic algae using electrochemical biosensors. Biosensors 10.

Park DL (1995). Detection of ciguatera and diarrheic shellfish toxins in finfish and shellfish with ciguatect kit. J AOAC Int 78:533-537.

Pisapia F, Holland WC, Hardison DR, Litaker RW, Fraga S, Nishimura T, Adachi M, Nguyen-Ngoc L, Sechet V, Amzil Z, Herrenknecht C, Hess P (2017). Toxicity screening of 13 *Gambierdiscus* strains using neuro-2a and erythrocyte lysis bioassays. Harmful Algae 63:173-183.

Reverté L, Toldrà A, Andree KB, Fraga S, de Falco G, Campàs M, Diogène J (2018). Assessment of cytotoxicity in ten strains of *Gambierdiscus australes* from Macaronesian Islands by neuro-2a cell-based assays. J Appl Phycol 30:2447-2461.

Roeder K, Erler K, Kibler S, Tester P, Van The H, Nguyen-Ngoc L, Gerdts G, Luckas B (2010). Characteristic profiles of ciguatera toxins in different strains of *Gambierdiscus* spp. Toxicon 56:731-738.

Rossignoli AE, Tudo A, Bravo I, Diaz PA, Diogene J, Riobo P (2020). Toxicity characterisation of *Gambierdiscus* species from the Canary Islands. Toxins (Basel) 12:134.

Toldrà A, Andree KB, Fernández-Tejedor M, Diogène J, Campàs M (2018). Dual quantitative PCR assay for identification and enumeration of *Karlodinium veneficum* and *Karlodinium armiger* combined with a simple and rapid DNA extraction method. J Appl Phycol 30:2435-2445.

Tudó À, Toldrà A, Andree KB, Rey M, Fernández-Tejedor M, Campàs M, Diogène J (2018). First report of *Gambierdiscus* in the Western Mediterranean Sea (Balearic Islands). Harmful Algae News.

Tudó À, Gaiani G, Varela MR, Tsumuraya T, Andree KB, Fernández-Tejedor M, Campàs M, Diogène J (2020a). Further advance of *Gambierdiscus* Species in the Canary Islands, with the First Report of *Gambierdiscus belizeanus*. Toxins 12:692.

Tudó À, Toldrà A, Rey M, Todolí I, Andree KB, Fernández-Tejedor M, Campàs M, Sureda FX, Diogène J (2020b). *Gambierdiscus* and *Fukuyoa* as potential indicators of ciguatera risk in the Balearic Islands. Harmful Algae 99:101913.

Vandersea MW, Kibler SR, Holland WC, Tester PA, Schultz TF, Faust MA, Holmes MJ, Chinain M, Litaker RW (2012). Development of semi-quantitative PCR assays for the detection and enumeration of *Gambierdiscus* species (Gonyaulacales, Dinophyceae). J Phycol 48: 902–915.

Conclusions

The achievements of this thesis draw the following conclusions:

- A rapid and simple method based on the use of a portable bead beating device was successfully developed for DNA extraction and applied to laboratory cultures of *Gambierdiscus/Fukuyoa* species and to field samples.
- A strategy based on DNA recombinase polymerase amplification using modified primers together with a sandwich hybridization assay using genera and species-specific capture probes was exploited to develop a colorimetric assay. This system successfully allowed the detection of *Gambierdiscus/Fukuyoa* genera and the discrimination between *G. australes* and *G. excentricus.* With this strategy, it was possible to detect as low as a third of a cell.
- An electrochemical biosensor for the simultaneous detection of *G. australes* and *G. excentricus* was successfully developed. The integration of maleimide-coated magnetic beads (MBs), with species-specific capture probes immobilized on their surface, improved the hybridization efficiency in comparison to the microtiter-plate format. The use of a redox mediator that results into an insoluble product after reaction with the enzyme label of the reporter probe helped in avoiding cross-reaction signals between electrodes of the array. The biosensor allowed the detection and discrimination of as low as 10 cells of both target species. The application of the fast DNA extraction strategy and the dual biosensor to the analysis of field samples allowed the identification of the target species in samples from Majorca and the first report of *G. excentricus* in the Balearic Islands.
- An electrochemical biosensor for the detection of four congeners belonging to two main groups of CTXs (CTX1B and CTX3C) was successfully developed. To achieve this, monoclonal antibodies that specifically recognize the left and right wings of the targeted CXTs were integrated in a sandwich configuration. The use of MBs as immobilization support for the capture antibodies provided an enlarged surface area for the binding of the antibodies and improved the washings steps. The biosensor was successfully applied to the analysis of fish samples from La Reunion naturally contaminated with CTXs, providing results that correlate well with mouse bioassay (MBA) and cell-based assay (CBA). Moreover, the crude extract and fractions of

an individual fished in Cyprus waters, which had resulted positive for CTXs with CBA, were screened with the same strategy, allowing the detection of CTXs.

- The development of a rapid CTXs extraction technique, which reduced the extraction time down to two minutes, was successfully applied to low concentrated pellets of microalgal samples. The biosensor allowed the successful screening of the extracts from laboratory cultures, including the discrimination between two groups of the main CTXs congeners (CTX1B and CTX3C). The application of these strategies (i.e. fast extraction and immunosensor) to the analysis of field samples showed the presence of CTXs traces in some samples. Particularly, in one of them CTXs quantifiable contents were detected, which were similar to those previously observed in the Great Caribbean Region, underlining the need and utility of rapid and easy-to-use tools to monitor potential ciguatera outbreaks.

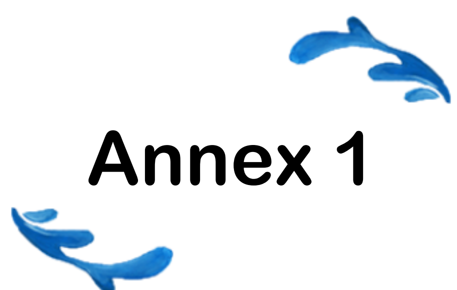
Future work:

- To develop a new technique for the simultaneous extraction and detection of DNA and CTXs from a sample.
- To develop a lateral flow dipstick assay for the detection of CTXs.
- To use antibodies in the clean-up step of natural samples that have to be tested with LC-MS/MS.
- To integrate the developed biosensors into portable devices for the *in situ* detection.

Potential applications:

- The use of the developed biosensor and lateral flow dipstick assay for the dual detection of *G. australes* and *G. excentricus* in routinely analysis of monitoring programs.
- The use of the developed immunosensor for a fast screening of fishes potentially contaminated with CTXs destined to be sold on markets.
- The implementation of the bioanalytical tools in inter-laboratory studies to obtain results from different areas of the world, particularly the ones endemic for ciguatera.

Annexes



Annex 1

Review

Magnetic Beads in Marine Toxin Detection: A Review

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Abstract: Due to the expanding occurrence of marine toxins, and their potential impact on human health, there is an increased need for tools for their rapid and efficient detection. We give an overview of the use of magnetic beads (MBs) for the detection of marine toxins in shellfish and fish samples, with an emphasis on their incorporation into electrochemical biosensors. The use of MBs as supports for the immobilization of toxins or antibodies, as signal amplifiers as well as for target pre-concentration, is reviewed. In addition, the exploitation of MBs in Systematic Evolution of Ligands by Exponential enrichment (SELEX) for the selection of aptamers is presented. These MB-based strategies have led to the development of sensitive, simple, reliable and robust analytical systems for the detection of toxins in natural samples, with applicability in seafood safety and human health protection.

Keywords: Magnetic bead; marine toxin; toxin capture; toxin detection; antibody; aptamer; immunoassay; immunosensor; electrochemical biosensor

1. Marine Toxins

Oceans and their resources have sustained nations for millennia, with seafood being a strong part of cultural identity and tradition. Marine toxins accumulate in shellfish, fish and other seafood, and, even if they do not all represent a threat for the hosting organism, they can be hazardous for human health, and have thus drawn attention from food safety agencies, the seafood industry and scientists worldwide [1]. The presence of marine toxins can have socio-economic impacts, including the closure of production and recreational areas, as well as enforcing changes in the diet of entire populations [2]. Diverse toxins cause different intoxications, which are grouped according to their effects: diarrheic shellfish poisoning (DSP), paralytic shellfish poisoning (PSP), amnesic shellfish poisoning (ASP), neurologic shellfish poisoning (NSP), ciguatera fish poisoning (CFP) and pufferfish poisoning [3]. The marine toxins responsible for these intoxications are produced by microalgae, except for pufferfish poisoning, in which the toxin producer is a bacterium [4].

In recent years, the use of traditional toxicity screening tests such as the mouse bioassay (MBA) is increasingly avoided due to their low sensitivity, low specificity and ethical problems. Chromatographic techniques coupled with several detection methods are powerful and accurate analysis tools, and are routinely used as reference methods for many marine toxins. However, the required instrumentation is expensive and requires trained personnel, and to address these shortcomings, the European Commission encourages the development and use of alternative or complementary methods [5], which are usually based on a functional or structural recognition of the toxin [6]. Cell-based assays (CBAs) are easy to perform, give an overall view of the toxicity of a sample and can detect the presence of unknown toxins. However, they show high variability, which hampers their harmonization, and may not be able to discriminate compounds that share the same mechanism of action. Some enzyme inhibition assays have

been developed, and these assays are relatively easy to apply, but may suffer from enzyme instability as well as from matrix effects, which may interfere with the response. Receptor-based assays (RBAs) are based on the structural recognition of ligands, but the isolation of receptors from animals is not a trivial task, and, additionally, the affinity may not correlate with the toxicity. Immunoassays, based on the affinity between antibodies and target antigens, show high sensitivity. Whilst the structural recognition may not be necessarily related to the toxicity, antibodies are easier to obtain than receptors, and are also more robust, facilitating an easier implementation of immunoassays, as well as immunosensors, which have the added potential benefit of being miniaturisable and portable [7,8].

2. Magnetic Beads

Magnetic beads (MBs) are particles that consist of magnetite (Fe_3O_4) or maghemite (mostly in the face-centered cubic crystal modification γ-Fe_2O_3) and they have a superparamagnetic or a ferromagnetic behaviour, depending on their size and magnetic content [9]. Superparamagnetism is a particular kind of magnetism that occurs in sufficiently small ferromagnetic or ferrimagnetic particles, which exhibit magnetic properties only when placed in a magnetic field, with no residual magnetism once the magnetic field is removed or switched off. Because of the absence of a remnant magnetization, the previously magnetized superstructure decomposes into single particles. Ferromagnetic magnetism, instead, keeps a magnetic moment even when the magnetic field is removed, not allowing superstructures to decompose.

According to Laurent and co-workers [10], numerous chemical methods can be used to synthesize MBs, such as microemulsions, sonochemical reactions, sol-gel syntheses, hydrothermal reactions, hydrolysis and thermolysis of precursors, electrospray syntheses and flow injection syntheses. All these methods have been used to prepare particles with a regular composition and small size. Nevertheless, the most common method for the production of magnetite and maghemite MBs is still the chemical co-precipitation of iron salts.

MBs of different materials, sizes and functionalizations are now commercially available, enabling their conjugation to a broad range of biomolecules or compounds though different reaction chemistries or affinity interactions [11–13].

3. Magnetic Beads in Marine Toxin Detection

The use of MBs, mainly superparamagnetic, in the development of immunoassays and immunosensors for food analysis and clinical diagnosis is garnering increasing interest [14–16], due to the various advantages that the use of MBs can entail, including an increased surface-to-volume ratio, improved assay kinetics, a higher washing efficiency and lower matrix effects. Herein, we describe the exploitation of MBs in different approaches related with the detection of marine toxins, classifying them according to their use as supports, signal amplifiers, capture agents and, finally, for the production of biorecognition molecules. Table 1 gives an overview of the MB uses and functionalizations taken in consideration for this manuscript.

Table 1. Overview of the magnetic bead (MB) uses and functionalizations for their applicability in the detection of marine toxins.

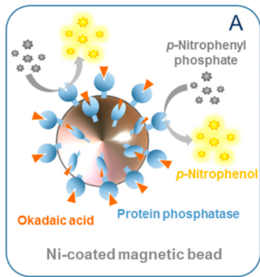
MB Use	Target	MB Functionalization	Conjugation to	Strategy	LOD	Applicability	Ref.
Support	OA	Streptavidin	Biotinylated OA	Colorimetric immunoassay Electrochemical immunosensor	0.8-1.99 μg/L 0.38-0.99 μg/L	Spiked mussels	[17]
Support	OA	Streptavidin	Biotinylated OA	Electrochemical immunosensor	0.15 μg/L	Spiked mussels	[18]
Support	OA	Carboxylic acid groups	OA-BSA	Fluorescence immunosensor	0.05 μg/L	-	[19]
Support	OA	Streptavidin	Biotinylated OA	Colorimetric immunoassay Fluorescence immunosensor	0.5 μg/L 0.05 μg/L	Spiked mussels	[20]
Support	OA	Protein G	Anti-OA mAb	Colorimetric immunoassay Electrochemical immunosensor	1 μg/L 0.5 μg/L	Spiked mussels	[21]
Support	OA	Ni-iminodiacetic acid	Hys tail of PP2A	Colorimetric enzyme assay	30.1 μg/L	Spiked mussels, wedge clams, flat oysters and Pacific oysters	[22]
Support	AZA	Protein G	Anti-AZA pAb	Colorimetric immunoassay Electrochemical immunoassay Electrochemical immunosensor	1.1 μg/L 1.0 μg/L 3.7 μg/L	Naturally-contaminated mussels	[23]
Support	TTX	Maleimide	TTX	Electrochemical immunosensor	1.2 μg/L	Pufferfish	[4]
Support	TTX	Polyethylene glycol	BSA-TTX	Electrochemical immunoassay	5 μg/L	Pufferfish	[24]
Support	TTX	Thiodiglycolic acid	Anti-TTX aptamer	Fluorescence aptamer assay	0.06 μg/L	Spiked human body fluids	[25]
Support	BTX-2/DTX-1	Epoxy groups	anti-BTX-2 mAb anti-DTX-1 mAb	Electrochemical immunoassay	1.8 ng/L 2.2 ng/L	Spiked mussels, razor clams and cockles	[26]
Support	STX	Avidin	Secondary Ab	Electrochemical immunosensor	1.2 ng/L	Spiked seawater and mussels	[27]
Support	STX	Protein G	Anti-STX pAb	Colorimetric immunoassay	~3 μg/L	-	[28]
Support	STX	Protein-G	Anti-STX pAb	Colorimetric immunoassay	~6 ng/L	Naturally-contaminated mussels	[29]
Support	CTX3C	Epoxy groups	Anti-CTX3C mAbs	Electrochemical immunoassay	0.09 ng/L	Spiked and naturally-contaminated fish	[30]
Signal amplifier	OA	Protein G	Anti-OA mAb	SPR immunosensor	1.2 μg/L	Naturally-contaminated mussels	[31]
Capture agent	PSP toxins	Glutaraldehyde	Anti-PSP mAb	HPLC	-	*Alexandrium tamarense* culture	[32]
Capture agent	STX	Protein G	Anti-STX mAb	LC–MS/MS	0.526 μg/L	Spiked human urine	[33]
Capture agent	OA	Protein G	Anti-OA mAb	LC–MS/MS	0.3 μg/L	Naturally-contaminated oysters, mussels, clams and scallops	[34]

Table 1. *Cont.*

Capture agent	DA	C8 alkyl groups	-	MALDI-TOF	-	Sea lion serum	[35]
Production of biorecognition molecules	CTX3C	Streptavidin	Biotinylated CTX3C fragment	Antibody phage display	-	-	[36]
Production of biorecognition molecules	STX	Epoxy groups	KLH-STX	Aptamer SELEX	-	-	[37]
Production of biorecognition molecules	GTX1/4	Amino groups	Carboxylated GTX1/4	Aptamer SELEX	-	-	[38]
Production of biorecognition molecules	PlTX	Carboxylic acid groups	PlTX	Aptamer SELEX; biolayer interferometry aptasensor	0.04 ng/L	Spiked shellfish and seawater	[39]
Production of biorecognition molecules	DA STX TTX	-	GO	Multiplex aptamer SELEX; fluorescence aptamer assay	0.45 µg/L 1.21 µg/L 0.39 µg/L	-	[40]
Production of biorecognition molecules	OA	Tosyl groups	Anti-OA $F(ab')^2$ fragment	Aptamer SELEX	0.33 µg/L	-	[41]

3.1. Magnetic Beads as Supports

The first report of the use MBs as a support for marine toxin detection was in the development of an immunosensor for okadaic acid (OA) [17] (Figure 1A). OA is a lipophilic marine toxin produced by microalgae of the genera *Dinophysis* and *Prorocentrum*. This toxin is accumulated in shellfish and, since its mode of action is related to the inhibition of protein phosphatases (PPs), it can cause DSP in humans. OA was conjugated to biotin and then captured on streptavidin-coated MBs. Once OA was immobilized on the MBs, a colorimetric indirect competitive enzyme-linked immunosorbent assay (ELISA) was performed, where OA in solution competed for interaction with an anti-OA monoclonal antibody (mAb). The authors tested two different sizes of MBs, achieving limits of detection (LODs) of 0.8 µg/L with 2.8 µm-diameter MBs and 1.99 µg/L with 1 µm-diameter MBs. The functionalized MBs were then exploited in an electrochemical immunosensor, where they were magnetically immobilized on screen-printed electrodes (SPEs), and, again, a competitive assay performed. Differential pulse voltammetry (DPV) was used to measure the oxidation of 1-naphthol resulting from the dephosphorylation of 1-naphthyl phosphate by the alkaline phosphatase (ALP) enzyme label, and slightly lower LODs were obtained, with the larger MBs again performing better (0.38 µg/L vs. 0.99 µg/L). It should be noted that whilst larger MBs imply a higher surface area, the amount of MBs used was 10-fold lower and the whole available surface area was lower when using the larger MBs. This immunosensor was then easily integrated into an automated flow-through system [18], one of the advantages of using MBs, achieving an improved LOD of 0.15 µg/L.



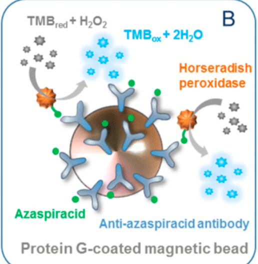


Figure 1. Examples of uses and functionalizations of MBs: (**A**) MBs as supports for enzymes, (**B**) MBs as supports for antibodies, and (**C**) MBs for the production of aptamers.

Moving towards the development of portable devices for field analysis, Pan and collaborators [19,20] described fluorescence immunosensors for the detection of OA. In the first work [19], carboxylic acid-modified MBs were used as a support for the immobilization of OA–bovine serum albumin (OA–BSA), which competed with OA in the sample to bind with an anti-OA mAb. The fluorescence of CdTe quantum dots (QDs) linked to the reporter antibody was detected using a portable flow cytometer (Moxi-Flow), facilitating on-site OA detection and quantification of OA, and achieving an LOD of 0.05 µg/L [19]. In the second work [20], the authors modified the system, using streptavidin-coated MBs with biotinylated OA and a secondary antibody labelled with R-phycoerythrin (R-PE) dye, again achieving an LOD of 0.05 µg/L.

Hayat and co-workers [21] also exploited MBs in a direct immunoassay/immunosensor format for the detection of OA. Instead of conjugating the toxin to the MBs, the anti-OA mAb was immobilized on protein G-coated MBs. OA labelled with horseradish peroxidase (HRP) was used as a tracer in the colorimetric assay, whilst, for the electrochemical immunosensor, no label was used. DPV measurements in a 1 mM $[Fe(CN)_6]^{3-/4-}$ solution showed that the interaction between the toxin and the antibody decreases the current peak of the ferri/ferrocyanide redox probe. Using this detection strategy,

they obtained an LOD of 0.5 μg/L, lower than that obtained with the colorimetric immunoassay (1 μg/L).

An interesting and different approach for the detection of OA is presented in the work of Garibo et al. [22]. In this work, the PP inhibition was measured to detect and quantify the toxin. The authors used genetically engineered PPs with extra-His tails to conjugate the enzymes to Ni-modified MBs. The colorimetric assay attained an LOD of 30.1 μg/L. Although this LOD was more than an order of magnitude higher than that achieved with free enzymes, the immobilization of the PP on the MBs provided higher enzyme activity stability, a crucial parameter, especially when working with these enzymes.

Azaspiracids (AZAs) are lipophilic marine toxins produced by microalgae of the genera *Azadinium* and *Amphiodioma*. Those toxins accumulate in shellfish, and the ingestion of contaminated seafood can lead to azaspiracid shellfish poisoning (AZP), first reported in 1995 [42]. Leonardo and co-workers [23] developed an MBs-based direct immunoassay for AZA detection (Figure 1B). Protein G-coated MBs were functionalized with anti-AZA polyclonal antibody (pAb), and free AZA competed with HRP-labelled AZA (HRP–AZA) for binding to the immobilized antibody in suspension, achieving LODs of 1.1 and 1.0 μg/L, using 3,3′, 5,5′-tetramethylbezidine (TMB) as an enzyme mediator and optical and electrochemical detection, respectively. Additionally, the assay was completed in just 15 min, due to the faster kinetics provided by the use of MBs in suspension. When the biorecognition was performed, immobilizing the Ab-MBs magnetically on the electrode surface, the LOD increased to 3.7 μg/L, which could be attributable to mass transfer limitations. Furthermore, naturally-contaminated mussels were analyzed, and results were similar to the ones obtained with liquid chromatography coupled to tandem mass spectrometry (LC–MS/MS), demonstrating the applicability of the system for monitoring purposes.

Tetrodotoxin (TTX) is a potent natural neurotoxin produced by bacteria that live in endosymbiosis with some other organisms such as pufferfish. Consumption of this contaminated animal may cause intoxication and even death, and the rapid and reliable detection of TTX in pufferfish is thus of enormous importance. Recently, an electrochemical MB-based immunosensor has been developed for the detection of TTX [4]. Oriented and stable TTX immobilization was achieved through the formation of a cysteine monolayer on maleimide-activated MBs, for the subsequent covalent biding of TTX. A competitive assay was again pursued, with TTX in solution competing with the immobilized TTX for binding to an anti-TTX mAb and using an HRP-labelled secondary antibody as a reporter antibody. The immunocomplexes were magnetically captured on an 8-electrode array, and using amperometric detection, an LOD of 1.2 μg/L was achieved. The authors applied the biosensor to the detection of TTX in muscle, skin and the internal organs of two juvenile pufferfishes (*Lagocephalus sceleratus)* from Greece, achieving a good degree of correlation with LC–MS/MS. It had previously been observed that the liver tissue matrix had a marked effect on assay performance, and this effect was almost completely eliminated due to the use of MBs as a support. This work thus demonstrates the advantages that MBs provide in terms of reduction of matrix effects. An alternative electrochemical immunoassay for TTX was described by Zhang and co-workers [24], who synthesized MBs and coated them with polyethylene glycol for subsequent reaction with BSA–TTX. After competition between immobilized TTX and free TTX for a primary anti-TTX antibody, and incubation with an enzyme-labelled secondary antibody, the enzyme product was electrochemically measured. The modification of the working electrode with ionic liquids and carbon nanotubes significantly avoided electrode surface fouling by the enzyme product and improved the sensitivity as compared to bare electrodes, achieving an LOD of 5 μg/L.

Aptamers have also been used for the detection of TTX, as described by Jin and co-workers [25], who conjugated an NH_2-terminated anti-TTX aptamer to thiodiglycolic acid-stabilized Fe_3O_4 MBs. Carxboxylated carbon dots (CDs) were then added, forming Fe_3O_4/aptamer/CDs nanocomposites. When excited at 780 nM, those nanocomposites were observed to have a decreased up-conversion fluorescence emission at 475 nm, attributed to the photo-induced electron transfer (PET) from the CDs

to the aptamer. The addition of TTX caused the unwinding of CDs from the aptamer and subsequent recovery of the up-conversion fluorescence. The system attained an LOD of 0.06 μg/L, and showed high selectivity when tested against other toxins (aflatoxin B_1 and B_2, botulin neurotoxin A and B and *Staphylococcus aureus* enterotoxin A and B), biomolecules (histidine, cysteine, uric acid, ascorbic acid, glucose, glutathione and thiohydracrylic acid) and anions (Cl^-, PO_4^{3-} and CO_3^{2-}) that could interfere in the analysis of human body fluids. The good recoveries obtained in the analysis of spiked gastric juice, serum and urine samples demonstrated the applicability of this aptamer-based optical assay.

Brevetoxin B (BTX-2) is a neurotoxin produced by microalgae such as *Ptychodiscus brevis* and *Gymnodinium breve*. This toxin accumulates in shellfish and, when ingested, can result in death. Additionally, aerosol exposure to BTX-2 during microalgae blooms can cause respiratory irritation [43]. This particular toxin together with dinophysistoxin-1 (DTX-1), an OA analog also responsible for DSP and produced by some *Prorocentrum* and *Dinophysis* species, were selected as targets for the development of a flow-through electrochemical immunoassay [26]. Anti-BTX-2 and anti-DTX-1 mAbs were co-immobilized on MBs. Tracers were synthesized by conjugation of the toxins with cadmium and copper nanoclusters. The incubation of the functionalized MBs with both toxins and their tracers, and the subsequent dissolution of the metal labels and injection into the detection cell, allowed the selective detection of the two toxins using square wave anodic stripping voltammetry, with no cross-reactivity observed. The system showed high cross reactivity with BTX-1, BTX-3, DTX-2 and DTX-3, as expected, and no false positive results from OA, pectenotoxin-6 (PTX-6) or yessotoxin (YTX). LODs of 1.8 ng/L and 2.2 ng/L were achieved for BTX-2 and DTX-1, respectively.

The PSP toxin group comprises saxitoxin (STX) and related compounds produced by marine dinoflagellates of *Alexandrium*, *Gymnodinium*, and *Pyrodinum* species. PSP toxins can accumulate in bivalves, crabs, lobsters and even carnivorous snails [44]. The ingestion of contaminated vectors causes neurotoxic illness that can result in paralysis and, at its acute expression, death. With this target in mind, Jin and co-workers [27] developed a magnetic electrochemical immunosensor for the detection of STX in seawater and seafood. The immunosensor used anti-STX antibody-functionalized MBs and palladium-doped graphitic carbon nitride nanoparticles (peroxidase mimetic) to generate the electrochemical signal. Unlike the other approaches described so far, the assay was non-competitive, because they took advantage of the electrostatic interaction between the electro-positive STX and the electro-negative palladium nanoparticles. The immunosensor successfully detected trace STX amounts in seawater and shellfish samples with an LOD of 1.2 ng/L. Moving towards compact analytical devices, Kim and Choi [28] proposed a lab-on-a-chip (LOC) system for the immunodetection of STX. The LOC system was composed of a sample chamber and a detection chamber connected via a channel. MBs functionalized with anti-STX antibodies were added to the sample chamber together with STX-HRP and the sample containing STX. After incubation, a magnet was used to transport the MBs from the sample chamber to the detection chamber, which had been previously filled with enzyme substrate. The LOD was around 3 μg/L, far below the regulatory level of PSP toxins (800 μg STX per kg shellfish). In 2017, Yu and Choi [29] improved the system by adding an extra washing chamber between the two existing ones, resulting in a decrease in the LOD to around 6 ng/L.

CFP is a human intoxication caused by the ingestion of contaminated fish and is a worldwide health problem. This disease is characterized by severe neurological, gastrointestinal and cardiovascular disorders. Causative toxins of CFP are produced by marine dinoflagellates of the genera *Gambierdiscus* and *Fukuyoa* and are known as ciguatoxins (CTXs). An electrochemical immunoassay for the detection of CTX3C was developed by Zhang et al. [30], where sample injection, incubation, capillary electrophoresis separation and electrochemical detection were all performed in a capillary system. An anti-CTX3C antibody was immobilized on MBs and injected into the capillary system, followed by the addition of CTX3C standard/contaminated samples. A rotating magnetic field was applied to increase mixing efficiency and molecular binding rates. An anti-CTX3C antibody linked to HRP-functionalized gold nanoparticles was then added and sandwich immunocomplexes were formed. Finally, the enzyme product was electrochemically detected, and the system achieved a very low LOD (0.09 ng/L),

almost 17,000 times lower than that obtained with high performance liquid chromatography coupled to mass spectrometry (HPLC–MS). The authors claim that the enhanced sensitivity can be attributed to the use of gold nanoparticles as multi-enzyme carriers, resulting in a high HRP/Ab molar ratio.

3.2. Magnetic Beads as Signal Amplifiers

One of the functionalities of MBs is their ability to amplify signals, as exemplified in the work of Garibo et al. [31], who described the development of a competitive surface plasmon resonance (SPR) optical immunosensor for OA. Protein G-coated MBs were used to immobilize anti-OA antibodies, whilst OA was immobilized on the sensor chip surface. The antibodies were added to the sensor together with a free OA standard/sample, and any binding of molecules to the immobilized OA generated a response proportional to the bound mass. SPR analysis demonstrated that, with conjugates, it is possible to attain similar responses to free antibodies, but using an 8-fold lower antibody concentration. The Ab–MBs resulted in a 3-fold lower LOD, even in the presence of mussel matrix (from 4.7 μg/L to 1.2 μg/L), demonstrating the ability of MBs to be used as signal amplifiers.

3.3. Magnetic Beads as Capture Agents

Immunomagnetic capture (IMC) represents an innovative technique for toxin extraction and purification from complex environmental or biological matrices and is much simpler and more rapid than the use of chromatographic columns. The first example of IMC with marine toxins was reported by Devlin and co-workers [32], who covalently immobilized an antibody to MBs using glutaraldehyde crosslinking for the immunoaffinity extraction of PSP toxins from cultures of the dinoflagellate *Alexandrium tamarense*. After steel ball bearing beating for cell lysis, HPLC measurements showed that toxin recovery increased with increasing amounts of MBs (up to 96.2%), and that the process could be completed within an hour. Recently, Bragg and collaborators [33] coupled IMC with LC–MS/MS for the extraction and detection of STX from human urine. The method showed advantages over conventional protocols, such as an improved selectivity (reducing matrix interference), a 5-fold increase in sensitivity, and requirement of only one third of the sample volume.

IMC combined with LC–MS/MS has also been used by Chen and collaborators [34], in this case for the extraction of OA from shellfish samples. MBs were able to capture the toxin in just 10 min, due to their use of suspension. Additionally, shellfish matrix effects were minimized, and recovery values between 82.2% and 95.5% were obtained for the analysis of oysters, mussels and scallops.

MBs have also been used as capture agents in the work of Neely et al. [35]. In their study, the researchers reported the exposure of C8-coated MBs to blood serum samples from California sea lions to identify patterns of domoic acid (DA) toxicosis. DA can cause ASP and can affect not only humans but also common predators that live in and around marine habitats. Detection of DA was achieved using matrix-assisted laser desorption ionization time-of-flight (MALDI-TOF) mass spectrometry. Artificial neuronal networks (ANN) were trained using MALDI-TOF data from serum analysis, and the obtained models were good predictors of acute DAT. The strategy resulted in a highly sensitive (100% negative predictive value) and a highly specific (100% positive predictive value) diagnostic tool.

3.4. Magnetic Beads to Produce Biorecognition Molecules

Marine toxins are not always easy to find and isolate from field samples. The limited availability of marine toxins has hindered the development of biorecognition molecules and, consequently, of systems for their detection. To address this problem, specifically for CTXs, the use of synthetic toxin fragments has been exploited in the production of antibodies [36]. In this work, streptavidin-coated MBs were used for the panning of phages. In the experiment, a biotinylated synthetic ABC-ring fragment of CTX3C (ABC-PEG-biotin) was incubated with a phage library, and then captured on the streptavidin-coated MBs together with the positive phages expressing hapten-binding antibodies. To select antibodies to the left side of CTX3C, elution was performed with a synthetic CTX3C fragment, instead of the scarce

CTX3C. Following three rounds of selection and amplification, the authors observed an increased recovery of eluted phages, as well as the enrichment of phages bearing Fab fragments. The gene fragments from the sorted phage were sub-cloned for the production of three soluble recombinant Fabs, which had dissociation constants (K_d) of about 10^{-5} M.

MBs have also been used for the production of aptamers, oligonucleotides able to bind to specific target molecules with high affinity and specificity and used as biorecognition molecules in bioanalysis. The in vitro process to obtain aptamers is termed systematic evolution of ligands by exponential enrichment (SELEX), and MBs are frequently used as a support and for the effective partitioning of bound and unbound DNA because they improve the binding kinetics and the washing steps. The first example was described by Handy and co-workers [37], who conjugated STX to keyhole limpet hemocyanin (KLH) using 2,2′-(ethylenedioxy)bis(ethylamine) (Jeffamine) as a spacer compound, for its subsequent covalent binding to epoxy-coated MBs (Figure 1C). The modified MBs were incubated with a random ssDNA library. Bound and unbound DNA were magnetically separated, and the bound ssDNA was eluted from the MBs, PCR-amplified and finally used to enrich the ssDNA library for the following round of selection. After 10 rounds, the PCR product was cloned and sequenced. Preliminary results using SPR showed the affinity of the selected aptamer for STX. A sensor chip modified with DA was used to evaluate the specificity of the aptamer towards this marine toxin, which often co-occurs with STX. Binding was not observed, further supporting that the selected aptamer was specific to STX. Gao and co-workers [38] used a SELEX with MBs to produce aptamers for gonyautoxins 1/4 (GTX1/4). They immobilized the GTX1/4-carboxylated derivative on amine-modified MBs via the EDC/NHS chemistry. In round 2, negative MBs were introduced to remove the ssDNA that bound non-specifically to improve the screening efficiency. In round 3, free competitive counter-molecules were added in the positive incubation system to improve the specificity of screening. After eight rounds of selection, appropriate sequences were obtained. However, these sequences were not further investigated. The same research group developed an aptamer for the detection of palytoxin (PlTX), a toxin initially isolated from soft corals and later found in shellfish, sea urchins and crabs, usually associated with *Ostreopsis* blooms [39]. Counter SELEX was performed against potential interferents, including OA, microcystin-LR (MC-LR), STX, and brevetoxin-A/B, resulting in a highly selective aptamer. The selected aptamer was used to develop an optical biosensor based on biolayer interferometry, where PlTX was immobilized on the biosensor surface, and competed with free PlTX for binding to HRP-labelled aptamer. The addition of 3,3′-diaminobenzidine substrate solution resulted in the formation of a precipitated polymeric product directly on the biosensor surface. Changes in the optical thickness and mass density of biosensor layer were measured, resulting in an LOD of 0.04 ng/L.

Gu and collaborators [40] developed a magnetic separation-based multiple SELEX to simultaneously select aptamers against three different marine biotoxins: DA, STX and TTX. The first 12 rounds entailed mixed screening against the three toxins, and the subsequent four rounds of single screening were against each individual toxin. Additionally to the multiplexing strategy, the authors provided the novelty of combining the advantages of MBs and graphene oxide (GO) for efficient partitioning. A fluorescence assay was developed to determine the affinity of the aptamers, showing K_d values of of 62, 44 and 61 nM for DA, TTX and STX, respectively. Additionally, two multi-target aptamers, which can bind with either DA or TTX, were also obtained.

Finally, an aptamer specific to the antigen binding site of a mAb against OA has been produced using MB–SELEX [41]. The aptamer produced following this strategy mimics the OA structure. In this approach, F(ab′)$_2$ fragments (obtained by pepsin digestion of the anti-OA mAb) were conjugated to MBs and subsequently incubated with the ssDNA library. Negative selection with bare MBs and six additional mAbs (against STX, BTX-2, TTX, DA, nodularin (NOD) and MC-LR) was applied to remove non-specifically bound ssDNA. The produced aptamer was used in two different immunoassays. In the first one, biotinylated aptamer competed with free OA for binding to immobilized anti-OA mAb, followed by the addition of streptavidin-HRP, with the aptamer thus acting as a tracer. In the

second assay, immobilized OA competed with the aptamer for binding to anti-OA mAb, which was subsequently detected using a secondary antibody.

4. Conclusions and Perspectives

Marine toxins play a crucial role in shellfish poisoning, and reliable, rapid and cost effective detection of very low concentrations of these toxins is critical. Currently, MBs have been used in the field of marine toxin detection as supports in assays and biosensors, capture agents for toxin pre-concentration and as tools to produce biorecognition molecules such as phages and aptamers. Because of their advantages in terms of increased surface-to-volume ratio, improved assay kinetics, increased washing efficiency and reduced matrix effects, efficient and highly sensitive analytical systems for the detection of marine toxins have been developed.

The use of MB-based strategies in marine environments can facilitate the confirmation of toxin presence in shellfish at the occurrence of harmful algal blooms (HABs), and speed up monitoring programs. However, to provide biotechnological tools for seafood safety and human health protection, it will be necessary to validate these MB-based approaches. Validation studies will include analyses of multiple samples, of different natures and from different geographic locations, some of them with multi-toxin profiles, and maybe with emerging toxins as challenging targets.

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References

1. Zhao, L.; Huang, Y.; Dong, Y.; Han, X.; Wang, S.; Liang, X. Aptamers and Aptasensors for Highly Specific Recognition and Sensitive Detection of Marine Biotoxins: Recent Advances and Perspectives. *Toxins* **2018**, *10*, 427. [CrossRef] [PubMed]
2. Rongo, T.; van Woesik, R. Socioeconomic consequences of ciguatera poisoning in Rarotonga, southern Cook Islands. *Harmful Algae* **2012**, *20*, 92–100. [CrossRef]
3. Campàs, M.; Garibo, D.; Prieto-Simón, B. Novel nanobiotechnological concepts in electrochemical biosensors for the analysis of toxins. *Analyst* **2012**, *137*, 1055–1067. [CrossRef] [PubMed]
4. Leonardo, S.; Kiparissis, S.; Rambla-Alegre, M.; Almarza, S.; Roque, A.; Andree, K.B.; Christidis, A.; Flores, C.; Caixach, J.; Campbell, K. Detection of tetrodotoxins in juvenile pufferfish Lagocephalus sceleratus (Gmelin, 1789) from the North Aegean Sea (Greece) by an electrochemical magnetic bead-based immunosensing tool. *Food Chem.* **2019**, *290*, 255–262. [CrossRef] [PubMed]
5. European Comission. Regulation (EU) No 15/2011 of 10 January 2011 amending Regulation (EC) No 2074/2005 as regards recognised testing methods for detecting marine biotoxins in live bivalve molluscs. *Off. J. Eur. Union* **2011**, *6*, 3–6.
6. Reverté, L.; Soliño, L.; Carnicer, O.; Diogène, J.; Campàs, M. Alternative methods for the detection of emerging marine toxins: Biosensors, biochemical assays and cell-based assays. *Mar. Drugs* **2014**, *12*, 5719–5763. [CrossRef] [PubMed]
7. Reverté, L.; Prieto-Simón, B.; Campàs, M. New advances in electrochemical biosensors for the detection of toxins: Nanomaterials, magnetic beads and microfluidics systems. A review. *Anal. Chim. Acta* **2016**, *908*, 8–21. [CrossRef] [PubMed]
8. Leonardo, S.; Toldrà, A.; Campàs, M. Trends and prospects on electrochemical biosensors for the detection of marine toxins. In *Recent Advances in the Analysis of Marine Toxins, Comprehensive Analytical Chemistry*, 1st ed.; Diogène, J., Campàs, M., Eds.; Elsevier: Amsterdam, The Netherlands, 2017; Volume 78, pp. 303–341. [CrossRef]
9. Ruffert, C. Magnetic bead—Magic bullet. *Micromachines* **2016**, *7*, 21. [CrossRef] [PubMed]

10. Laurent, S.; Forge, D.; Port, M.; Roch, A.; Robic, C.; Vander Elst, L.; Muller, R.N. Magnetic iron oxide nanoparticles: Synthesis, stabilization, vectorization, physicochemical characterizations, and biological applications. *Chem. Rev.* **2008**, *108*, 2064–2110. [CrossRef] [PubMed]
11. Wu, W.; Wu, Z.; Yu, T.; Jiang, C.; Kim, W.-S. Recent progress on magnetic iron oxide nanoparticles: Synthesis, surface functional strategies and biomedical applications. *Sci. Technol. Adv. Mater.* **2015**, *16*, 023501. [CrossRef] [PubMed]
12. Chen, Z.; Wu, C.; Zhang, Z.; Wu, W.; Wang, X.; Yu, Z. Synthesis, functionalization, and nanomedical applications of functional magnetic nanoparticles. *Chin. Chem. Lett.* **2018**, *29*, 1601–1608. [CrossRef]
13. Duan, M.; Shapter, J.G.; Qi, W.; Yang, S.; Gao, G. Recent progress in magnetic nanoparticles: Synthesis, properties, and applications. *Nanotechnology* **2018**, *29*, 452001. [CrossRef] [PubMed]
14. Cardoso, V.F.; Francesko, A.; Ribeiro, C.; Bañobre-López, M.; Martins, P.; Lanceros-Mendez, S. Advances in magnetic nanoparticles for biomedical applications. *Adv. Healthc. Mater.* **2018**, *7*, 1700845. [CrossRef] [PubMed]
15. Xianyu, Y.; Wang, Q.; Chen, Y. Magnetic particles-enabled biosensors for point-of-care testing. *TrAC Trends Anal. Chem.* **2018**, *106*, 213–224. [CrossRef]
16. Pastucha, M.; Farka, Z.; Lacina, K.; Mikušová, Z.; Skládal, P. Magnetic nanoparticles for smart electrochemical immunoassays: A review on recent developments. *Microchim. Acta* **2019**, *186*, 312. [CrossRef] [PubMed]
17. Hayat, A.; Barthelmebs, L.; Marty, J.-L. Enzyme-linked immunosensor based on super paramagnetic nanobeads for easy and rapid detection of okadaic acid. *Anal. Chim. Acta* **2011**, *690*, 248–252. [CrossRef] [PubMed]
18. Dominguez, R.B.; Hayat, A.; Sassolas, A.; Alonso, G.A.; Munoz, R.; Marty, J.-L. Automated flow-through amperometric immunosensor for highly sensitive and on-line detection of okadaic acid in mussel sample. *Talanta* **2012**, *99*, 232–237. [CrossRef] [PubMed]
19. Pan, Y.; Zhou, J.; Su, K.; Hu, N.; Wang, P. A novel quantum dot fluorescence immunosensor based on magnetic beads and portable flow cytometry for detection of okadaic acid. *Procedia Technol.* **2017**, *27*, 214–216. [CrossRef]
20. Pan, Y.; Wei, X.; Liang, T.; Zhou, J.; Wan, H.; Hu, N.; Wang, P. A magnetic beads-based portable flow cytometry immunosensor for in-situ detection of marine biotoxin. *Biomed. Microdevices* **2018**, *20*, 60. [CrossRef] [PubMed]
21. Hayat, A.; Barthelmebs, L.; Sassolas, A.; Marty, J.-L. Development of a novel label-free amperometric immunosensor for the detection of okadaic acid. *Analytica Chim. Acta* **2012**, *724*, 92–97. [CrossRef] [PubMed]
22. Garibo, D.; Devic, E.; Marty, J.-L.; Diogène, J.; Unzueta, I.; Blázquez, M.; Campàs, M. Conjugation of genetically engineered protein phosphatases to magnetic particles for okadaic acid detection. *J. Biotechnol.* **2012**, *157*, 89–95. [CrossRef] [PubMed]
23. Leonardo, S.; Rambla-Alegre, M.; Samdal, I.A.; Miles, C.O.; Kilcoyne, J.; Diogène, J.; O'Sullivan, C.K.; Campàs, M. Immunorecognition magnetic supports for the development of an electrochemical immunoassay for azaspiracid detection in mussels. *Biosens. Bioelectron.* **2017**, *92*, 200–206. [CrossRef] [PubMed]
24. Zhang, Y.; Fan, Y.; Wu, J.; Wang, X.; Liu, Y. An Amperometric Immunosensor based on an ionic liquid and single-walled carbon nanotube composite electrode for detection of Tetrodotoxin in pufferfish. *J. Agric. Food Chem.* **2016**, *64*, 6888–6894. [CrossRef] [PubMed]
25. Jin, H.; Gui, R.; Sun, J.; Wang, Y. Facilely self-assembled magnetic nanoparticles/aptamer/carbon dots nanocomposites for highly sensitive up-conversion fluorescence turn-on detection of tetrodotoxin. *Talanta* **2018**, *176*, 277–283. [CrossRef] [PubMed]
26. Zhang, B.; Hou, L.; Tang, D.; Liu, B.; Li, J.; Chen, G. Simultaneous multiplexed stripping voltammetric monitoring of marine toxins in seafood based on distinguishable metal nanocluster-labeled molecular tags. *J. Agric. Food Chem.* **2012**, *60*, 8974–8982. [CrossRef] [PubMed]
27. Jin, X.; Chen, J.; Zeng, X.; Xu, L.; Wu, Y.; Fu, F. A signal-on magnetic electrochemical immunosensor for ultra-sensitive detection of saxitoxin using palladium-doped graphitic carbon nitride-based non-competitive strategy. *Biosens. Bioelectron.* **2019**, *128*, 45–51. [CrossRef] [PubMed]
28. Kim, M.-H.; Choi, S.-J. Immunoassay of paralytic shellfish toxins by moving magnetic particles in a stationary liquid-phase lab-on-a-chip. *Biosens. Bioelectron.* **2015**, *66*, 136–140. [CrossRef] [PubMed]
29. Yu, E.; Choi, S.-J. Development of an improved stationary liquid-phase lab-on-a-chip for the field monitoring of paralytic shellfish toxins. *BioChip J.* **2017**, *11*, 30–38. [CrossRef]

30. Zhang, Z.; Zhang, C.; Luan, W.; Li, X.; Liu, Y.; Luo, X. Ultrasensitive and accelerated detection of ciguatoxin by capillary electrophoresis via on-line sandwich immunoassay with rotating magnetic field and nanoparticles signal enhancement. *Anal. Chim. Acta* **2015**, *888*, 27–35. [CrossRef] [PubMed]
31. Garibo, D.; Campbell, K.; Casanova, A.; De La Iglesia, P.; Fernández-Tejedor, M.; Diogène, J.; Elliott, C.; Campàs, M. SPR immunosensor for the detection of okadaic acid in mussels using magnetic particles as antibody carriers. *Sensors and Actuators B Chem.* **2014**, *190*, 822–828. [CrossRef]
32. Devlin, R.; Campbell, K.; Kawatsu, K.; Elliott, C. Physical and immunoaffinity extraction of paralytic shellfish poisoning toxins from cultures of the dinoflagellate Alexandrium tamarense. *Harmful Algae* **2011**, *10*, 542–548. [CrossRef]
33. Bragg, W.A.; Garrett, A.; Hamelin, E.I.; Coleman, R.M.; Campbell, K.; Elliott, C.T.; Johnson, R.C. Quantitation of saxitoxin in human urine using immunocapture extraction and LC–MS. *Bioanalysis* **2018**, *10*, 229–239. [CrossRef] [PubMed]
34. Chen, J.; Tan, Z.; Wu, H.; Peng, J.; Zhai, Y.; Guo, M. Selective enrichment and quantification of okadaic acid in shellfish using an immunomagnetic-bead-based liquid chromatography with tandem mass spectrometry assay. *J. Sep. Sci.* **2019**, *42*, 1423–1431. [CrossRef] [PubMed]
35. Neely, B.A.; Soper, J.L.; Greig, D.J.; Carlin, K.P.; Favre, E.G.; Gulland, F.M.; Almeida, J.S.; Janech, M.G. Serum profiling by MALDI-TOF mass spectrometry as a diagnostic tool for domoic acid toxicosis in California sea lions. *Proteome Sci.* **2012**, *10*, 18. [CrossRef] [PubMed]
36. Nagumo, Y.; Oguri, H.; Tsumoto, K.; Shindo, Y.; Hirama, M.; Tsumuraya, T.; Fujii, I.; Tomioka, Y.; Mizugaki, M.; Kumagai, I. Phage-display selection of antibodies to the left end of CTX3C using synthetic fragments. *J. Immunol. Methods* **2004**, *289*, 137–146. [CrossRef] [PubMed]
37. Handy, S.M.; Yakes, B.J.; DeGrasse, J.A.; Campbell, K.; Elliott, C.T.; Kanyuck, K.M.; DeGrasse, S.L. First report of the use of a saxitoxin–protein conjugate to develop a DNA aptamer to a small molecule toxin. *Toxicon* **2013**, *61*, 30–37. [CrossRef] [PubMed]
38. Gao, S.; Hu, B.; Zheng, X.; Cao, Y.; Liu, D.; Sun, M.; Jiao, B.; Wang, L. Gonyautoxin 1/4 aptamers with high-affinity and high-specificity: From efficient selection to aptasensor application. *Biosens. Bioelectron.* **2016**, *79*, 938–944. [CrossRef] [PubMed]
39. Gao, S.; Zheng, X.; Hu, B.; Sun, M.; Wu, J.; Jiao, B.; Wang, L. Enzyme-linked, aptamer-based, competitive biolayer interferometry biosensor for palytoxin. *Biosens. Bioelectron.* **2017**, *89*, 952–958. [CrossRef] [PubMed]
40. Gu, H.; Duan, N.; Xia, Y.; Hun, X.; Wang, H.; Wang, Z. Magnetic Separation-Based Multiple SELEX for Effectively Selecting Aptamers against Saxitoxin, Domoic Acid, and Tetrodotoxin. *J. Agric. Food Chem.* **2018**, *66*, 9801–9809. [CrossRef] [PubMed]
41. Lin, C.; Liu, Z.-S.; Wang, D.-X.; Li, L.; Hu, P.; Gong, S.; Li, Y.-S.; Cui, C.; Wu, Z.-C.; Gao, Y. Generation of internal-image functional aptamers of okadaic acid via magnetic-bead SELEX. *Mar. Drugs* **2015**, *13*, 7433–7445. [CrossRef] [PubMed]
42. McMahon, T.; Silke, J. Winter toxicity of unknown aetiology in mussels. *Harmful Algae News* **1996**, *14*, 2.
43. Mello, D.F.; De Oliveira, E.S.; Vieira, R.C.; Simoes, E.; Trevisan, R.; Dafre, A.L.; Barracco, M.A. Cellular and transcriptional responses of Crassostrea gigas hemocytes exposed in vitro to brevetoxin (PbTx-2). *Mar. Drugs* **2012**, *10*, 583–597. [CrossRef] [PubMed]
44. Deeds, J.; Landsberg, J.; Etheridge, S.; Pitcher, G.; Longan, S. Non-traditional vectors for paralytic shellfish poisoning. *Mar. Drugs* **2008**, *6*, 308–348. [CrossRef] [PubMed]

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Annex 2

Addressing ciguatera risk using biosensors for the detection of *Gambierdiscus* and ciguatoxins

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Abstract

Ciguatera fish poisoning is a foodborne disease that affects human health since ancient time. It is caused by the ingestion of seafood contaminated with ciguatoxins (CTXs), potent marine neurotoxins produced by microalgae of the genera *Gambierdiscus* and *Fukuyoa.* These genera are endemic of the tropical and subtropical areas of the world but in recent times are found more and more in temperate areas, such as the Mediterranean Sea. In ciguatera fish poisoning process several species of fishes, sharks and marine invertebrates are involved, making its management a difficult task. Moreover, identify contaminated seafood is complicated by the nonexistence of difference between the intoxicated and non-intoxicated one. To cope with this problem, biosensors have been recently developed for the detection not only of CTXs in fish and algal samples but also for the DNA of the microalgae producers. These tools are fast, robust, reliable, easy to use and they could represent a step forward into the *in situ* detection, helping in the ciguatera management and protecting human health.

1. Introduction

Ciguatera fish poisoning (CFP) is a foodborne disease that can cause gastrointestinal, cardiological and neurological symptoms that can last weeks, months or even years and in some cases led to death (Hamilton et al. 2010). CFP occurs when ciguatoxins (CTXs) and precursors enter into food webs being grazed by herbivorous fishes and further bio-accumulated and bio-transformed in carnivores fishes. CTXs are a group of cyclic polyether lipophilic compounds produced by microalgae of the genera *Gambierdiscus* and *Fukuyoa* (Chinain et al. 2021). In the CFP process more than 400 fish species and sharks (Diogène et al. 2017) are implicated (Bagnis et al. 1970) with some being more likely to contain CTXs than others (Halstead 1988). Moreover, it seems that several species of marine invertebrates may be involved in CFP pathways such as sea urchins (Darius et al. 2018) lobster and octopus (Mak et al. 2013), giant clams (Rongo and van Woesik 2011; Roué et al. 2016) and sea stars (Silva et al. 2015). The discrimination between contaminated and uncontaminated specimens is an important challenge since toxic specimens do not look, smell or taste any differently from non-toxic ones. This issue led, in areas endemic for CFP, to the decrease in fish consumption (Nellis and Barnard

1986) to drastic modification in dietary habits (Rongo and van Woesik 2011), to ban the sale of certain high-risk species, causing important financial losses (Sanchez-Henao et al. 2019). Furthermore, in 2020 the International Association for Medical Assistance to Travelers (IAMAT) labeled several countries as "ciguatera at-risk destinations", making CFP an important issue that if not managed correctly could affect the tourism sector, important source of income for endemic populations.

CFP is an extremely complex phenomenon to manage, from the detection of CTXs in natural samples to the diagnosis in patients (some symptoms can be easily misunderstood for other food poisoning). Thus, taking into consideration that an antidote for CFP has not been found yet, the efforts of the scientific community must focus on the prevention, by providing fast and reliable tools for the detection not only of CTXs in fish samples, but also of their precursors directly in *Gambierdiscus* and *Fukuyoa* genera collected in the environment. In this chapter the development of such tools will be presented and discussed together with the known methods to identify CTXs in fish or algal samples and to detect DNA of the microalgae producers.

2. *Gambierdiscus* and *Fukuyoa* global distribution with particular focus on the Mediterranean and Macaronesian regions

Until 1995, *Gambierdiscus* was considered as a monotypic taxon with just one species named *G. toxicus* (Adachi and Fukuyo 1979). Further studies over the past decade resulted in the identification of 18 different *Gambierdiscus* *(G. australes, G. balechii, G. belizeanus, G. caribaeus, G. carolinianus, G. carpenteri, G. cheloniae, G. excentricus, G. holmesii, G. honu, G. jejuensis, G. lapillus, G. lewesii, G. pacificus, G. polynesiensis, G. scabrosus, G. silvae* and *G. toxicus)* (Chinain et al. 1999; Litaker et al. 2009; Fraga et al. 2011; Nishimura et al. 2014; Rhodes et al. 2017; Jang et al. 2018; Kretzschmar et al. 2019) and 4 *Fukuyoa* species (*F. paulensis, F. ruetlzeri, F. yasumotoi, F. koreensis;* Gómez et al. 2015; Li et al. 2021). Among them, 14 are considered able to produce CTXs *(G. australes, G. balechii, G. belizeanus, G. caribaeus, G. carolinianus, G. carpenteri, G. excentricus, G. pacificus, G. polynesiensis, G. scabrosus, G. silvae, G. toxicus, F. paulensis* and *F. ruetlzeri*) (Tester et al., 2020) with different tests and techniques (Chinain et al. 2010a; Fraga et al. 2011; Rhodes et al. 2014; Litaker et al. 2017; Pisapia et al. 2017; Longo et al. 2019; Rossignoli et al. 2020).

Gambierdiscus and *Fukuyoa* genera are endemic of the subtropical areas of the world (35°N and 35°S) (Bienfang et al. 2010; Chinain et al. 2021). In more recent times, they have been identified in temperate areas such as Korea, Japan, New Zealand (Jeong et al. 2012; Nishimura et al. 2014; Rhodes et al. 2017), Gulf of Mexico, coast of North Carolina, Brazil (Gómez et al. 2015; Litaker et al. 2009; Litaker et al. 2017) and also the Macaronesia region (Fraga et al. 2011;

Kaufmann and Böhm-Beck 2013; Fraga and Rodriguez 2014) and the Mediterranean Sea (Aligizaki and Nikolaidis 2008; Aligizaki et al. 2009; Laza-Martínez et al. 2016; Tudó et al. 2018, 2020). The region that hosts the major diversity of *Gambierdiscus* and *Fukuyoa* species is the Pacific, where the presence of 18 out of the 21 currently recognized species has been reported. Thus, it is not surprising that several archipelagos of this region, such as French Polynesia and Cook Islands, are identified as biodiversity "hotspots" of *Gambierdiscus* (Chinain et al. 2021). In addition, the Caribbean region also presents a huge variety of *Gambierdiscus* and *Fukuyoa* species, and it is quite common to find the co-occurrence of 5 or 6 species (Tester et al. 2013). The fact that *G. excentricus* and *G. silvae*, two of the most CTX-producing species, are not as frequently found as other species draws the attention. The observable patchy distributional pattern has been related to their thermal tolerance (Chinain et al. 2021). Another curious circumstance can be observed in the distribution of *G. australes*, which is globally distributed with the exception of the Caribbean region, and the reasons behind these findings are still unknown. Different is the situation of the Indian Ocean, where the distribution of the genera is poorly reported, especially in the coastal areas of Africa. Additionally, most records reported the species as *G. toxicus*, since the identification was mainly performed with microscopy techniques (Turquet 1998; Lugomela 2006) and species confirmation with genetic tools would be advisable. Even if molecular studies identified the presence of few species, like *G. australes* and *G. belizeanus* (Lavenu et al. 2018), more studies are needed to have a correct species composition of this region. Another recently found "hotspot" of *Gambierdiscus* is the northern Macaronesian region, with the Canary Island hosting the highest biodiversity and the highest number of CTX-producing species (*G. australes, G. caribaeus, G. carolinianus, G. excentricus* and *G. silvae*) (Fraga et al. 2011; Fraga and Rodriguez 2014; Pisapia et al. 2017; Rodríguez et al. 2017; Reverté et al. 2018). Hence, the variety of species found made Rodriguez and coworkers (2017) think that *Gambierdiscus* settlement in the region can be dated to ancient time. Actually, a first report of *Gambierdiscus* in Cabo Verde can be attributed to Silva (1956), reported as *Goniodoma* (Fraga et al. 2011). Recently, Soler Onis identified several cells of *G. excentricus* in the waters of the Cabo Verde archipelago, confirming the presence of the genus (Soler Onis et al. 2019).

Despite the fact that the settlement of a *Gambierdiscus* and *Fukuyoa* species and the finding event can be separated by several decades, there is a general concern that the geographic range of these two genera, and especially of the CTX-producing species, will expand as a consequence of the rise of sea surface temperature (Tester et al. 2010). According to Parsons et al. (2010), a significant modification in the distribution and the abundances of ciguateric

species is to be expected, with some species becoming more dominant over others.

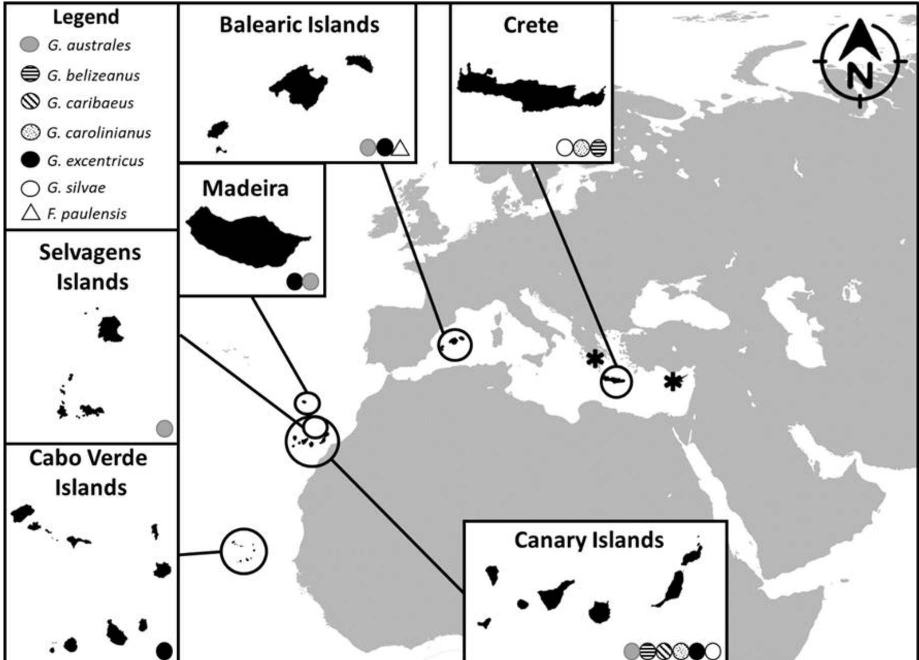


Figure 2. *Gambierdiscus* and *Fukuyoa* distribution in Mediterranean and Macaronesian waters. Symbol * indicates the places (Greece and Cyprus) in which the presence of both *Gambierdiscus* and *Fukuyoa* was reported only at genus level. For the global distribution, see Tester et al. (2018).

3. Ciguatoxins

CTXs are secondary metabolites produced by the marine benthic dinoflagellate of the genera *Gambierdiscus* and *Fukuyoa*. CTXs are cyclic polyether compounds with a rigid structure formed by 13-14 rings connected with ether bonds. CTXs target the binding site 5 of the voltage-gated Na^+ channels (Lombet et al. 1987), inducing effects at the cellular and physiological levels, such as membrane excitability, release of neurotransmitters (Molgó et al. 1990), increase of intracellular calcium (Molgó et al. 1993) and blockage of voltage potassium channels (Hidalgo et al. 2002). The affinity of the different congeners of CTXs for the binding site on the voltage-dependent Na^+ channels is proportional to their toxicity in mice (Lewis 1994).

Up to date, 34 different CTX congeners have been described and grouped in Pacific (P-CTX) (22 congeners), Caribbean (C-CTX) (12 congeners) and Indian (I-CTX) (no congeners described yet), according to their geographical origin (Longo et al. 2019). CTX1B was the first one to be identified in 1990 by

Murata and coworkers (Murata et al. 1990), followed by the description of many other congeners. Additionally, in order to classify the different congeners of P-CTXs, Legrand et al. (1998) proposed to distinguish them into two different groups according to the number of carbons and the structure of the E ring (7 in the CTX1B group and 8 in the CTX3C group) and to the presence (CTX1B) or absence (CTX3C) of the 4-carbon side chain of the left wing (Figure 3). Afterwards, two CTXs from the Caribbean Sea (C-CTXs) were isolated by Vernoux and Lewis (1997) and identified structurally in 1998 (Lewis et al. 1998). Subsequently, other congeners were identified by Pottier et al. (Pottier et al. 2002). Also, six Indian Ocean CTXs (I-CTXs) were isolated (Hamilton et al. 2002; Diogène et al 2017), but their structural determination remains undescribed. Alongside with the CTXs bioaccumulation in fish flesh and through the marine food webs, CTXs undergo metabolization processes in fish (Ikehara et al. 2017), resulting in more toxic compounds, as observed in fish samples from the Pacific area (Chinain et al. 2010b). The occurrence of these different toxins in fish and microalgal samples can vary. Nevertheless, P-CTX-1 (CTX1B) is found as dominant in toxin profiles in the carnivorous fishes of the Pacific (Lewis et al. 1991). The toxicity of CTXs in mice (i.p.) is equivalent to an LD_{50} 0.25, 2.3 and 0.9 µg/kg for P-CTX-1, P-CTX-2 and P-CTX-3, respectively (Lewis et al. 1991), classifying them as extremely potent marine toxins. Generally, P-CTXs are more potent than C-CTXs (LD_{50} of 3.6 and 1 µg/kg for C-CTX-1 and C-CTX-2) and I-CTXs (5 µg/kg). In humans, it has been estimated that no more than 1 ng P-CTX-1 per kg of body weight is needed to cause the occurrence of mild CFP symptoms (Lehane and Lewis 2000). Moreover, these toxins are heat resistant, so they cannot be deteriorated by cooking processes (Abraham et al. 2012).

CTX1B : R = OH
54-deoxCTX1B : R = H

CTX3C : R = H
51-hydroxyCTX3C : R = OH

Figure 3. Structure of the two main groups of CTXs congeners: CTX1B and CTX3C.

The United States Food and Drug Administration (US FDA) proposed guidance levels of ≤0.01 μg/kg of CTX1B and ≤0.1 μg/kg of C-CTX-1 equivalent toxicity in fish, and these values represent the only existing suggested threshold. In fact, New Zealand and Australia provide general guidelines (FZAN 2006) and Japan (MHWL 1953, 2001) banned from the market several species associated with ciguatera. In European markets, no fish product containing CTXs can be sold (Regulation (EC) No. 853/2004), but no regulatory limits have been established and no suggestion regarding the analytical methodology to use is given, although the European Food Safety Authority (EFSA) has adopted the FDA guidance levels for CTXs (EFSA, 2010). Thus, the creation of fast, reliable and easy to use tools for the detection of even small quantities of CTXs in fish and algal samples can be of outmost help for the managing of CFP.

4. Methods for *Gambierdiscus* and *Fukuyoa* detection

The presence of highly toxic *Gambierdiscus* and *Fukuyoa* species in a given area is likely to contribute to the final toxic profile in fishes. Therefore, document the presence of these ones, that might not be dominant in terms of cell concentration, but whose contribution in the environmental flux of CTXs is noticeable is of extreme importance (Longo et al. 2019). Thus, it should not surprise the reader that scientists all over the world focused their efforts mainly in the detection of these CTX producing species in field samples. The most known and used technique is light microscopy, followed by electron

microscopy. These strategies are still used, but it is almost impossible to differentiate between species using them alone. Hence, the use of genetic sequencing is almost mandatory to correctly identify to the species level (Bravo et al. 2019). In fact, in every study concerning the detection of species, especially in environmental samples, the confirmation with sequencing is highly requested to support the findings obtained with other methods. Among the existing sequencing procedures certainly the Sanger has been the most used since its invention in 1977. Other molecular techniques are more and more implied in the identification of microalgal species in field samples. In fact, quantitative polymerase chain reaction (qPCR) has been used on several occasions for the identification and quantification of *Gambierdiscus/Fukuyoa genera, G. belizeanus, G. caribaeus, G. carolinianus, G. carpenteri* and *G. ruetzleri, G. australes, G. scabrosus, G. excentricus, G. silvae*, *G. lapillus* and *F. paulensis* targeting the D1-D2 regions (Smith et al. 2017), the D1-D3 region (Vandersea et al. 2012; Litaker et al. 2019; Kretzschmar et al. 2019), or the D8-D10 (Nishimura et al. 2016; Kretzschmar et al. 2019) of the large subunit (LSU) ribosomal gene. This method is strong and reliable, but it requires the use of a thermocycler, is laboratory based and time consuming. As a solution to these drawbacks, our group used an isothermal technique that, with the use of modified primers, allowed obtaining, in 30 min and at a constant temperature of 37 °C, an amplified product flanked with oligonucleotides tails. Then, a sandwich hybridization assay was performed, and thanks to the use of capture probes and a horseradish peroxidase (HRP)-labeled reporter probe, it was possible to differentiate between *Gambierdiscus/Fukuyoa* and other microalgae genera and among two toxin-producing species (*G. australes* and *G. excentricus*) and their congeneric species (Gaiani et al. 2021a). Despite its advantageous features, this technique is not commonly used, mostly because the kit needed to perform the test is expensive and its efficiency gets lower and lower once opened, compromising the inter-day reproducibility. Meanwhile, researchers have focused on the development of PCR-based techniques that could be implemented into portable devices for the *in situ* detection of microalgae. Recently, a PCR-lateral flow assay (PCR-LFA) was developed targeting the same two CTX producing species (Ginés et al., 2021). The assay requires less than 1.30 h to be performed, PCR included. Again, tailed primers are used and specific detection is achieved with the aid of capture probes and single-chain Cro proteins conjugated with carbon nanoparticles used as labels. This technique represents a step forward towards field analysis, although further studies, including the screening of natural samples, need to be performed. The advantage of using modified primers, whose use results in tailed amplified products, is undoubtable, and for this reason, this strategy has been chosen to develop biosensors, which are fast, robust, easy to use and reliable tools that can pave the way of the *Gambierdiscus* species detection directly in the field.

5. Methods for ciguatoxin detection

Ciguatera is a threat for human health at least since the sixteenth century when the first intoxication was dated. Therefore, a huge variety of methods have been developed to detect CTXs and therefore identify contaminated fishes. Up to date, the techniques developed include native tests, animal mortality tests, cell-based assays (CBAs), receptor-binding assays (RBAs), immunological assays and instrumental analysis (such as LC-MS/MS) (Hoffman et al. 1983, Chinain et al. 2010b, Reverté et al., 2014; Diogène and Campàs, 2017; Pasinszki et al. 2020).

Several animal tests have been developed throughout the years and even though none of these is applied nowadays for the detection of CTXs or other marine toxins, there is one that is still in use: the mouse bioassay (MBA) (ANSES, 2015). The MBA is useful since it provides a compositive toxicological response, which is very convenient in case of samples with unknow toxicity. Apart from sharing the limitation of the other animal-based tests, it has a limit of detection that is approximately 0.56 ng/g for P-CTX-1B (EFSA 2010), meaning that it does not attain the suggested FDA threshold. Therefore, part of the scientific community switched to the use of assays based on mammalian cell, instead of entire animals.

The CBAs developed for CTXs detection are based on the activity of these toxins on neuronal potassium and voltage-gated sodium channels (VGSCs) (Lewis and Vetter 2016) and involve a huge assortment of cells and tissues, from blood, used for the development of hemolytic assays (Shimojo and Iwaoka 2000), the guinea pig ileums (Endean et al. 1993), the guinea pig atrium (Hokama et al. 1994), frog nerve fibers (Benoit et al. 1986) and crayfish nerve cords (Miller et al. 1986). Despite this variety of available tests, nowadays the most used test is the mouse neuroblastoma cell assay (N2a CBA) (Manger et al. 1993). This test has demonstrated to be very sensitive and provides a composite toxicity response for the several existing CTXs. Additionally, the test is easy to perform and interpret. Briefly, it is based on the colorimetric detection of metabolically active N2a cells exposed to CTX in presence of ouabain/veratridine (Manger et al. 1993). The detection of CTXs requires the addition of veratridine, that is a VGSC activator with a different binding site than CTX, and ouabain, a sodium/potassium pump inhibitor. The combined effect of these three substances together increases the concentration of intracellular sodium, which has a negative effect on cell viability and can be measured as a function of CTX concentration. The amount of toxin is measured with the MTT-based CBA, in which the MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) is added in each well, reduced by mitochondrial dehydrogenase activity into a formazan product, which is later solubilized and whose absorbance intensity is proportional to the number of live cells and so inversely proportional to the concentration of CTXs, within a certain range. Even if the limit of quantification (LOQ) is different in each experiment, in general it is lower than the clinically relevant toxin levels in fish tissue, and of the FDA suggested threshold. However, a consensus protocol for the MTT-based CBA is still lacking since users throughout the last decade made customized changes to the assay (Viallon et al. 2020). The modifications

included almost every aspect of the test, starting from the cell seeding densities, the cell layer viability, the MTT incubation time, up to the ouabain/veratridine treatment. Other N2a CBAs have been developed. For example, in the study of Fairey et al. (1997), they used N2a cells that expressed c-*fos*-Luciferase reporter gene. The c-*fos* is a response gene and a sensitive biomarker that easily localize the effects of toxins. Detection is achieved with luciferase-catalyzed light generation and a luminometer for quantification. Additionally, cell lines other than N2a have been exploited for application in CBA. In particular, the human neuroblastoma cell line SH-SY5Y has been used to develop another fluorescent assay (Lewis et al. 2016). In this test, cells were loaded with a dye containing calcium adsorbed into the cytoplasm, and then incubated with veratridine and subsequently with CTXs. Fluorescence responses to CTXs were measured as the increase of calcium ion influx into cells with a plate reader. These fluorescent assays are not commonly used due to the costs of the fluorescent dye, the need of specialized equipment, and the sensitivity to maitotoxin presence, which can affect enormously the outcome of the test. Indeed, the interfering effects caused by maitotoxins, other toxic compound or the natural matrix itself (i.e. fish or algal extracts) can induce an over or under estimation of the CTXs content. Additionally, the cytotoxicity assays respond similarly to all the toxins that block VGSCs (i.e. brevetoxin) and, therefore, it will be impossible to distinguish one from another.

In order to focus more on the affinity of CTXs for their binding site on the VGSC, RBAs have been developed. Since CTXs share with brevetoxins the same binding site on the VGSC (i.e. binding site 5) but with a higher affinity, they can be considered as competitors of brevetoxin binding (Fairey et al. 1997, Bottein Dechraoui et al. 2005). Therefore, measuring the competition binding of a radioactively labeled brevetoxin ([3H]-brevetoxin-3) and CTXs for the receptor sites in a membrane can be used to estimate the amount of CTXs in an extract. Hence, the concentration of the labeled brevetoxin (that is maintained constant) should decrease after the addition of CTXs, and a competition dose-response curve can be obtained. This screening method has been widely used, but it is highly sophisticated, making the comparison between laboratories quite complicated. Thus, Díaz-Asencio and coworkers (Díaz-Asencio et al. 2018) made the effort to provide guidance on its quality control checks for the analysis of environmental samples, reaching an LOD of 0.75 ng/g of P-CTX-3C in fish samples in their optimized assay. However, these assays imply the use of radioactive compounds. To avoid the use of instable radioactive compounds, a fluorescence-based RBA has been developed, where CTXs compete with a fluorescently labeled brevetoxin (brevetoxin-2) (McCall et al. 2014). Following these studies, Hardison and coworkers developed a fluorescent RBA using a brevetoxin-2 labeled with BODIPY® (Hardison et al. 2016), which provides a LOD of 0.075 ng/g of P-CTX-3C equivalents. Moreover, a commercial kit for CTXs based on this study has been marketed by Sea Tox Research Inc. (Wilmington, NC, USA https://www.seatoxresearch.com/testing-kits/) and can be used as screening tool for fish extracts.

Despite the undoubtable utility of the kit described above, it does not allow to know which CTXs are inside a sample. The best solution to obtain toxin profiles is to separate the toxins and HPLC is the method to perform this task. Since most CTXs do not have a characteristic chromophore group in their structure (i.e. alternating single and double bounds), they do not strongly absorb radiation over the UV/VIS region, and therefore spectroscopy is not viable for their detection. Indeed, the trials with classical HPLC method that uses UV detector showed not enough sensitivity to detect the presence of low concentrations of CTXs (Caillaud et al. 2010). Therefore, the HPLC with fluorescent detection has been tried, since some CTX congeners have a primary hydroxyl group available for fluorescent labelling. Even if this technique showed better sensitivity than the previous one, it does not detect CTXs at the recommended level (0.01 µg/kg). Additionally, it does not detect CTXs without a primary hydroxyl group (i.e. P-CTX-3C). Therefore, in order to increase the sensitivity and specificity of the system, Lewis and Jones (1997) combined the HPLC technique with tandem mass spectrometry (HPLC-MS/MS) for the detection of CTXs. Then, Lewis and coworkers (1999) combined an electrospray triple quadrupole mass spectrometer with a gradient reverse-phased HPLC and, with this technique, a limit of detection of 0.04 ppb and 0.1 ppb for P-CTX1 and C-CTX1 was achieved (Lewis et al. 1999). Right after this first trials, LC-MS/MS become one of the most used, if not the most used, techniques for detecting and identify CTXs. It must be underlined that CBA is the most used technique to perform sample screening, even though LC-MS/MS is the one that actually confirms the presence of CTXs. Although instrumental analysis techniques are highly sensitive, their application to monitoring programs is hampered by the cost of the machinery, the time needed to prepare the samples for the analysis and the need of highly trained personnel to perform the assays. Additionally, the analysis of CTXs in natural samples is limited by the lack of CTX standards, certified materials and the chemical complexity of the CTX compounds.

These limitations have also hindered the development of immunoassays, based on antibodies (Abs). These assays take advantage from the high specificity of the antigen-antibody reaction. The first group to produce anti-CTXs antibodies was the one of Hokama and coworkers (1977). In their work, they produced an anti-CTX polyclonal Ab (pAb) and labelled it with a radioactive compound to subsequently perform a radioimmunoassay directly on fish tissues from the Hawaiian Islands (Hokama et al. 1977; Kimura et al. 1982). The same pAb was labelled horseradish peroxidase and exploited in an immunoassay also for fish extracts (Hokama et al. 1983). The authors decided to simplify the enzyme immunoassay by formatting it into a faster stick test that did not require any instrumentation (Hokama 1985). These last findings were used to build two commercial kits named Cigua-Check (Hokama 1985; Hokama et al. 1987) and Ciguatect (Park 1995). Although these achievements represent an advance for the development of easy-to-use tests, since no extraction whatsoever was needed to perform the assay, these assays showed cross-reactivity with okadaic acid and brevetoxin (Hokama et al. 1987; Hokama et al. 1989. This cross-reactivity together with the low sensitivity led to false positive and false

negative results, respectively (Bienfang et al. 2011). Thus, the only fish that was reported as ciguateric up to date in the Mediterranean, which was analyzed with the Cigua-Check kit (Bentur and Spanier 2007), is still pending of confirmation. Due to the disadvantages represented by the use of these pAbs, Hokama and coworkers (1990) decided to focus on the production of monoclonal antibodies (mAbs), that were subsequently used in a similar system but using colored latex beads for the labelling of the mAbs. Another approach to produce mAbs was based on the use of synthetic haptens instead of natural CTXs. The first work related to the use of such technique is the one of Campora and coworkers (Campora et al. 2008), which developed a sandwich enzyme linked immunosorbent assay ELISA, using one specific Ab for the left wing of P-CTX1B and one specific Ab for the right wing labeled with HRP. No cross-reactivity was observed with other marine toxins such as brevetoxin-3, okadaic acid or domoic acid. Subsequently, Tsumuraya and coworkers immunized mice with haptens that mimic the left and right wing of the four principal congeners of pacific CTXs, CXT1B, 54-deoxy-CTX1B, CTX3C and 51-hydroxy-CTX3C. The resulting antibodies were used to develop colorimetric sandwich ELISAs. The assays performed with these mAbs demonstrate the high specificity and sensitivity that was expected, showing no cross-reactivity with other marine toxins such as okadaic acid, maitotoxin, brevetoxin A and brevetoxin B (Oguri et al. 2003; Nagumo et al. 2004; Tsumuraya et al. 2006, 2010, 2012). Additionally, the previously described mAbs have been used to develop a fluorescent ELISA, whose LOD was as low as 1 pg/mL for both CTX1B and CTX3C group. Moreover, CTX1B was spiked into a fish extract at the suggested threshold and then detected with the presented technique (Tsumuraya et al. 2018; Tsumuraya and Hirama 2019). Based on this fluorescent technique, a kit named “CTX-ELISATM 1B” for the detection of the CTX1B group of congeners was marketed and can be bought from Fujifilm Wako Corporation (Osaka, Japan). Since the results obtained with this strategy seemed to be very encouraging, the mAbs produced by the group of Tsumuraya were integrated in the development of a biosensor, a rapid, reliable and cheap tool for the screening of fish and algal samples.

6. Biosensors

Biosensors constitute practical and reliable tools to detect biological and chemical hazards. They are composed of a biorecognition element that interacts specifically with a target molecule, and a transducer that converts the biorecognition event into a quantifiable signal, both in intimate contact. The biorecognition element is the one that gives specificity to the system, and it could be an enzyme, antibody, oligonucleotide, aptamer, receptor, whole cell, bacteria, microorganism, animal or vegetal tissue. The transducer can be electrochemical, optical, gravimetric, thermometric, according to the type of signal they transform in a measurable unit. Even if biosensors represent an interesting and useful tool for the detection of different type of analytes, they have been rarely used to detect DNA of toxin-producing microalgae. This has

been the case for *Karenia brevis* (LaGier et al. 2007), *Karlodinium armiger* (Magriñá et al. 2019), *Ostreopsis ovata* (Toldrà et al. 2019) and for some species of *Gambierdiscus (G. australes, G. excentricus* and *G. silvae), Coolia (C. monotis, C. tropicalis* and *C.* cf. *canariensis), Ostreopsis* genus and *Prorocentrum lima* (Medlin et al. 2020). On the contrary, several biosensors have been developed for the detection of marine toxins, such as surface plasmon resonance immunosensors (palytoxins, tetrodotoxins), surface plasmon resonance receptor-based biosensors (palytoxins), electrochemical immunosensors (tetrodotoxins, okadaic acid, azaspiracids, domoic acid, saxitoxins, palytoxins, brevetoxins), electrochemical enzyme-based sensors (okadaic acid), electrochemical aptasensors (okadaic acid, brevetoxin-2, saxitoxin, tetrodotoxins), electrochemical cell-based biosensors (palytoxin), electrochemiluminescence immunosensors (palytoxins) (for more details see Reverté et al. 2014; Leonardo et al. 2017; Campàs et al. 2021), although none of them targeted CTXs. In the following paragraphs, the existing biosensors for the detection of *Gambierdiscus* DNA and CTXs will be described and commented.

6.1. Biosensor for the detection of DNA from *Gambierdiscus*

The current increase in the reports of *Gambierdiscus* species (Aligizaki and Nikolaidis 2008; Tudó et al. 2018; Gaiani et al. 2021b) in Mediterranean and Macaronesian waters has raised the need to have quick and fast tools to detect those species directly in the field. In recent years, rapid and reliable molecular-based biosensors for the detection and enumeration of marine microalgae species have been developed as an alternative to the traditional light microscopy technique. In their work, Medlin et al. (2020) designed probes for the detection of several species of the genera *Gambierdiscus, Ostreopsis, Coolia* and *Prorocentrum.* These probes were used in the development of sandwich hybridization assays, where streptavidin-coated magnetic beads (MBs) were used for capture probe immobilization and HRP as a label to target DNA or RNA. Oligocomplexes were immobilized on the surface of screen-printed carbon electrodes (SPCEs) with the use of a customized case containing a magnet placed underneath the working electrode and immersed in an electrochemical cell containing 1mM hydroquinone (HQ) under constant agitation). Measurements were performed with amperometry. The reduction current intensity was proportional to the concentration of the RNA/DNA target and, consequently, to the number of microalgal cells. The specificity towards *G. australes, G. excentricus* and *G. silvae,* species that are commonly found in the Canary Islands, was tested. The LOD achieved was close to 1 pM of RNA, but no corresponding quantification in terms of cell abundance was established. Nevertheless, for the other dinoflagellates targeted in the study, the number of cells corresponding to 1 pM of RNA ranged from 10 to 444 cultivated cells, so probably the LOD for *Gambierdiscus* species would be similar or even lower.

The technique presented by Medlin and coworkers is fast, since no PCR step is performed (although it would be necessary to detect DNA from field samples), cost effective and reliable, but it has been tested only with synthetic DNA and it has not been applied yet to the analysis of genomic DNA/RNA or the screening of field samples.

More recently, our group (Gaiani et al. 2021b) described the first electrochemical biosensor for the simultaneous detection of the two CTX producing species *G. australes* and *G. excentricus* (Figure 4). Similarly to Medlin et al. (2020), we used a sandwich configuration, although the strategy was different. We designed species-specific capture probes for *G. australes* and *G. excentricus*, which were immobilized on the surface of maleimide-coated MBs. Subsequently, PCR was performed using primers for both *G. australes* and *G. excentricus* at the same time. These particular primers included tails that, after PCR, gave amplified products flanked with single-stranded oligonucleotides at each end. Afterwards, the amplified products were incubated with the MBs modified with the specific capture probes, and then an HRP-labelled reporter probe was added. For the electrochemical detection, the *G. australes* oligocomplexes were immobilized on one of the working electrodes of a dual electrode array, and the *G. excentricus* oligocomplexes were immobilized on the other one. As in Medlin et al. (2020), a customized plastic case with magnets placed underneath the working electrodes was used for MBs immobilization. Nevertheless, our electrodes were used in a horizontal configuration, which requires smaller sample volumes to operate. Finally, 3,3′,5,5′-tetramethylbenzidine (TMB) Enhanced One Component HRP Membrane Substrate was added and incubated for 10 min. The use of this particular compound allowed the simultaneous detection of amplified DNA on both electrodes because, when oxidized, it precipitates on the electrode on which the chemical reaction is happening, without interference with the other one. Measurements were performed with amperometry. The reduction current intensity was proportional to the amount of amplified product and consequently to the number of microalgal cells present in the samples. In our work, the LOD of the system was studied starting from a 10^4 cell pellet, performing 1/10 serial dilutions and extracting each pellet dilution with a newly developed fast technique, based on a portable bead beater device and combined with magnetic beads for DNA capture. Since the DNA extraction part is crucial, in order to assess its efficiency, DNA dilutions (i.e. extraction of DNA from 10^4 cells, and subsequent 1/10 serial dilutions) were also tested and compared. In both cases, an LOD of 10 cells was reached for the target species. Additionally, the simultaneous detection of these two species at different cell concentrations and ratios was successfully achieved. Finally, field samples collected in Majorca (Balearic Islands, Spain) were screened, obtaining results similar to the estimations provided by light microscopy. In comparison with Medlin et al. (2020), the strategy presented by our group is longer and less species were targeted. Nevertheless, not only did it allow the detection of genomic DNA extracted from laboratory cultures of target species, but also the screening of

field samples, including the first report of *G. excentricus* in the Balearic Islands waters. The detection strategy proposed combined with the fast extraction technique represent an important step forward in practical application of *in situ* detection of toxin producing species, providing punctual warnings of CTXs presence in an area and so facilitating quick management decisions. Moreover, this system can be easily modified for the simultaneous detection of other microalgae species (pending the design of tailed primers that do not cross-react with each other), and so it could be useful for the assessment of other marine-related diseases.

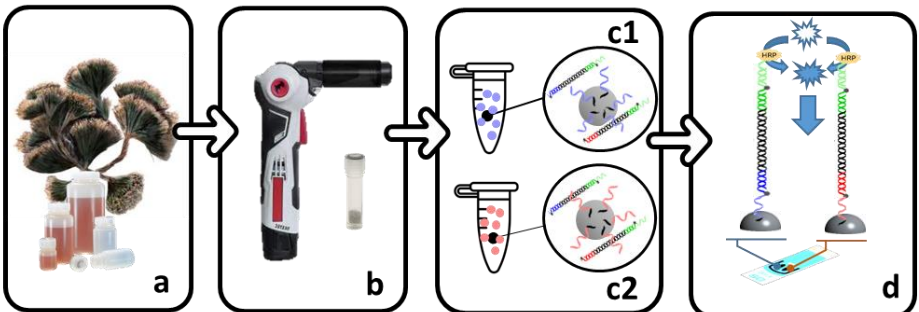


Figure 4. Schematic representation of the strategy developed for the dual detection of *G. australes* and *G. excentricus* (Gaiani et al. 2021b). Field samples collection (a) was followed by a fast DNA extraction procedure (b) and amplification with multiplex PCR procedure. Amplified products were exposed to the capture probes specific for *G. excentricus* (c1) and *G. australes* (c2) conjugated with maleimide-coated magnetic beads and to HRP-labeled reporter probe. Electrochemical detection was achieved by immobilizing the oligocomplexes on the working electrodes of a dual array, adding TMB substrate and measuring the reduction current with amperometry (d).

6.2. Biosensors for the detection of CTXs

Ciguatera symptoms can appear between 1 and 48 hours after the ingestion by the consumer, which means that the ingestion and the symptoms can occur in different places, making difficult spotting ciguatera outbreaks. Therefore, the development of fast, reliable and easy-to-use tools for the screening of fish extracts are of extreme interest, not only for scientific purposes but also for fisheries and public health. In the next paragraphs, a description of the few existing biosensors for CTXs is provided (Table 1).

6.2.1. Detection of CTXs in fish samples

The detection of CTXs in fish samples is a challenging task. Therefore, researchers are trying to focus on the development of simple, fast and cheap systems for sample screening and quantification. Biosensors, which additionally can be made portable, may be the solution, which would require

analytical instrumentation only as a confirmation technique. Recently, the first biosensor for CTXs detection was developed by our group (Leonardo et al. 2020), targeting the detection of four congeners belonging to the CTX1B and the CTX3C groups. Three different mAbs, two capture ones, which specifically bind to the right wing of the CTX1B and 54-deoxyCTX1B (3G8) and of the CTX3C and 51-hydroxyCTX3C (10C9), and a detector one, which has either a specific bind or a cross-reactivity with the left wing (8H4) of all the four congeners, were used (Oguri et al. 2003; Nagumo et al. 2004; Tsumuraya et al. 2006, 2010, 2012, 2018) (Figure 5). MBs were used for the immobilization of the capture mAbs. Then, the mAb-functionalized MBs were exposed to CTX standards (CTX1B or 51-OH-CTX3C) or extracts of fish naturally contaminated with CTXs, followed by the addition of the detector antibody previously biotinylated. Subsequently, polyHRP-streptavidin was incubated and, finally, the immunocomplexes were placed on the working electrodes of an eight-electrode array. Again, a plastic support with magnets underneath each working electrode was used to block MBs in the right position. Then, TMB liquid substrate was incubated, and the reduction current intensity was measured with amperometry. The LODs obtained were 1.96 pg/mL of CTX1B and 3.59 pg/mL of 51-OH-CTX3C. The effects of the fish matrix on the detection of CTX1B congener were evaluated and recovery values calculated. Additionally, an extract of *Variola louti*, negative for CTXs, was spiked with CTX1B at the threshold value suggested as safety guidance level by the FDA (0.01 μg/kg), and then screened with the immunosensor, which successfully detect this concentration of CTXs. Finally, fishes naturally contaminated from La Réunion island were tested and the amount of CTX congeners detected correlated well with the results obtained with the MBA and CBA analysis. Even if this strategy allows to detect only four of the many CTXs existing congeners, the detection was not affected by the presence of marine toxins other than these four CTXs, thanks to the high specificity of the antibodies. Therefore, with this strategy, samples do not require many purification steps (to remove other marine toxins), shortening the assay time.

This immunosensor strategy has been simplified even more in one of the last works of our group (Campàs et al., 2022). In this work, capture antibodies were immobilized directly on carbon electrodes modified with multiwalled carbon nanotubes instead that on magnetic beads. The sandwich assay was then performed and amperometric signals were measured with a ready-to-go smartphone potentiostat. The achieved LOD was 0.001 μg/kg of CTX1B, ten times lower than the FDA suggested threshold. In addition, recovery values around 100% were obtained, indicating that the fish flesh matrix did not interfere with the performance of the assay, which represents a step forward in comparison to the previous work. However, it is fair to mention that this was probably due to the extraction protocol, rather than the biosensor configuration,

which resulted in very clean samples but involved many purification steps. Therefore, in front of a fish suspected for CTXs a compromise would have to be made: a long extraction protocol that gives a more purified extract but that may involve CTXs losses, or a shorter extraction that may cause matrix effects. Probably, to take the best decision, also the facilities at disposal for the assay execution would have to be considered. In fact, the long extraction procedure would require more sophisticated instruments and reagents that are not always available. However, in this work, a long extraction protocol was applied to fish samples from Japan and Fiji and the extracts were analyzed with the biosensor, sandwich ELISA, CBA and LC-MS/MS, obtaining comparable results. It is important to explain that the correlation between CBA and immunosensor was excellent, and the quantifications provided by this last strategy were only slightly lower than the ones by CBA, in contrast to what observed in the previous work. This cannot be due to the quality of the extracts, the same for both assays, and probably the geographic origins of the fishes analyzed in the different works may also play a role. In fact, in this last work, the fishes were from the Pacific Ocean, and so most likely to contain P-CTXs, which are the specific target of the antibodies used. Anyhow, the strategies proposed by our group are fast, easy to perform and reliable, and they definitely represent a step forward into the development of portable devices for the *in situ* detection of CTXs, especially the last one, in which the instruments required for the analysis are a compact potentiostat and a smartphone. Therefore, they can be easily implemented in monitoring systems.

6.2.2. Detection of CTXs in algal samples

After the success in detecting CTXs in fish samples, our group decided to extend the strategy to microalgal producers. Therefore, several strains of *Gambierdiscus* and *Fukuyoa* were tested in order to investigate the differences in toxin production among species (Gaiani et al. 2020). In this work, 20.000 cells from 9 *Gambierdiscus* and 4 *Fukuyoa* strains were cultured, extracted and analyzed with the strategy previously developed (Leonardo et al. 2020). Nevertheless, since the capture antibodies (3G8 and 10C9) targeted two different groups of CTX congeners, they were used combined together, but also separately (Figure 5). Our results showed the presence of CTX congeners in 11 out of the 13 strains analyzed. A higher CTX content was detected when the two capture antibodies were combined together, in comparison to the detection achieved with just one. A predominance of CTX1B equiv. was observed in 4 out of 6 *G. excentricus* strains (0.06 to 0.21 fg/cell), and 1 out of 4 *F. paulensis* strains (0.33 fg/cell). On the other hand, *G. australes* and the other 2 *G. excentricus* strains showed a higher abundance of CTX3C equiv. (0.16 fg/cell and 0.04-3.54 fg/cell, respectively). The unique strain of *G. caribaeus* tested showed an equal amount of both the congeners (0.13 fg/cell). Additionally, the

same microalgal extracts were screened with CBA, which identified CTX-like activity only in 4 out of the 6 *G. excentricus* strains. It should be kept in mind that the strategy, although allows to detect only four CTX congeners, is not affected by the presence of the other toxic compounds produced by *Gambierdiscus* and *Fukuyoa*, such as maitotoxins, and so provides reliable results. In fact, maitotoxins are known to affect in the execution of the CBA, if no pretreatment of the extract is performed. Moreover, in this study, a new fast CTXs extraction technique was developed, allowing to operate with as low as 20.000 cells of *Gambierdiscus*, an amount that can be easily found in natural samples, making it suitable for the screening of field samples. The biosensor also allowed the identification for the first time of two different CTXs congeners in the same extracts, giving new information about the *Gambierdiscus* and *Fukuyoa* toxin profiles. For this reason, this strategy was chosen to characterize a *G. belizeanus* strain first reported in the Canary Islands, detecting the production of congeners of both the CTX1B and CTX3C groups (Tudó et al. 2020b).

In order to make further advances in the direction of *in situ* detection of CTXs congeners, this strategy was also directly applied to the analysis of field samples (Gaiani et al. 2021b). In this work, macroalgae substrates were collected in Majorca, extracted, and then exposed to both capture mAbs simultaneously, to maximize the probabilities to detect CTXs. Results showed CTX contents in one sample (13.35 ± 0.5 pg CTX1B equiv./cell) and traces of CTX (below LOQ) in 3 out of 9 analyzed samples, finally demonstrating that the system is suitable for the screening of field samples. Even if these are preliminary results and certainly further studies and optimizations are needed, they underline the efficiency and the applicability of biosensing tools for monitoring programs, contributing to the evaluation of ciguatera risk and possible prevention of outbreaks.

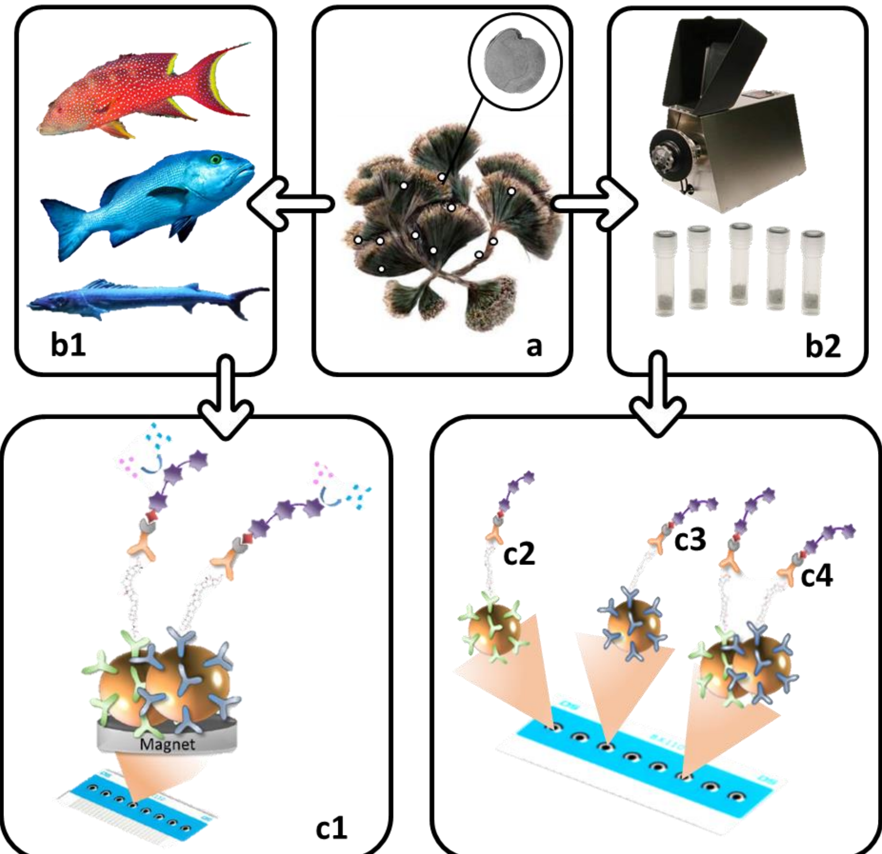


Figure 5. Schematic representation of the biosensor for the detection of the four target CTXs congeners in fish and algal samples (Leonardo et al. 2020; Gaiani et al 2020). Epiphytic Gambierdiscus species (a) were either grazed by herbivorous fishes (b1), which were sampled and extracted, or collected, cultivated and then extracted with a fast extraction technique (b2). Fish extracts were exposed to both capture antibodies (3G8 and 10C9) (c1). Algal extracts were incubated with the capture antibodies separately (c2, c3) and combined (c4). The biotinylated detector (8H4) and poly-HRP streptavidin were added and the immunocomplexes were immobilized on the electrode of an 8-electrode array. Detection was achieved by adding TMB substrate and measuring the reduction current with amperometry.

7. Conclusions

CFP is one of the most common seafood borne diseases, whose real incidence is difficult to estimate. Many factors can co-occur during a CFP event, making its prediction very difficult. The most important feature for a correct CFP management in a specific area is the discrimination between toxin and non-toxin producing *Gambierdiscus/Fukuyoa* species, and toxic and non-toxic fish specimens. Several strategies have been developed during the years to determine the CTX content in an extract either from microalgae or fish, which involve costly and time-consuming procedures. Additionally, these strategies

require the use of standards that, in the case of CTXs, are scarce and expensive. In this picture, there is an extreme need for fast and reliable bioanalytical devices able to detect the DNA of toxin producing species and CTXs in fish and algal samples ease up CFP managing and risk assessment. Biosensors could be the answer to this need. They are sensitive, specific, robust, rapid, cost-effective and do not need highly trained personnel to be operated.

The existence of biosensors that detect CTXs in fish samples at a level even lower than the suggested guideline is of outmost interest, and would be helpful in discriminating contaminated specimens, and consequently allowing to take quick and correct managing decisions. Moreover, the successful detection of CTXs in low cell concentrated microalgal pellets obtained from *Gambierdiscus* cultures can help gaining important information about the toxin producing behavior of these species without requiring large scale cultures. When the ability of detecting CTXs from few cells is exploited in the analysis of field samples, the utility of the biosensors is even more evident. In fact, the application of these techniques to the analysis of monitoring programs samples could help in quickly identify areas of CTXs pick up for marine specimens. Additionally, the instrumentation needed for the analysis can be easily miniaturized at a relatively low cost, and so these strategies can be easily integrated into portable devices, as demonstrated with the last work performed by our group. Additionally, the existing biosensors able to detect CTX producing microalgae species in field samples at low cell concentrations would be extremely useful to know their geographical distribution and consequently were fishes can be intoxicated. This information could help to more rationally design the sampling strategy and therefore improve the CFP management. In conclusion, the studies presented in this chapter underline the efficiency and simplicity of the biosensors, together with their applicability in the screening of natural samples. Considering this, the use of the biosensors for CTX detection can be seen as a promising screening method, complementary to the expensive instrumental techniques, which would be required for confirmation purposes. Up to date, the use of such devices is still not routinely, as further validation studies, but sure, the integration of biosensors in the monitoring of fish and environmental samples coming from the regions endemic for ciguatera can be extremely helpful in spotting in advance an outbreak.

Table 2. Existing biosensors for the detection of *Gambierdiscus* species and CTXs.

Biosensor type	Target	Immobilization support	Electrochemical technique	LOD	Samples	Ref.
Electrochemical DNA-based sensor	*G. australes* *G. excentricus* *G. silvae*	Biotinylated capture probes conjugated to streptavidin-coated MBs and immobilized on screen-printed carbon electrodes	Amperometry	1pM of RNA (10 to 444 cells)	RNA or synthetic DNA	(Medlin et al. 2020)
Electrochemical DNA-based sensor	*G. australes* *G. excentricus*	Thiolated capture probes conjugated to maleimide-coated MBs and immobilized on screen-printed carbon electrodes	Amperometry	10 cells	Genomic DNA of microalgae cultures; field samples from the Balearic Islands	(Gaiani et al 2021b)
Electrochemical immunosensor	CTX1B 54-deoxyCTX1B CTX3C 51-hydroxyCTX3C	Abs conjugated to carboxylic-acid-modified MBs and immobilized on screen-printed carbon electrodes	Amperometry	1.96 pg/mL of CTX1B 3.59 pg/mL of 51-OH-CTX3C	Fishes from La Réunion *Gambierdiscus/Fukuyoa* cultures Field samples from the Balearic Islands	(Leonardo et al. 2020) (Gaiani et al. 2020) (Gaiani et al. 2021b)
Electrochemical immunosensor	CTX1B 54-deoxyCTX1B CTX3C 51-hydroxyCTX3C	Abs immobilized on multiwalled carbon nanotubes-modified screen-printed carbon electrodes	Amperometry	6 pg/mL of CTX1B	Fishes from Fijii and Japan	(Campàs et al. 2022)

References

Abraham A, Jester ELE, Granade HR, Plakas SM, Dickey RW (2012). Caribbean ciguatoxin profile in raw and cooked fish implicated in ciguatera. Food Chem 131:192-198. https://doi.org/10.1016/j.foodchem.2011.08.059

Adachi R, Fukuyo Y (1979). The thecal structure of a marine toxic dinoflagellate *Gambierdiscus toxicus* gen. et sp. nov. collected in a ciguatera-endemic area. Bull Jpn Soc Sci Fish 45:67-71. https://doi.org/10.2331/suisan.45.67

Aligizaki K, Nikolaidis G (2008). Morphological identification of two tropical dinoflagellates of the genera *Gambierdiscus* and *Sinophysis* in the Mediterranean Sea. J Biol Res 9:75-82

Aligizaki K, Nikolaidis G, Katikou P, Baxevanis AD, Abatzopoulos TJ (2009). Potentially toxic epiphytic *Prorocentrum* (Dinophyceae) species in Greek coastal waters. Harmful Algae 8:299-311. https://doi.org/10.1016/j.hal.2008.07.002

Agence nationale de sécurité sanitaire de l'alimentation, de l'environnement et du travail (ANSES) (2015). Contamination des requins, notamment tigre et bouledogue, par des ciguatoxines: occurrence, méthodes analytiques, cas humains rapportés et éléments d'éthologie. Avis de l'Anses Rapport d'expertise collective. Avis de l'Anses Saisine n° 2013-SA-0198.

Bagnis R, Berglund F, Elias PS, Van Esch GJ (1970). Halstead BW, and Kojima K. Problems of toxicans in marine food products. Bull Wld Hlth Org 42:69-88

Benoit E, Legrand AM, Dubois JM (1986). Effects of ciguatoxin on current and voltage clamped frog myelinated nerve fibre. Toxicon 24:357-364. http://doi.org/10.1016/0041-0101(86)90195-9

Bentur Y, Spanier E (2007). Ciguatoxin-like substances in edible fish on the eastern Mediterranean, Clin Toxicol 45: 695-700. http://doi.org/10.1080/15563650701502865

Bienfang P, Oben B, DeFelice S, Moeller P, Huncik K, Oben P, Toonen R, Daly-Engel T, Bowen B (2010). Ciguatera: the detection of neurotoxins in carnivorous reef fish from the coast of Cameroon, Afr J Mar Sci 30:533-540. https://doi.org/10.2989/AJMS.2008.30.3.8.642

Bienfang P, DeFelice S, Dowling A.(2011). Quantitative evaluation of commercially available test kit for ciguatera in fish. Food Sci Nutr 2:594-598. http://doi.org/10.4236/fns.2011.26083

Bottein Dechraoui MY, Tiedeken JA, Persad R, Wang Z, Granade H, Dickey RW, Ramsdell JS (2005). Use of two detection methods to discriminate ciguatoxins from brevetoxins: Application to great barracuda from Florida Keys. Toxicon 46:261-270. https://doi.org/10.1016/j.toxicon.2005.04.006

Bravo I, Rodriguez F, Ramilo I, Rial P, Fraga S (2019). Ciguatera-causing dinoflagellate *Gambierdiscus* spp.(Dinophyceae) in a subtropical

region of North Atlantic Ocean (Canary Islands): morphological characterization and biogeography. Toxins 11:423. https://doi.org/10.3390/toxins11070423

Caillaud A, de la Iglesia P, Darius HT, Pauillac S, Aligizaki K, Fraga S, Chinain M, Diogene J (2010). Update on methodologies available for ciguatoxin determination: perspectives to confront the onset of ciguatera fish poisoning in Europe. Mar Drugs 8:1838-1907. http://doi.org/10.3390/md8061838

Campàs M, Alkassar M, Gaiani G, Leonardo S, Rambla-Alegre M, Diogène J (2021). Chapter seven-The wide spectrum of methods available to study marine neurotoxins. In: Novelli A, Fernández-Sánchez MT, Aschner M, Costa LG (eds.) Advances in Neurotoxicology series, Elsevier, pp 275-315. https://doi.org/10.1016/bs.ant.2021.03.005.

Campàs M, Leonardo S, Oshiro N, Kuniyoshi K, Tsumuraya T, Hirama M, Diogène J (2022). A smartphone-based portable biosensor to assess ciguatoxin i fish from the Pacific Ocean. Food Chem, 374:131687.

Campora CE, Hokama Y, Yabusaki K, Isobe M (2008). Development of an enzyme-linked immunosorbent assay for the detection of ciguatoxin in fish tissue using chicken immunoglobulin Y. J Clin Lab Anal 22:239-245. http://doi.org/10.1002/jcla.20256

Chinain M, Darius HT, Ung A, Cruchet P, Wang Z, Ponton D, Laurent D, Pauillac S (2010a). Growth and toxin production in the ciguatera-causing dinoflagellate *Gambierdiscus polynesiensis* (Dinophyceae) in culture. Toxicon 56:739-750. https://doi.org/10.1016/j.toxicon.2009.06.013

Chinain M, Darius HT, Ung A, Fouc MT, Revel T, Cruchet P, Pauillac S, Laurent D (2010b). Ciguatera risk management in French Polynesia: the case study of Raivavae Island (Australes Archipelago). Toxicon 56:674-690. https://doi.org/10.1016/j.toxicon.2009.05.032

Chinain M, Faust MA, Pauillac S (1999). Morphology and molecular analyses of three toxic species of *Gambierdiscus* (Dinophyceae): *G. pacificus*, sp. nov., *G. australes*, sp. nov., and *G. polynesiensis*, sp. nov. J Phycol 35:1282-1296. https://doi.org/10.1046/j.1529-8817.1999.3561282.x

Chinain M, Gatti CMI, Darius HT, Quod JP, Tester PA (2021). Ciguatera poisonings: a global review of occurrences and trends. Harmful Algae 102:101873. https://doi.org/10.1016/j.hal.2020.101873

Darius HT, Roue M, Sibat M, Viallon J, Gatti C, Vandersea MW, Tester PA, Litaker RW, Amzil Z, Hess P, Chinain M (2018). Toxicological investigations on the sea urchin *Tripneustes gratilla* (Toxopneustidae, Echinoid) from Anaho Bay (Nuku Hiva, French Polynesia): evidence for the presence of Pacific ciguatoxins. Mar Drugs. https://doi.org/10.3390/md16040122

Díaz-Asencio L, Clausing RJ, Rañada ML, Alonso-Hernández CM, Bottein M-YD (2018). A radioligand receptor binding assay for ciguatoxin monitoring in environmental samples: method development and

determination of quality control criteria. J Environ Radioact 192:289-294. https://doi.org/10.1016/j.jenvrad.2018.06.019

Diogène J, Campàs M (2017). Recent advances in the analysis of marine toxins. Vol 78. Comprehensive Analytical Chemistry series, Amsterdam, The Netherlands, Elsevier.

Diogene J, Reverte L, Rambla-Alegre M, Del Rio V, de la Iglesia P, Campàs M, Palacios O, Flores C, Caixach J, Ralijaona C, Razanajatovo I, Pirog A, Magalon H, Arnich N, Turquet J (2017). Identification of ciguatoxins in a shark involved in a fatal food poisoning in the Indian Ocean. Sci Rep 7:8240. https://doi.org/10.1038/s41598-017-08682-8

Endean R, Griffith JK, Robins JJ, Monks SA (1993). Multiple toxins in a specimen of the narrow-barred Spanish mackerel, *Scomberomorus commersoni*. Toxicon 31:195-204.http://doi.org/10.1016/0041-0101(93)90286-r

EFSA Panel on Contaminants in the Food Chain (2010). Scientific Opinion on marine biotoxins in shellfish emerging toxins: Ciguatoxin group. EFSA J. 8:1627, 38 pp. https://doi.org/10.2903/j.efsa.2010.1627.

Fairey ER, Edmunds JSG, Ramsdell JS (1997). A cell-based assay for brevetoxins, saxitoxins, and ciguatoxins using a stably expressed c-*fos*–luciferase reporter gene. Anal Biochem 251:129-132. http://doi.org/10.1006/abio.1997.2264

Fraga S, Rodríguez F, Caillaud A, Diogène J, Raho N, Zapata M (2011). *Gambierdiscus excentricus* sp. nov.(Dinophyceae), a benthic toxic dinoflagellate from the Canary Islands (NE Atlantic Ocean). Harmful Algae 11:10-22. https://doi.org/10.1016/j.hal.2011.06.013

Fraga S, Rodriguez F (2014). Genus *Gambierdiscus* in the Canary Islands (NE Atlantic Ocean) with description of *Gambierdiscus silvae* sp. nov., a new potentially toxic epiphytic benthic dinoflagellate. Protist 165:839-853 doi:https://doi.org/10.1016/j.protis.2014.09.003

Food Standards Australia New Zealand (FZAN) (2006). A guide to the Australian Primary Production and Processing Standard for Seafood, Safe Seafood Australia. 2nd edn, Canberra

Gaiani G, Leonardo S, Tudó À, Toldrà A, Rey M, Andree KB, Tsumuraya T, Hirama M, Diogène J, O'Sullivan CK (2020). Rapid detection of ciguatoxins in *Gambierdiscus* and *Fukuyoa* with immunosensing tools. Ecotox. and Environm. Saf. 204:111004. https://doi.org/10.1016/j.ecoenv.2020.111004

Gaiani G, Toldrà A, Andree KB, Rey M, Diogène J, Alcaraz C, O’Sullivan CK, Campàs M (2021a). Detection of *Gambierdiscus* and *Fukuyoa* single cells using recombinase polymerase amplification combined with a sandwich hybridization assay. J Appl Phycol http://doi.org/10.1007/s10811-021-02447-7

Gaiani G, Cucchi F, Toldrà A, Andree KB, Rey M, Tsumuraya T, O'Sullivan CK, Diogène J, Campàs M (2021b). Electrochemical biosensor for the dual detection of *Gambierdiscus australes* and *Gambierdiscus excentricus* in field samples. First report of *G. excentricus* in the

Balearic Islands. Sci Total Environ 806:150915. http://doi.org/10.1016/j.scitotenv.2021.150915

Ginés I, Gaiani G, Ruhela A, Skoruridou V, Campàs M, Masip L (2021). Nucleic acid lateral flow dipstick assay for the duplex detection of *Gambierdiscus australes* and *Gambierdiscus excentricus*. Harmuf Algae 110:102135. https://doi.org/10.1016/j.hal.2021.102135

Gómez F, Qiu D, Lopes RM, Lin S (2015). *Fukuyoa paulensis* gen. et sp. nov., a new genus for the globular species of the dinoflagellate *Gambierdiscus* (Dinophyceae). PLoS One 10. https://doi.org/10.1371/journal.pone.0119676

Halstead BW (1967). Poisonous and venomous marine animals of the world. US Government Printing Office, Washington, DC, USA.

Hamilton B, Hurbungs M, Vernoux J-P, Jones A, Lewis RJ (2002). Isolation and characterisation of Indian Ocean ciguatoxin. Toxicon 40:685-693. http://doi.org/10.1016/s0041-0101(01)00259-8.

Hamilton B, Whittle N, Shaw G, Eaglesham G, Moore MR, Lewis RJ (2010). Human fatality associated with Pacific ciguatoxin contaminated fish. Toxicon 56:668-673. https://doi:org/10.1016/j.toxicon.2009.06.007

Hardison DR, Holland WC, McCall JR, Bourdelais AJ, Baden DG, Darius HT, Chinain M, Tester PA, Shea D, Flores Quintana HA (2016). Fluorescent receptor binding assay for detecting ciguatoxins in fish. PLoS One 11:e0153348. https://doi.org/10.1371/journal.pone.0153348

Hidalgo J, Liberona JL, Molgó J, Jaimovich E (2002). Pacific ciguatoxin-1b effect over Na^+ and K^+ currents, inositol 1, 4, 5-triphosphate content and intracellular Ca^{2+} signals in cultured rat myotubes. Br J Pharmacol 137:1055-1062. http://doi.org/10.1038/sj.bjp.0704980

Hoffman PA, Granade HR, McMillan JP (1983). The mouse ciguatoxin bioassay: a dose-response curve and symptomatology analysis. Toxicon 21:363-369. http://doi.org/ https://doi.org/10.1016/0041-0101(83)90092-2

Hokama Y, Banner AH, Boylan DB (1977). A radioimmunoassay for the detection of ciguatoxin. Toxicon 15:317-325. https://doi.org/10.1016/0041-0101(77)90014-9

Hokama Y, Abad MA, Kimura LH (1983). A rapid enzyme-immunoassay for the detection of ciguatoxin in contaminated fish tissues. Toxicon 21:817-824. https://doi.org/10.1016/0041-0101(83)90070-3

Hokama Y (1985). A rapid, simplified enzyme immunoassay stick test for the detection of ciguatoxin and related polyethers from fish tissues. Toxicon 23:939-946. https://doi.org/10.1016/0041-0101(85)90386-1

Hokama Y, Shirai LK, Iwamoto LM, Kobayashi MN, Goto CS, Nakagawa LK (1987). Assessment of a rapid enzyme immunoassay stick test for the detection of ciguatoxin and related polyether toxins in fish tissues. Biol Bull 172:144-153. https://doi.org/10.2307/1541615

Hokama Y, Honda SAA, Asahina AY, Fong JML, Matsumoto CM, Gallacher TS (1989). Cross-reactivity of ciguatoxin, okadaic acid, and polyethers

with monoclonal antibodies. Food Agr Immunol1:29-35. https://doi.org/10.1080/09540108909354672

Hokama Y (1990). Simplified solid-phase immunobead assay for detection of ciguatoxin and related polyethers. J of clinical laboratory analysis 4:213-217. http://doi.org/ 10.1002/jcla.1860040313

Hokama Y, Asahina AY, Titus E, Ichinotsubo D, Chun S, Hong TWP, Shirai JL, Asuncion DA, Miyahara JT (1994). Assessment of ciguateric fish in Hawaii by immunological, mouse toxicity and guinea pig atrial assays. Mem Queensl Mus Brisbane 34:489-496

IAMAT, 2017. Ciguatera Fish Poisoning. https://www.iamat.org/risks/ciguatera-fish- poisoning. Last accessed 08/11/2021.

Ikehara T, Kuniyoshi K, Oshiro N, Yasumoto T (2017). Biooxidation of ciguatoxins leads to species-specific toxin profiles. Toxins (Basel) 9:205. http://doi.org/10.3390/toxins9070205

Jang SH, Jeong HJ, Yoo YD (2018). *Gambierdiscus jejuensis* sp. nov., an epiphytic dinoflagellate from the waters of Jeju Island, Korea, effect of temperature on the growth, and its global distribution. Harmful Algae 80:149-157. http://doi.org/10.1016/j.hal.2018.11.007

Jeong HJ, Lim AS, Jang SH, Yih WH, Kang NS, Lee SY, Yoo YD, Kim HS (2012). First report of the epiphytic dinoflagellate *Gambierdiscus caribaeus* in the temperate waters off Jeju Island, Korea: morphology and molecular characterization. J Eukaryot Microbiol 59:637-650

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

Kimura LH, Abad MA, Hokama Y (1982). Evaluation of the radioimmunoassay (RIA) for detection of ciguatoxin (CTX) in fish tissues. J Fish Biol 21:671-680. https://doi.org/10.1111/j.1095-8649.1982.tb02871.x

Kretzschmar AL, Larsson ME, Hoppenrath M, Doblin MA, Murray SA (2019). Characterisation of two toxic *Gambierdiscus* spp. (Gonyaulacales, Dinophyceae) from the Great Barrier Reef (Australia): *G. lewisii* sp. nov. and *G. holmesii* sp. nov. Protist 170. https://doi.org/10.1016/j.protis.2019.125699

LaGier MJ, Fell JW, Goodwin KD (2007). Electrochemical detection of harmful algae and other microbial contaminants in coastal waters using hand-held biosensors. Mar Pollut Bull 54:757-770. https://doi.org/10.1016/j.marpolbul.2006.12.017

Lavenu L, Chomérat N, Díaz-Asencio L, Gerry C, Belmont, C. , Hollanda S, Tunin- Ley A, Dechraoui Bottein MY (2018). *Gambierdiscus* from Seychelles: morphology, molecular identification and toxicity. In: 18th International Conference on Harmful Algae 2018. p 497.

Laza-Martínez A, David H, Riobó P, Miguel I, Orive E (2016). Characterization of a strain of *Fukuyoa paulensis* (Dinophyceae) from

the Western Mediterranean Sea. J Eukaryot Microbiol 63:481-497. https://doi.org/10.1111/jeu.12292

Lehane L, Lewis RJ (2000). Ciguatera: recent advances but the risk remains. Int. J Food Microbiol 61:91-125. http://doi.org/10.1016/S0168-1605(00)00382-2

Legrand AM, Teai T,Cruchet P, Satake M,Murata K,Yasumoto T (1998). Two structural types of ciguatoxin involved in ciguatera fish poisoning in French Polynesia. In: Reguera B, Blanco J, Fernandez ML, Wyatt T (eds). VIII International Conference on Harmful Algae. Xunta de Galicia and International Oceanographic Commission of UNESCO, Paris, France, pp 473–475.

Leonardo, S, Toldrà, A and Campàs, M (2017). Trends and prospects on electrochemical biosensors for the detection of marine toxins. In: Diogène J, Campàs M (eds). Recent advances in the analysis of marine toxins, comprehensive analytical chemistry, Elsevier, pp 303-341.

Leonardo S, Gaiani G, Tsumuraya T, Hirama M, Turquet J, Sagristà N, Rambla-Alegre M, Flores C, Caixach J, Diogene J (2020). Addressing the analytical challenges for the detection of ciguatoxins using an electrochemical biosensor. Anal Chem. http://doi.org/10.1021/acs.analchem.9b04499

Lewis RJ, Sellin M, Poli MA, Norton RS, MacLeod JK, Sheil MM (1991). Purification and characterization of ciguatoxins from moray eel (*Lycodontis javanicus*, Muraenidae). Toxicon 29:1115-1127. http://doi.org/10.1016/0041-0101(91)90209-a

Lewis RJ, Jones A (1997). Characterization of ciguatoxins and ciguatoxin congeners present in ciguateric fish by gradient reverse-phase high-performance liquid chromatography/mass spectrometry. Toxicon 35:159-168. https://doi.org/10.1016/S0041-0101(96)00132-8

Lewis RJ, Vernoux J-P, Brereton IM (1998). Structure of Caribbean ciguatoxin isolated from *Caranx latus*. J Am Chem Soc 120:5914-5920. https://doi.org/10.1021/ja980389e

Lewis RJ, Jones A, Vernoux J-P (1999). HPLC/tandem electrospray mass spectrometry for the determination of sub-ppb levels of Pacific and Caribbean ciguatoxins in crude extracts of fish. Anal Chem 71:247-250. http://doi.org/10.1021/ac980598h

Lewis RJ, Inserra M, Vetter I, Holland WC, Hardison DR, Tester PA, Litaker RW (2016). Rapid extraction and identification of maitotoxin and ciguatoxin-like toxins from Caribbean and Pacific *Gambierdiscus* using a new functional bioassay. PLoS One 11. http://doi.org/10.1371/journal.pone.0160006

Lewis RJ, Vetter I (2016). Ciguatoxin and Ciguatera. In: Gopalakrishnakone P, Haddad VJ, Tubaro A, Kim E, Kem WR (eds). Marine and Freshwater Toxins, Toxinology. Springer Science+Business Media: Dordrecht, The Netherlands, pp 71-92.

Li Z, Park JS, Kang NS, Chomérat N, Mertens KN, Gu H, Lee KW, Kim KH, Baek SH, Shin K, Han KH, Son MH, Shin HH (2021). A new

potentially toxic dinoflagellate Fukuyoa koreansis sp. nov. (Gonyaulacales, Dinophyceae) from Korean coastal waters: Morphology, phylogeny, and effects of temperature and salinity on growth. Harmful Algae 109:102107. http://doi.org/10.1016/j.hal.2021.102107.

Litaker RW, Vandersea MW, Faust MA, Kibler SR, Chinain M, Holmes MJ, Holland WC, Tester PA (2009). Taxonomy of Gambierdiscus including four new species, Gambierdiscus caribaeus, Gambierdiscus carolinianus, Gambierdiscus carpenteri and Gambierdiscus ruetzleri (Gonyaulacales, Dinophyceae). Phycologia 48:344-390. https://doi.org/10.2216/07-15.1

Litaker RW, Holland WC, Hardison DR, Pisapia F, Hess P, Kibler SR, Tester PA (2017). Ciguatoxicity of *Gambierdiscus* and *Fukuyoa* species from the Caribbean and Gulf of Mexico. PLoS One 12:e0185776. https://doi.org/10.1371/journal.pone.0185776

Litaker RW, Tester PA, Vandersea MW (2019). Species-specific PCR assays for *Gambierdiscus excentricus* and *Gambierdiscus silvae* (Gonyaulacales, Dinophyceae). J Phycol 55:730-732. https://doi.org/10.1111/jpy.12852

Lombet A, Bidard J-N, Lazdunski M (1987). Ciguatoxin and brevetoxins share a common receptor site on the neuronal voltage-dependent Na^+ channel. FEBS letters 219:355-359. https://doi.org/10.1016/0014-5793(87)80252-1

Longo S, Sibat M, Viallon J, Darius HT, Hess P, Chinain M (2019). Intraspecific variability in the toxin production and toxin profiles of in vitro cultures of *Gambierdiscus polynesiensis* (Dinophyceae) from French Polynesia. Toxins (Basel) 11:735. https://doi.org/10.3390/toxins11120735

Lugomela C (2006). Autecology of the Toxic Dinoflagellate *Gambierdiscus toxicus* Adachi et Fukuyo (Dinophyceae) in Central Coastal Areas of Tanzania. Western Indian Ocean J Mar Sci 5:213-221

Magriñá I, Toldrà A, Campàs M, Ortiz M, Simonova A, Katakis I, Hocek M, O'Sullivan CK (2019). Electrochemical genosensor for the direct detection of tailed PCR amplicons incorporating ferrocene labelled dATP. Biosens Bioelectron 134:76-82. https://doi.org/10.1016/j.bios.2019.03.060

Mak YL, Wai T-C, Murphy MB, Chan WH, Wu JJ, Lam JCW, Chan LL, Lam PKS (2013). Pacific ciguatoxins in food web components of coral reef systems in the Republic of Kiribati. Environ Sci Technol 47:14070-14079. doi:https://doi.org/10.1021/es403175d

Manger RL, Leja LS, Lee SY, Hungerford JM, Wekell MM (1993). Tetrazolium-based cell bioassay for neurotoxins active on voltage-sensitive sodium channels: semiautomated assay for saxitoxins, brevetoxins, and ciguatoxins. Anal Biochem 214:190-194. doi:10.1006/abio.1993.1476

McCall JR, Jacocks HM, Niven SC, Poli MA, Baden DG, Bourdelais AJ (2014). Development and utilization of a fluorescence-based receptor-binding assay for the site 5 voltage-sensitive sodium channel ligands brevetoxin and ciguatoxin. J AOAC Int 97:307-315. http://doi.org/10.5740/jaoacint.sgemccall

Medlin LK, Gamella M, Mengs G, Serafín V, Campuzano S, Pingarrón JM (2020). Advances in the detection of toxic algae using electrochemical biosensors. Biosensors 10. http://doi.org/10.3390/bios10120207

Miller DM, Tindall DR, Tibbs B (1986). Ciguatera-type toxins: Bioassay using crayfish nerve cord (Abstract1103). Fed Proc Abstr 45:344.

Ministry of Health W, and Labour (MHWL) (1953). A ban on domestic sales of barracuda. MHWL notification No. 20. vol 20.

Ministry of Health W, and Labour (MHWL) (2001). Handling of ciguatera fish, Office memorandum, by MHWL to heads of quarantine stations.

Molgó J, Cornelia JX, Legrand AM (1990). Ciguatoxin enhances quantal transmitter release from frog motor nerve terminals. Br J Pharmacol 99:695-700. http://doi.org/10.1111/j.1476-5381.1990.tb12991.x.

Molgó J, Shimahara T, Legrand AM (1993). Ciguatoxin, extracted from poisonous morays eels, causes sodium-dependent calcium mobilization in NG108-15 neuroblastoma× glioma hybrid cells. Neurosci Lett 158:147-150. http://doi.org/10.1016/0304-3940(93)90250-o

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

Nagumo Y, Oguri H, Tsumoto K, Shindo Y, Hirama M, Tsumuraya T, Fujii I, Tomioka Y, Mizugaki M, Kumagai I (2004). Phage-display selection of antibodies to the left end of CTX3C using synthetic fragments. J Immunol Methods 289:137-146.http://doi.org/10.1016/j.jim.2004.04.003

Nellis DW, Barnard GW (1986). Ciguatera: a legal and social overview. Mar Fish Rev 48:2-5

Nishimura T, Sato S, Tawong W, Sakanari H, Yamaguchi H, Adachi M (2014). Morphology of *Gambierdiscus scabrosus* sp. nov.(Gonyaulacales): a new epiphytic toxic dinoflagellate from coastal areas of Japan. J Phycol 50:506-514. https://doi.org/10.1111/jpy.12175

Nishimura T, Hariganeya N, Tawong W, Sakanari H, Yamaguchi H, Adachi M (2016). Quantitative PCR assay for detection and enumeration of ciguatera-causing dinoflagellate *Gambierdiscus* spp.(Gonyaulacales) in coastal areas of Japan. Harmful Algae 52:11-22. https://doi.org/10.1016/j.hal.2015.11.018

Oguri H, Hirama M, Tsumuraya T, Fujii I, Maruyama M, Uehara H, Nagumo Y (2003). Synthesis-based approach toward direct sandwich immunoassay for ciguatoxin CTX3C. J Am Chem Soc 125:7608-7612. http://doi.org/10.1021/ja034990a

Park DL (1995). Detection of ciguatera and diarrheic shellfish toxins in finfish and shellfish with ciguatect kit. J AOAC Int 78:533-537. https://doi.org/10.1093/jaoac/78.2.533

Parsons ML, Settlemier CJ, Bienfang PK (2010). A simple model capable of simulating the population dynamics of *Gambierdiscus*, the benthic dinoflagellate responsible for ciguatera fish poisoning. Harmful Algae 10:71-80. https://doi.org/10.1016/j.hal.2010.07.002

Pasinszki T, Lako J, Dennis TE (2020). Advances in detecting ciguatoxins in fish. Toxins 12:494. https://doi.org/10.3390/toxins12080494

Pisapia F, Holland WC, Hardison DR, Litaker RW, Fraga S, Nishimura T, Adachi M, Nguyen-Ngoc L, Sechet V, Amzil Z, Herrenknecht C, Hess P (2017). Toxicity screening of 13 *Gambierdiscus* strains using neuro-2a and erythrocyte lysis bioassays. Harmful Algae 63:173-183. https://doi.org/10.1016/j.hal.2017.02.005

Pottier I, Vernoux J-P, Jones A, Lewis RJ (2002). Characterisation of multiple Caribbean ciguatoxins and congeners in individual specimens of horse-eye jack (*Caranx latus*) by high-performance liquid chromatography/mass spectrometry. Toxicon 40:929-939. http://doi.org/10.1016/s0041-0101(02)00088-0

Reverté L, Soliño L, Carnicer O, Diogène J, Campàs M (2014). Alternative methods for the detection of emerging marine toxins: biosensors, biochemical assays and cell-based assays. Mar Drugs.12:5719-63. https://doi.org/10.3390/md12125719

Reverté L, Toldrà A, Andree KB, Fraga S, de Falco G, Campàs M, Diogène J (2018). Assessment of cytotoxicity in ten strains of *Gambierdiscus australes* from Macaronesian Islands by neuro-2a cell-based assays. J Appl Phycol 30:2447-2461. https://doi.org/10.1007/s10811-018-1456-8.

Rhodes L, Harwood T, Smith K, Argyle P, Munday R (2014). Production of ciguatoxin and maitotoxin by strains of *Gambierdiscus australes*, *G. pacificus* and *G. polynesiensis* (Dinophyceae) isolated from Rarotonga, Cook Islands. Harmful Algae 39:185-190. https://doi.org/10.1016/j.hal.2014.07.018

Rhodes LL, Smith KF, Murray S, Harwood DT, Trnski T, Munday R (2017). The Epiphytic Genus *Gambierdiscus* (Dinophyceae) in the Kermadec Islands and Zealandia Regions of the Southwestern Pacific and the associated risk of ciguatera fish poisoning. Mar Drugs 15:219. https://doi.org/10.3390/md15070219

Rodríguez F, Fraga S, Ramilo I, Rial P, Figueroa RI, Riobó P, Bravo I (2017). Canary Islands (NE Atlantic) as a biodiversity 'hotspot'of *Gambierdiscus*: implications for future trends of ciguatera in the area. Harmful Algae 67:131-143 doi:https://doi.org/10.1016/j.hal.2017.06.009

Rongo T, van Woesik R (2011). Ciguatera poisoning in Rarotonga, southern Cook islands. Harmful Algae 10:345-355. https://doi.org/10.1016/j.hal.2010.11.005

Rossignoli AE, Tudo A, Bravo I, Diaz PA, Diogene J, Riobo P (2020). Toxicity characterisation of *Gambierdiscus* species from the Canary Islands. Toxins (Basel) 12:134. https://doi.org/10.3390/toxins12020134

Roué M, Darius HT, Picot S, Ung A, Viallon J, Gaertner-Mazouni N, Sibat M, Amzil Z, Chinain M (2016). Evidence of the bioaccumulation of ciguatoxins in giant clams (*Tridacna maxima*) exposed to *Gambierdiscus* spp. cells. Harmful Algae 57:78-87. https://doi.org/10.1016/j.hal.2016.05.007

Sanchez-Henao JA, García-Álvarez N, Fernández A, Saavedra P, Sergent FS, Padilla D, Acosta-Hernández B, Suárez MM, Diogène J, Real F (2019). Predictive score and probability of CTX-like toxicity in fish samples from the official control of ciguatera in the Canary Islands. Sci Total Environ 673:576-584. https://doi.org/10.1016/j.scitotenv.2019.03.445

Shimojo RY, Iwaoka WT (2000). A rapid hemolysis assay for the detection of sodium channel-specific marine toxins. Toxicology 154:1-7. http://doi.org/10.1016/s0300-483x(00)00242-0

Silva SE (1956). Contribution al'etude du microplankton de dakar et des regions maritimes voisines. Bull Inst Fr Afr Noire Ser A Sci Nat 18:335-71.

Silva M, Rodriguez I, Barreiro A, Kaufmann M, Isabel Neto A, Hassouani M, Sabour B, Alfonso A, Botana LM, Vasconcelos V (2015). First report of ciguatoxins in two starfish species: *Ophidiaster ophidianus* and *Marthasterias glacialis*. Toxins (Basel) 7:3740-3757. https://doi.org/10.3390/toxins7093740

Smith KF, Biessy L, Argyle PA, Trnski T, Halafihi T, Rhodes LL (2017). Molecular identification of *Gambierdiscus* and *Fukuyoa* (Dinophyceae) from environmental samples. Mar Drugs 15:243. doi:https://doi.org/10.3390/md15080243

Soler Onis E, Fernández Zabala J, Ramírez Corbera AS (2019). First records of *Gambierdiscus excentricus* and *Ostreopsis lenticularis* in the Cape Verde Archipelago (Macaronesia, Central Eastern Atlanctic). IMS Newsletter.

Tester PA, Feldman RL, Nau AW, Kibler SR, Litaker RW (2010). Ciguatera fish poisoning and sea surface temperatures in the Caribbean Sea and the West Indies. Toxicon 56:698-710. https://doi.org/10.1016/j.toxicon.2010.02.026

Tester PA, Vandersea MW, Buckel CA, Kibler SR, Holland WC, Davenport ED, Clark RD, Edwards KF, Taylor JC, Vander Pluym JL (2013). *Gambierdiscus* (Dinophyceae) species diversity in the flower garden banks national marine sanctuary, Northern Gulf of Mexico, USA. Harmful Algae 29:1-9. https://doi.org/10.1016/j.hal.2013.07.001

Tester PA, Wickliffe L, Jossart J, Rhodes L, Enevoldsen H, Adachi M, Nishimura T, Rodriguez F, Chinain M, Litaker, W (2018). Global distribution of the genera *Gambierdiscus* and *Fukuyoa*. In: Hess P (ed). Harmful Algae 2018 - From ecosystems to socioecosystems. Proceedings of the 18th Intl. Conf. on Harmful Algae. Nantes, International Society for the Study of Harmful Algae, pp 138-143.

Tester PA, Berdalet E, Litaker RW, (2020). Climate change and benthic harmful microalgae. Harmful Algae 91, 101655. https://doi.org/10.1016/j.hal.2019.101655

Toldrà A, Alcaraz C, Diogène J, O'Sullivan CK, Campàs M (2019). Detection of *Ostreopsis* cf. *ovata* in environmental samples using an electrochemical DNA-based biosensor. Sci Total Environ 689:655-661. https://doi.org/10.1016/j.scitotenv.2019.06.448

Tsumuraya T, Fujii I, Inoue M, Tatami A, Miyazaki K, Hirama M (2006). Production of monoclonal antibodies for sandwich immunoassay detection of ciguatoxin 51-hydroxyCTX3C. Toxicon 48:287-294. http://doi.org/10.1016/j.toxicon.2006.05.014

Tsumuraya T, Fujii I, Hirama M (2010). Production of monoclonal antibodies for sandwich immunoassay detection of Pacific ciguatoxins. Toxicon 56:797-803. http://doi.org/10.1016/j.toxicon.2009.06.003

Tsumuraya T, Takeuchi K, Yamashita S, Fujii I, Hirama M (2012). Development of a monoclonal antibody against the left wing of ciguatoxin CTX1B: thiol strategy and detection using a sandwich ELISA. Toxicon 60:348-357. http://doi.org/10.1016/j.toxicon.2012.04.347

Tsumuraya T, Sato T, Hirama M, Fujii I (2018). Highly sensitive and practical fluorescent sandwich ELISA for ciguatoxins. Anal Chem 90:7318-7324. http://doi.org/10.1021/acs.analchem.8b00519

Tsumuraya T, Hirama M (2019). Rationally designed synthetic haptens to generate anti-ciguatoxin monoclonal antibodies, and development of a practical sandwich ELISA to detect ciguatoxins. Toxins 11:533. https://doi.org/10.3390/toxins11090533

Tudó À, Toldrà A, Andree KB, Rey M, Fernández-Tejedor M, Campàs M, Diogène J (2018). First report of *Gambierdiscus* in the Western Mediterranean Sea (Balearic Islands). Harmful Algae News.

Tudó À, Toldrà A, Rey M, Todolí I, Andree KB, Fernández-Tejedor M, Campàs M, Sureda FX, Diogène J (2020a). *Gambierdiscus* and *Fukuyoa* as potential indicators of ciguatera risk in the Balearic Islands. Harmful Algae 99:101913. https://doi.org/10.1016/j.hal.2020.101913

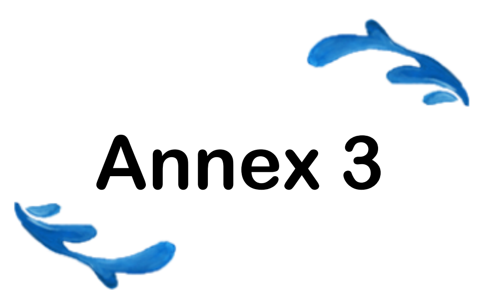
Tudó À, Gaiani G, Rey Varela M, Tsumuraya T, Andree KB, Fernández-Tejedor M, Campàs M, Diogène J (2020b). Further advance of *Gambierdiscus* species in the Canary Islands, with the first report of *Gambierdiscus belizeanus*. Toxins 12:692. https://doi.org/10.3390/toxins12110692

Turquet J (1998). Assemblage of benthic dinoflagellates and monitoring of harmful species in Reunion Island, SW Indian Ocean, 1993-1996. Harmful Algae, pp 44-47.

Vandersea MW, Kibler SR, Holland WC, Tester PA, Schultz TF, Faust MA, Holmes MJ, Chinain M, Wayne Litaker R (2012). Development of semi-quantitative pcr assays for the detection and enumeration of *Gambierdiscus* species (gonyaulacales, dinophyceae). J Phycol 48:902-915. http://doi.org/10.1111/j.1529-8817.2012.01146.x.

Vernoux J-P, Lewis RJ (1997). Isolation and characterisation of Caribbean ciguatoxins from the horse-eye jack (*Caranx latus*). Toxicon 35:889-900

Viallon J, Chinain M, Darius HT (2020). Revisiting the neuroblastoma cell-based assay (CBA-N2a) for the improved detection of marine toxins active on voltage gated sodium channels (VGSCs). Toxins 12:281. https://doi.org/10.3390/toxins12050281



Annex 3

Article

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 that has been known since ancient times to be found mainly in tropical and subtropical areas, which occurs when fish or very rarely invertebrates contaminated with ciguatoxins (CTXs) are consumed. The genus of marine benthic dinoflagellates *Gambierdiscus* produces CTX precursors. 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. In this study, *G. belizeanus* is reported for the first time in the Canary Islands. *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 resulting in *G. belizeanus* producing 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 (CBA); immunoassay; immunosensor

Key Contribution: *G. belizeanus* is reported for the first time in the Canary Islands (El Hierro). *G. belizeanus* produced CTX1B and CTX3C series of congeners. CTX-like toxicity of *G. australes*, *G. excentricus* and *G. caribaeus* was re-assessed.

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 and contribute 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. Referring to *G. belizeanus*, it 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. Results and Discussion

2.1. 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 1 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, and 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* appears, but it is 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 or 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.

2.2. 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 1.

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]. However, 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 the cells of Bravo et al. [32]. In reference to *G. excentricus*, all measurements are in accordance with the original description Fraga et al. [30], and the values were similar to Bravo et al. [32] and Hoppenrath et al. [45].

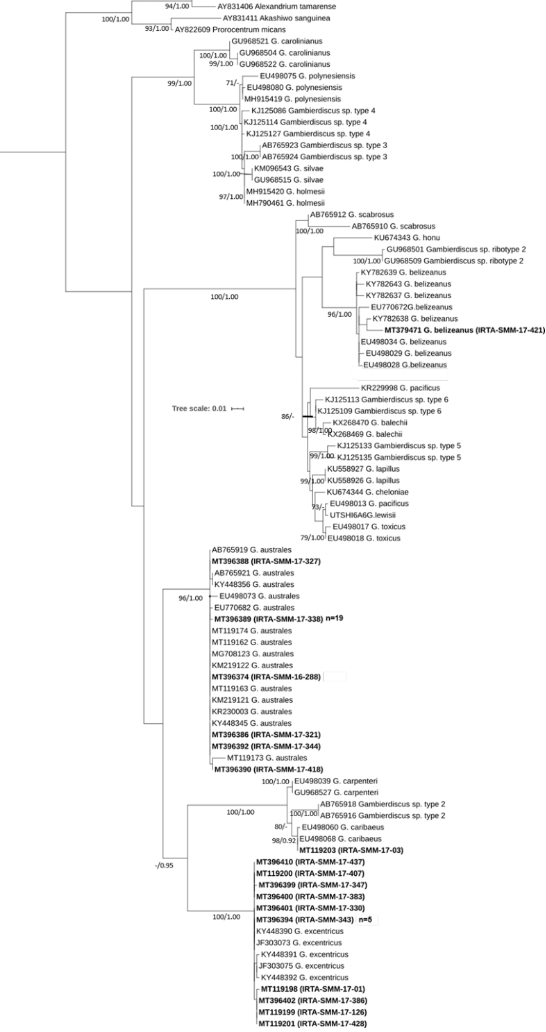

Figure 1. Phylogenetic tree of the LSU D8-D10 region (rDNA) using Maximum Likelihood analysis. Sequences in bold represent the strains of this study. The number of clones (*n*) with the same haplotypes is shown in parentheses. Values at nodes represent bootstrap values (≥70) and the Bayesian posterior probability (≥0.95) (bt/pp).

Table 1. Morphological sizes average of depth and width (±SD) of *Gambierdiscus* species of this study measured with 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)

Morphological Characterization of *G. belizeanus*

Cells were anterior–posteriorly compressed. The plate formula of *G. belizeanus* was Po, 4′, 0a, 6′′, 6c, ?s, 5′′′, 0p, 2′′′′ based on Fraga et al. [30]. The cells were heavily aerolated (Figure 2A–C). Limits of the thecae are well defined by intercalary bands. These two latter characteristics are typical of *G. belizeanus* [38,40,44]. The 2′ plate is rectangular (Figure 2A), and the 2′′′′ plate is pentagonal (Figure 2B). Figure 2D shows the apical pore plate (Po).

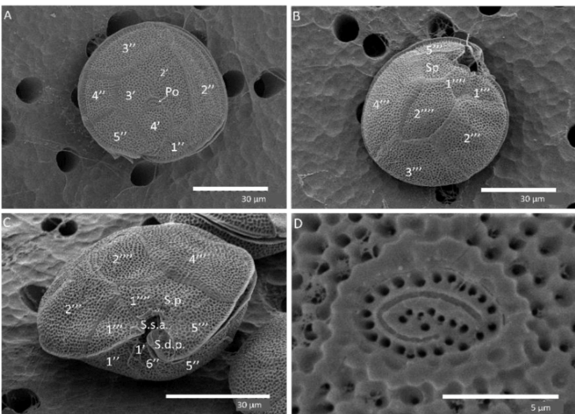

Figure 2. SEM images of *G. belizeanus* (IRTA-SMM-17-421): apical (**A**), antapical (**B**), ventral (**C**) views, detail of Po plate and pores (**D**).

2.3. *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 3. 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.

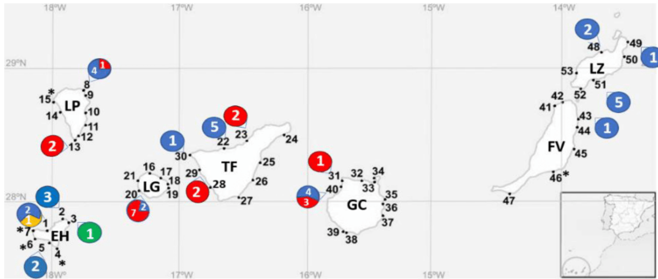

Figure 3. Distribution of each species in the stations of the Canary Islands during 2016–2017. Station numbers are represented in bold. The presence of *Gambierdiscus* species determined with molecular analysis is presented with a circle and includes the number strains identified for each species. The asterisk represents the presence of *Gambierdiscus* sp. Colors of circles are for *G. australes* (blue), *G. excentricus* (red), *G. caribaeus* (green), and *G. belizeanus* (yellow). EH (El Hierro), FV (Fuerteventura), GC (Gran Canaria), LG (La Gomera), LP (La Palma), LZ (Lanzarote), and TF (Tenerife).

Before 2004, the 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 obtained from 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. 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 and October 2015. In the study of 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, which was contrary to what had been previously considered as a recent introduction [46]. In October 2017, Bravo and collaborators [32] performed systematic samplings in La Gomera and La Palma and 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 the identification of cultured strains. 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, the 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 (Tester et al., 2020). Hence, it is expected that high abundances and more diversity of *Gambierdiscus* species would be found during August–October.

The distributions of *G. australes* and *G. excentricus* observed in the current study were similar to those reported by Rodriguez et al. [33] and Bravo et al. [32]. Table 2 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. These data are consistent since *G. australes* is the *Gambierdiscus* species with the widest optimal temperature range: between 19 and 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. Physiological data have not been reported for *G. excentricus*, even though 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, the 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 tolerance [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. 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, this 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 a 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.

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 *Gambierdiscus* cell densities under the influence of the SST in the eastern islands is still to be studied.

Table 2. Literature review of the distribution of the species in the Canary Islands. EH (El Hierro), FV (Fuerteventura), GC (Gran Canaria), LG (La Gomera), LP (La Palma), LZ (Lanzarote), and TF (Tenerife).

Species	Islands	Date of Sampling (References)
G. australes	LZ, FV, GC	September–October 2015 [33] October 2016 (this study)
	EH	September–October 2015 [33] October 2017 (this study)
	TF	2013 [31] September–October 2015 [33] October 2017 (this study)
	LG	October 2017 ([33] and this study)
	LP	October 2017 (this study)
G. belizeanus	EH	October 2017 (This study)
G. caribaeus	EH	September–October 2015 [33] October 2015 [55] October 2017 (this study)
	LG	October 2017 [32]
G. carolinianus	TF	September–October 2015 [33]
G. excentricus	TF	March 2004 [30] 2013 [31] September–October 2015 [33] October 2017 (This study)
	LZ	September–October 2015 [33]
	FV	September–October 2015 [33]
	GC	September–October 2015 [33] April 2017, October 2017 (this study)
	LP	March 2004 [30] October 2017 ([33] and this study)
	LG	March 2004 [30] October 2017 ([33] and this study)
G. silvae	TF	2013 [31] September–October 2015 [33]
	GC	Winter 2010 [31]
	LG	October 2017 [32]

2.4. Evaluation of CTX-Like Toxicity with the Neuro-2a CBA

The evaluation of the CTX-like toxicity was conducted for 41 extracts from cultures harvested in 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 standard was CTX1B [59], which is a CTX typically 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*. For these islands only one strain was tested. If these islands were not considered in the analysis, then the one-way ANOVA was not significant. Table 3 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.

Table 3. Ciguatoxin (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 Figure 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

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 4.

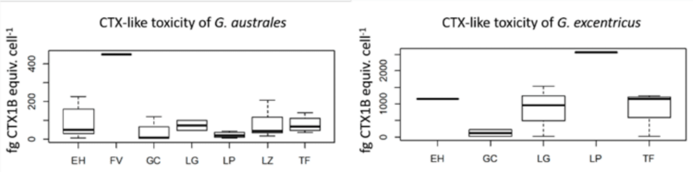

Figure 4. Distribution of CTX-like toxicity of *G. australes* and *G. excentricus* according to island of origin. EH (El Hierro), FV (Fuerteventura), GC (Gran Canaria), LG (La Gomera), LP (La Palma), LZ (Lanzarote) and TF (Tenerife).

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/quantification of CTX-like toxicity impossible. This effect was likely due to the presence of MTX4, which was also 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* was 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. [61] 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. australes* (VGO1252) with 107 fg CTX1B equiv. cell^{-1}, *G. carolinianus* (VGO1197) with 101 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 [25,37]. *G. caribaeus* showed no CTX-like toxicity

by CBA [66]. Referring to *G. belizeanus*, 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 [67], and *G. belizeanus* is 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, suggests that the contribution of *G. belizeanus* to CP of the Canary Islands may be negligible, although more strains should be evaluated.

The literature shows that 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.

2.5. 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 recognized 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 higher toxin content (0.35 ± 0.04 fg CTX1B equiv. cell^{-1}). Both immunochemical tools provide similar CTX quantifications 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, the 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 toward improved characterization of the different *Gambierdiscus* species.

2.6. Future Research Strategies to Understand CP in the Canary Islands

During the last years, the official control of CTX-like toxicity evaluation of the harvested fish in the Canary Islands has been used as the first step to prevent CP cases [21,69]. The official control obliges all analyzed 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 it 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 analyze 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 their interactions. Hence big gaps in the detection of fish and microalgal toxins still exist [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.

3. 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) show that data in the geographical and temporal scale is still scarce. Similar to the previous studies, the evaluation of the CTX-like toxicity shows that *G. excentricus* and *G. australes* are the species which could more contributeto CP. 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 the 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.

4. Methods

4.1. Reagents and Equipment

CTX1B standard solution was obtained from Prof. Richard J. Lewis (The Queensland University, Australia) [60] 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, fetal 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 (2×109 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 [79–85]. A microplate Reader KC4 (BIO-TEK Instruments, Inc., Vermont, VT, 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 (PalmSens, 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 (PalmSens, Houte, Netherlands).

4.2. 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, and Lanzarote were sampled in October 2016 and La Palma, Tenerife, El Hierro, and La Gomera were sampled in October 2017. Additionally, samplings were carried out in Gran Canaria in April 2017. Sampling locations are shown on a map in Figure 5, and Table 4 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).

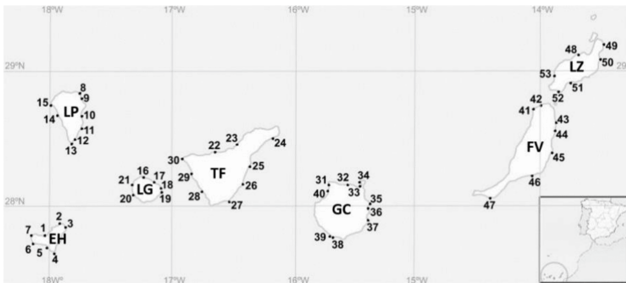

Figure 5. Sampling stations in the Canary Islands for the current study. Numbers correspond to locations described in Table 1. EH (El Hierro), FV (Fuerteventura), GC (Gran Canaria), LG (La Gomera), LP (La Palma), LZ (Lanzarote), and TF (Tenerife).

4.3. Isolation and Culturing

Samples were observed under a light microscope (Leica Microsystems GmbH, Germany) to isolate *Gambierdiscus* and *Fukuyoa* cells by the capillary method [86]. Each dinoflagellate cell was placed individually in one well of an untreated Nunc 24 well plate (Thermo Fisher Scientific) with 1 mL of modified ES medium [87]. 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 a 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 QSL-2100 Radiometer (Biospherical Instruments, San Diego, CA, USA).

Table 4. Description of the sampling stations of the present study in the Canary Islands. Ref.: correspond to the locations in Figure 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

4.4. Molecular Identification

To identify the fifty-two cultures at the species level, the D8-D10 region of the LSU rDNA was used [43,44]. 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. [88]. 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 [43]. Amplifications were carried out in a Mastercycler nexus gradient thermal cycler (Eppendorf, Spain) as it was described in Reverté et al. [61]. 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 [89] 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 [90] and by Bayesian inference (BI) using Mr. Bayes v.3.2.2 [91].

The model of evolutionary reconstruction for ML analysis was estimated using JModelTest 2.1.10 [92]. 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 [93].

4.5. 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 [94]. 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. [68], 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 [95]. The thecae were described following the nomenclature proposed by Fraga et al. [30]. 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 an 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 mold 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 and 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.

4.6. 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 and 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 1×10^5–1.6×10^6 cells of each strain. Cell pellets were kept with absolute methanol (1 mL of methanol for 1×10^{-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 s on and 2 s 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 by Turbovap (Caliper, Hopkinton, MA, USA) at 40 °C. Dried extract was dissolved with methanol and kept at −20 °C until toxin evaluation.

4.7. 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^+ [96] and veratridine, which activates the VGSC, enhancing the influx of Na^+ [97]. 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 [98].

The neuro-2a CBA and the data analysis were conducted following the protocol described in Caillaud et al. [99]. 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 6000 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 [98]. Data analysis was performed using SigmaPlot software 12.0 (Systat Software Inc., San Jose, CA, 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 [100].

4.8. 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 [83], and 10C9 mAb binds to the left wing of CTX3C and 51-hydroxyCTX3C [81]. The 8H4 mAb, used as a reporter antibody, binds to the right wing of all the four congeners [101]. For this reason, quantifications 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 quantifications can be provided either in fg $cell^{-1}$ of CTX1B equiv. or 51-hydroxyCTX3C equiv. In this work, the obtained quantifications 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. [68]. 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 [83,85]. Figure 6 shows the structure of the four CTX congeners (CTX1B and CTX3C congeners) that the antibodies recognize. 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 [101]. 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 6. Structure of CTX1B and CTX3C congeners recognized by the antibodies used in this work.

Supplementary Materials: The following are available online at http://www.mdpi.com/2072-6651/12/11/692/s1, Table S1: supplementary material, Table S2: supplementary material, Table S3: Cell viability and CTX-like toxicity after the exposure of neuro-2a cells to *Gambierdiscus* spp. extracts with (O/V$^+$) and without (O/V$^-$) ouabain and veratridine treatment.

Author Contributions: À.T., G.G., M.C. and J.D., M.F.-T. and T.T. designed the experiments. À.T. and M.R.V. carried out the samplings, the microalgae isolation and scale up the cultures. À.T. and G.G. performed the experiments. À.T. and K.B.A. carried out the identification of isolates by molecular tools. À.T., M.R.V. and M.F.-T. characterized the species by light and electron microscope. À.T., M.C., J.D., G.G., M.R.V., K.B.A. and M.F.-T. analyzed and interpreted the data. À.T. wrote the paper. All authors contributed to editing the paper. All authors have read and agreed to the published version of the manuscript.

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References

1. Adachi, R.; Fukuyo, Y. The thecal structure of a marine toxic dinoflagellate *Gambierdiscus toxicus* gen. et spec. nov. collected in a ciguatera-endemic area. *Bull. Jpn. Soc. Sci. Fish.* **1979**, *45*, 67–71. [CrossRef]
2. Murata, M.; Ishibashi, Y.; Fukui, M.; Yasumoto, T.; Legrand, A.M. Structures and Configurations of Ciguatoxin from the Moray Eel *Gymnothorax javanicus* and Its Likely Precursor from the Dinoflagellate *Gambierdiscus toxicus*. *J. Am. Chem. Soc.* **1990**, *112*, 4380–4386. [CrossRef]

82. Oguri, H.; Tanaka, S.-I.; Hishiyama, S.; Oishi, T.; Hirama, M.; Tumuraya, T.; Tomioka, Y.; Mizugaki, M. Designed hapten aimed at anti-ciguatoxin monoclonal antibody: Synthesis, immunization and discrimination of the C2 configuration. *Syntesis* **1999**, *S1*, 1431–1436. [CrossRef]
83. Tsumuraya, T.; Takeuchi, K.; Yamashita, S.; Fujii, I.; Hirama, M. Development of a monoclonal antibody against the left wing of ciguatoxin CTX1B: Thiol strategy and detection using a sandwich ELISA. *Toxicon* **2012**, *60*, 348–357. [CrossRef]
84. Tsumuraya, T.; Fujii, I.; Hirama, M. Production of monoclonal antibodies for sandwich immunoassay detection of Pacific ciguatoxins. *Toxicon* **2010**, *56*, 797–803. [CrossRef]
85. Tsumuraya, T.; Fujii, I.; Inoue, M.; Tatami, A.; Miyazaki, K.; Hirama, M. Production of monoclonal antibodies for sandwich immunoassay detection of ciguatoxin 51-hydroxyCTX3C. *Toxicon* **2006**, *48*, 287–294. [CrossRef]
86. Hoshaw, R.W.; Rosowski, J.R. *Handbook of Phycological Methods: Culture Methods and Growth Measurements*; Stein, J.R., Ed.; Cambridge University Press: London, UK; New York, NY, USA, 1973; p. 448.
87. Provasoli, L. Media and prospects of the cultivation of marine algae. In *Culure and Collection of Algae Proceedings*; Japanese Society of Plant Physiology: Hakone, Japan, 1968; pp. 63–75.
88. Toldrà, A.; Andree, K.B.; Fernández-Tejedor, M.; Diogène, J.; Campàs, M. Dual quantitative PCR assay for identification and enumeration of *Karlodinium veneficum* and *Karlodinium armiger* combined with a simple and rapid DNA extraction method. *J. Appl. Phycol.* **2018**, *30*, 2435–2445. [CrossRef]
89. Rozewicki, J.; Li, S.; Amada, K.M.; Standley, D.M.; Katoh, K. MAFFT-DASH: Integrated protein sequence and structural alignment. *Nucleic Acids Res.* **2019**, *47*, W5–W10. [CrossRef]
90. Stamatakis, A. RAxML version 8: A tool for phylogenetic analysis and post-analysis of large phylogenies. *Bioinformatics* **2014**, *30*, 1312–1313. [CrossRef] [PubMed]
91. Huelsenbeck, J.P.; Ronquist, F. MRBAYES: Bayesian inference of phylogenetic trees. *Bioinformatics* **2001**, *17*, 754–755. [CrossRef]
92. Darriba, D.; Taboada, G.L.; Doallo, R.; Posada, D. jModelTest 2: More models, new heuristics and high-performance computing Europe PMC Funders Group. *Nat. Methods* **2012**, *9*, 772. [CrossRef]
93. Rambaut, A.; Drummond, A.J.; Xie, D.; Baele, G.; Suchard, M.A. Posterior summarization in Bayesian phylogenetics using Tracer 1.7. *Syst. Biol.* **2018**, *67*, 901. [CrossRef] [PubMed]
94. Bomber, J.W.; Tindall, D.R.; Miller, D.M. Genetic variability in toxin potencies among seventeen clones of *Gambierdiscus toxicus* (Dinophyceae). *J. Phycol.* **1989**, *25*, 617–625. [CrossRef]
95. Fritz, L.; Triemer, R.E. A Rapid simple technique utilizing calcofluor white M2R for the visualization of dinofalgellate thecal plates. *J. Phycol.* **1985**, *21*, 662–664. [CrossRef]
96. Catterall, W.A.; Nirenberg, M. Sodium uptake associated with activation of action potential ionophores of cultured neuroblastoma and muscle cells. *Proc. Natl. Acad. Sci. USA* **1973**, *70*, 3759–3763. [CrossRef]
97. Catterall, W.A. Molecular Properties of Voltage-Sensitive Sodium Channels. In *New Insights into Cell and Membrane Transport Processes*; Springer: Boston, MA, USA, 1986; pp. 3–20.
98. Manger, R.L.; Leja, L.S.; Lee, S.Y.; Hungerforg, J.M.; Wekell, M.M. Tetrazolium-based cell bioassay for neurotoxins active on voltage-sensitive sodium channels: Semiautomated assay for saxitoxins, brevetoxins, and ciguatoxins. *Anal. Biochem.* **1993**, *214*, 190–194. [CrossRef]
99. Caillaud, A.; Cañete, E.; de la Iglesia, P.; Giménez, G.; Diogène, J. Cell-based assay coupled with chromatographic fractioning: A strategy for marine toxins detection in natural samples. *Toxicol. Vitr.* **2009**, *23*, 1591–1596. [CrossRef] [PubMed]
100. R Core Team. *R: A Language and Environment for Statistical Computing*; R Foundation for Statistical Computing: Vienna, Austria, 2017.
101. Tsumuraya, T.; Sato, T.; Hirama, M.; Fujii, I. Highly Sensitive and Practical Fluorescent Sandwich ELISA for Ciguatoxins. *Anal. Chem.* **2018**, *90*, 7318–7324. [CrossRef]

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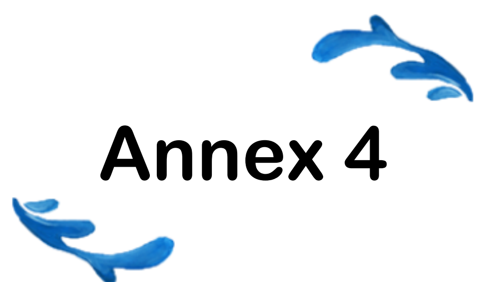
64. Estevez, P.; Sibat, M.; Leao, J.M.; Tudó, A.; Rambla-Alegre, M.; Aligizak, K.; Gago-Martinez, A.; Diogène, J.; Hess, P. Use of Mass Spectrometry to determine the Diversity of Toxins Produced by *Gambierdiscus* and *Fukuyoa* Species from Balearic Islands and Crete (Mediterranean Sea) and the Canary Islands (Northeast Atlantic). *Toxins (Basel)* **2020**, *12*, 305. [CrossRef]
65. Caillaud, A.; Yasumoto, T.; Diogène, J. Detection and quantification of maitotoxin-like compounds using a neuroblastoma (Neuro-2a) cell based assay. Application to the screening of maitotoxin-like compounds in Gambierdiscus spp. *Toxicon* **2010**, *56*, 36–44. [CrossRef]
66. Lewis, R.J.; Inserra, M.; Vetter, I.; Holland, W.C.; Hardison, D.R.; Tester, P.A.; Litaker, R.W. Rapid extraction and identification of maitotoxin and ciguatoxin-like toxins from Caribbean and pacific Gambierdiscus using a new functional bioassay. *PLoS ONE* **2016**, *11*, e0160006. [CrossRef] [PubMed]
67. De Haro, L.; Pommier, P.; Valli, M. Emergence of Imported Ciguatera in Europe: Report of 18 Cases at the Poison Control Centre of Marseille. *J. Toxicol. Clin. Toxicol.* **2003**, *41*, 927–930. [CrossRef] [PubMed]
68. Gaiani, G.; Leonardo, S.; Tudó, À.; Toldrà, A.; Rey, M.; Andree, K.B.; Tsumuraya, T.; Hirama, M.; Diogène, J.; O'Sullivan, C.K.; et al. Rapid extraction of ciguatoxins from *Gambierdiscus* and *Fukuyoa* and detection with immunosensing tools. *Ecotoxicol. Environ. Saf.* **2020**, *204*, 111004. [CrossRef]
69. Sanchez-Henao, A.; García-álvarez, N.; Silva, F.; Estévez, P.; Gago-martínez, A.; Martín, F.; Ramos-sosa, M.; Fernández, A.; Diogène, J.; Real, F. Presence of CTXs in moray eels and dusky groupers in the marine environment of the Canary Islands. *Aquat. Toxicol.* **2020**, *221*, 105427. [CrossRef] [PubMed]
70. Tibiriçá, C.E.J.A.; Sibat, M.; Fernandes, L.F.; Bilien, G.; Chomérat, N.; Hess, P.; Mafra, L.L., Jr. Diversity and Toxicity of the Genus *Coolia* Meunier in Brazil, and Detection of 44-methyl Gambierone in *Coolia tropicalis*. *Toxins* **2020**, *12*, 327. [CrossRef] [PubMed]
71. Rodríguez, I.; Genta-Jouve, G.; Alfonso, C.; Calabro, K.; Alonso, E.; Sánchez, J.A.; Alfonso, A.; Thomas, O.P.; Botana, L.M. Gambierone, a Ladder-Shaped Polyether from the Dinoflagellate *Gambierdiscus belizeanus*. *Org. Lett.* **2015**, *17*, 2392–2395. [CrossRef]
72. Boente-Juncal, A.; Álvarez, M.; Antelo, Á.; Rodríguez, I.; Calabro, K.; Vale, C.; Thomas, O.P.; Botana, L.M. Structure elucidation and biological evaluation of maitotoxin-3, a homologue of gambierone, from *Gambierdiscus belizeanus*. *Toxins (Basel)* **2019**, *11*, 79. [CrossRef]
73. Murray, J.S.; Nishimura, T.; Finch, S.C.; Rhodes, L.L.; Puddick, J.; Harwood, D.T.; Larsson, M.E.; Doblin, M.A.; Leung, P.; Yan, M.; et al. The role of 44-methylgambierone in ciguatera fish poisoning: Acute toxicity, production by marine microalgae and its potential as a biomarker for *Gambierdiscus* spp. *Harmful Algae* **2020**, *97*, 101853. [CrossRef]
74. Reverté, L.; Soliño, L.; Carnicer, O.; Diogène, J.; Campàs, M. Alternative methods for the detection of emerging marine toxins: Biosensors, biochemical assays and cell-based assays. *Mar. Drugs* **2014**, *12*, 5719–5963. [CrossRef]
75. Caillaud, A.; De La Iglesia, P.; Darius, H.T.; Pauillac, S.; Aligizaki, K.; Fraga, S.; Chinain, M.; Diogène, J. Update on methodologies available for ciguatoxin determination: Perspectives to confront the onset of ciguatera fish poisoning in Europe. *Mar. Drugs* **2010**, *8*, 1838–1907. [CrossRef]
76. Kretzschmar, A.L.; Verma, A.; Kohli, G.; Murray, S. Development of a quantitative PCR assay for the detection and enumeration of a potentially ciguatoxin-producing dinoflagellate, *Gambierdiscus lapillus* (Gonyaulacales, Dinophyceae). *PLoS ONE* **2019**, *14*, 1–18. [CrossRef]
77. Smith, K.F.; Biessy, L.; Argyle, P.A.; Trnski, T.; Halafihi, T.; Rhodes, L.L. Molecular identification of *Gambierdiscus* and *Fukuyoa* (Dinophyceae) from environmental samples. *Mar. Drugs* **2017**, *15*, 243. [CrossRef] [PubMed]
78. Fernández-Zabala, J.; Tuya, F.; Amorim, A.; Soler-Onís, E. Benthic dinoflagellates: Testing the reliability of the artificial substrate method in the Macaronesian region. *Harmful Algae* **2019**, *87*, 101634. [CrossRef] [PubMed]
79. Nagumo, Y.; Oguri, H.; Tsumoto, K.; Shindo, Y.; Hirama, M.; Tsumuraya, T.; Fujii, I.; Tomioka, Y.; Mizugaki, M.; Kumagai, I. Phage-display selection of antibodies to the left end of CTX3C using synthetic fragments. *J. Immunol. Methods* **2004**, *289*, 137–146. [CrossRef]
80. Nagumo, Y.; Oguri, H.; Shindo, Y.; Sasaki, S.-Y.; Oishi, T.; Hirama, M.; Tomioka, Y.; Mizugaki, M.; Tsumuraya, T. Concise synthesis of ciguatoxin ABC-ring fragments and surface plasmon resonance study of the interaction of their BSA conjugates with monoclonal antibodies. *Bioorg. Med. Chem. Lett.* **2001**, *11*, 2037–2040. [CrossRef]
81. Oguri, H.; Hirama, M.; Tsumuraya, T.; Fujii, I.; Maruyama, M.; Uehara, H.; Nagumo, Y. Synthesis-based approach toward direct sandwich immunoassay for ciguatoxin CTX3C. *J. Am. Chem. Soc.* **2003**, *125*, 7608–7612. [CrossRef]

43. Chinain, M.; Faust, M.A.; Pauillac, S. Morphology and molecular analyses of three toxic species of *Gambierdiscus* (Dinophyceae): *G. pacificus*, sp nov., *G. australes*, sp nov., and *G. polynesiensis*, sp nov. *J. Phycol.* **1999**, *35*, 1282–1296. [CrossRef]
44. Litaker, R.W.; Vandersea, M.W.; Faust, M.A.; Kibler, S.R.; Chinain, M.; Holmes, M.J.; Holland, W.C.; Tester, P.A. Taxonomy of *Gambierdiscus* including four new species, *Gambierdiscus caribaeus*, *Gambierdiscus carolinianus*, *Gambierdiscus carpenteri* and *Gambierdiscus ruetzleri* (Gonyaulacales, Dinophyceae). *Phycologia* **2009**, *48*, 344–390. [CrossRef]
45. Hoppenrath, M.; Kretzschmar, A.L.; Kaufmann, M.J.; Murray, S.A. Morphological and molecular phylogenetic identification and record verification of *Gambierdiscus excentricus* (Dinophyceae) from Madeira Island (NE Atlantic Ocean). *Mar. Biodivers. Rec.* **2019**, *12*, 1–9. [CrossRef]
46. Aligizaki, K.; Nikolaidis, G.; Fraga, S. Is *Gambierdiscus* expanding to new areas? *Harmful Algae News* **2008**, *36*, 6–7.
47. Kibler, S.R.; Litaker, R.W.; Holland, W.C.; Vandersea, M.W.; Tester, P.A. Growth of eight *Gambierdiscus* (Dinophyceae) species: Effects of temperature, salinity and irradiance. *Harmful Algae* **2012**, *19*, 1–14. [CrossRef]
48. Xu, Y.; Richlen, M.L.; Liefer, J.D.; Robertson, A.; Kulis, D.; Smith, T.B.; Parsons, M.L.; Anderson, D.M. Influence of Environmental Variables on *Gambierdiscus* spp. (Dinophyceae) Growth and Distribution. *PLoS ONE* **2016**, *11*, e0153197. [CrossRef] [PubMed]
49. Espino, F.; Tuya, F.; Rosario, A.; Bosch, N.E.; Coca, J.; Gonz, A.J.; Rosario, F.; Otero-ferrer, F.J.; Moreno, Á.C.; Haroun, R. Geographical Range Extension of the Spotfin burrfish, *Chilomycterus reticulatus* (L. 1758), in the Canary Islands: A Response to Ocean Warming? *Diversity* **2019**, *11*, 230. [CrossRef]
50. PLOCAN Oceanic Platform of the Canary Islands (PLOCAN). Available online: http://obsplatforms.plocan.eu/climatology (accessed on 3 June 2020).
51. Yoshimatsu, T.; Yamaguchi, H.; Iwamoto, H.; Nishimura, T.; Adachi, M. Effects of temperature, salinity and their interaction on growth of Japanese *Gambierdiscus* spp. (Dinophyceae). *Harmful Algae* **2014**, *35*, 29–37. [CrossRef]
52. Litaker, R.W.; Tester, P.A.; Vandersea, M.W. Species-specific PCR assays for *Gambierdiscus excentricus* and *Gambierdiscus silvae* (Gonyaulacales, Dinophyceae). *J. Phycol.* **2019**, *55*, 730–732. [CrossRef]
53. Tawong, W.; Yoshimatsu, T.; Yamaguchi, H.; Adachi, M. Temperature and salinity effects and toxicity of <i>*Gambierdiscus caribaeus* (Dinophyceae) from Thailand. *Phycologia* **2016**, *55*, 274–278. [CrossRef]
54. Tuya, F.; Haroun, R.J. Spatial patterns and response to wave exposure of shallow water algal assemblages across the Canarian Archipelago: A multi-scaled approach. *Mar. Ecol. Prog. Ser.* **2006**, *311*, 15–28. [CrossRef]
55. Soler-Onis, E.; Fernández-Zabala, J.; Ojeda-Rodriguez, A.; Amorim, A. Bloom of *Gambierdiscus caribaeus* in the temperate-subtropical waters of El Hierro, Canary Islands (North East Atlantic). *Harmful Algae News* **2016**, *55*, 14–16.
56. Vélez-Belchí, P.; Gonzalez-Carballo, M.; Perez-Hernández, M.D.; Hernández-Guerra, A. Open ocean temperature and salinity trends. *Oceanogr. Biol. Featur. Canar. Curr. Large Mar. Ecosyst.* **2015**, *13*, 299–308.
57. Kibler, S.R.; Tester, P.A.; Kunkel, K.E.; Moore, S.K.; Litaker, R.W. Effects of ocean warming on growth and distribution of dinoflagellates associated with ciguatera fish poisoning in the Caribbean. *Ecol. Modell.* **2015**, *316*, 194–210. [CrossRef]
58. Ikehara, T.; Kuniyoshi, K.; Oshiro, N.; Yasumoto, T. Biooxidation of ciguatoxins leads to species-specific toxin profiles. *Toxins (Basel)* **2017**, *9*, 205. [CrossRef] [PubMed]
59. Yogi, K.; Sakugawa, S.; Oshiro, N.; Ikehara, T.; Sugiyama, K.; Yasumoto, T. Determination of toxins involved in ciguatera fish poisoning in the Pacific by LC/MS. *J. AOAC Int.* **2014**, *97*, 398–402. [CrossRef] [PubMed]
60. Lewis, R.J.; Sellin, M.; Poli, M.A.; Norton, R.S.; MacLeod, J.K.; Sheil, M.M. Purification and characterization of ciguatoxins from moray eel (*Lycodontis javanicus*, Muraenidae). *Toxicon* **1991**, *29*, 1115–1127. [CrossRef]
61. Reverté, L.; Toldrà, A.; Andree, K.B.; Fraga, S.; de Falco, G.; Campàs, M.; Diogène, J. Assessment of cytotoxicity in ten strains of *Gambierdiscus australes* from Macaronesian Islands by neuro-2a cell-based assays. *J. Appl. Phycol.* **2018**, *30*, 2447–2461. [CrossRef]
62. Paz, B.; Riobó, P.; Franco, J.M. Preliminary study for rapid determination of phycotoxins in microalgae whole cells using matrix-assisted laser desorption/ionization time-of-flight mass spectrometry. *Rapid Commun. Mass Spectrom.* **2011**, *25*, 3627–3639. [CrossRef]
63. Pisapia, F.; Sibat, M.L.; Herrenknecht, C.; Lhaute, K.; Gaiani, G.; Ferron, P.J.; Fessard, V.; Fraga, S.; Nascimento, S.M.; Litaker, R.W.; et al. Maitotoxin-4, a novel MTX analog produced by *Gambierdiscus* excentricus. *Mar. Drugs* **2017**, *15*, 220. [CrossRef]

23. Rhodes, L.; Harwood, T.; Smith, K.; Argyle, P.; Munday, R. Production of ciguatoxin and maitotoxin by strains of *Gambierdiscus australes*, *G. pacificus* and *G. polynesiensis* (Dinophyceae) isolated from Rarotonga, Cook Islands. *Harmful Algae* **2014**, *39*, 185–190. [CrossRef]
24. Pisapia, F.; Holland, W.C.; Hardison, D.R.; Litaker, R.W.; Fraga, S.; Nishimura, T.; Adachi, M.; Nguyen-Ngoc, L.; Séchet, V.; Amzil, Z.; et al. Toxicity screening of 13 *Gambierdiscus* strains using neuro-2a and erythrocyte lysis bioassays. *Harmful Algae* **2017**, *63*, 173–183. [CrossRef] [PubMed]
25. Rossignoli, A.E.; Tudó, A.; Bravo, I.; Díaz, P.A.; Diogène, J.; Riobó, P. Toxicity characterisation of *Gambierdiscus* species from the canary Islands. *Toxins (Basel)* **2020**, *12*, 134. [CrossRef] [PubMed]
26. Tester, P.A.; Litaker, R.W.; Berdalet, E. Climate change and harmful benthic microalgae. *Harmful Algae* **2020**, *91*, 101655. [CrossRef]
27. Navarro-Pérez, E.; Barton, E.D. Seasonal and interannual variability of the Canary Current. *Sci. Mar.* **2001**, *65*, 205–213. [CrossRef]
28. Davenport, R.; Neuer, S.; Helmke, P.; Perez-Marrero, J.; Llinas, O. Primary productivity in the northern Canary Islands region as inferred from SeaWiFS imagery. *Deep. Res. Part II Top. Stud. Oceanogr.* **2002**, *49*, 3481–3496. [CrossRef]
29. Fraga, S.; Riobó, P.; Diogène, J.; Paz, B.; Franco, J.M. Toxic and potenially toxic benthic dinoflagellates observed in Macaronesia (NE Atlantic Archipelago). In Proceedings of the Abstract Book of the 11th International Conference on Harmful Algae, Capetown, South Africa, 15–19 November 2004; p. 115.
30. Fraga, S.; Rodríguez, F.; Caillaud, A.; Diogène, J.; Raho, N.; Zapata, M. *Gambierdiscus excentricus* sp. nov. (Dinophyceae), a benthic toxic dinoflagellate from the Canary Islands (NE Atlantic Ocean). *Harmful Algae* **2011**, *11*, 10–22. [CrossRef]
31. Fraga, S.; Rodríguez, F. Genus *Gambierdiscus* in the Canary Islands (NE Atlantic Ocean) with Description of *Gambierdiscus silvae* sp. nov., a New Potentially Toxic Epiphytic Benthic Dinoflagellate. *Protist* **2014**, *165*, 839–853. [CrossRef]
32. Bravo, I.; Rodriguez, F.; Ramilo, I.; Rial, P.; Fraga, S. Ciguatera-causing dinoflagellate *Gambierdiscus* spp. (Dinophyceae) in a subtropical region of North Atlantic Ocean (Canary Islands): Morphological characterization and biogeography. *Toxins (Basel)* **2019**, *11*, 423. [CrossRef]
33. Rodríguez, F.; Fraga, S.; Ramilo, I.; Rial, P.; Figueroa, R.I.; Riobó, P.; Bravo, I. "Canary Islands (NE Atlantic) as a biodiversity 'hotspot' of *Gambierdiscus*: Implications for future trends of ciguatera in the area". *Harmful Algae* **2017**, *67*, 131–143.
34. Faust, M.A. Observation of sand-dwelling toxic dinoflagellates (Dinophyceae) from widely differing sites, including two new species. *J. Phycol.* **1995**, *31*, 996–1003. [CrossRef]
35. Díaz-Asencio, L.; Clausing, R.J.; Vandersea, M.; Chamero-Lago, D.; Gómez-Batista, M.; Hernández-Albernas, J.I.; Chomérat, N.; Rojas-Abrahantes, G.; Litaker, R.W.; Tester, P.; et al. Ciguatoxin occurrence in food-web components of a cuban coral reef ecosystem: Risk-assessment implications. *Toxins (Basel)* **2019**, *11*, 722. [CrossRef] [PubMed]
36. Richlen, M.L.; Morton, S.L.; Barber, P.H.; Lobel, P.S. Phylogeography, morphological variation and taxonomy of the toxic dinoflagellate *Gambierdiscus toxicus* (Dinophyceae). *Harmful Algae* **2008**, *7*, 614–629. [CrossRef]
37. Litaker, R.W.; Holland, W.C.; Hardison, D.R.; Pisapia, F.; Hess, P.; Kibler, S.R.; Tester, P.A. Ciguatoxicity of *Gambierdiscus* and *Fukuyoa* species from the Caribbean and Gulf of Mexico. *PLoS ONE* **2017**, *12*, 1–19. [CrossRef]
38. Catania, D.; Richlen, M.L.; Mak, Y.L.; Morton, S.L.; Laban, E.H.; Xu, Y.; Anderson, D.M.; Chan, L.L.; Berumen, M.L. The prevalence of benthic dinoflagellates associated with ciguatera fish poisoning in the central Red Sea. *Harmful Algae* **2017**, *68*, 206–216. [CrossRef] [PubMed]
39. Murray, S.; Momigliano, P.; Heimann, K.; Blair, D. Molecular phylogenetics and morphology of *Gambierdiscus yasumotoi* from tropical eastern Australia. *Harmful Algae* **2014**, *39*, 242–252. [CrossRef]
40. Leaw, C.P.; Lim, P.T.; Tan, T.H.; Tuan-Halim, T.N.; Cheng, K.W.; Ng, B.K.; Usup, G. First report of the benthic dinoflagellate, *Gambierdiscus belizeanus* (Gonyaulacales: Dinophyceae) for the east coast of Sabah, Malaysian Borneo. *Phycol. Res.* **2011**, *59*, 143–146. [CrossRef]
41. Xu, Y.; Richlen, M.L.; Morton, S.L.; Mak, Y.L.; Chan, L.L.; Tekiau, A.; Anderson, D.M. Distribution, abundance and diversity of *Gambierdiscus* spp. from a ciguatera-endemic area in Marakei, Republic of Kiribati. *Harmful Algae* **2014**, *34*, 56–68. [CrossRef]
42. Bravo, I.; Figueroa, R.I.; Fraga, S. Cellular and nuclear morphological variability within a single species of the toxigenic dinoflagellate genus *Gambierdiscus*: Relationship to life-cycle processes. *Harmful Algae* **2014**, *40*, 1–8. [CrossRef]

3. Parsons, M.L.; Settlemier, C.J.; Ballauer, J.M. An examination of the epiphytic nature of *Gambierdiscus toxicus*, a dinoflagellate involved in ciguatera fish poisoning. *Harmful Algae* **2011**, *10*, 598–605. [CrossRef] [PubMed]
4. FAO; WHO. *Report of the Expert Meeting on Ciguatera Poisoning*; Food Safety and Quality: Rome, Italy, 2020; ISBN 9789240006294.
5. Molgó, J.; Shimahara, T.; Legrand, A.M. Ciguatoxin, extracted from poisonous morays eels, causes sodium-dependent calcium mobilization in NG108-15 neuroblastoma × glioma hybrid cells. *Neurosci. Lett.* **1993**, *158*, 147–150. [CrossRef]
6. Nicholson, G.M.; Lewis, R.J. Ciguatoxins: Cyclic polyether modulators of voltage-gated ion channel function. *Mar. Drugs* **2006**, *4*, 82–118. [CrossRef]
7. Gillespie, N.C.; Lewis, R.J.; Pearn, J.H.; Bourke, A.T.; Holmes, M.J.; Bourke, J.B.; Shields, W.J. Ciguatera in Australia. Occurrence, clinical features, pathophysiology and management. *Med. J. Aust.* **1986**, *145*, 584–590. [CrossRef]
8. Friedman, M.A.; Fernandez, M.; Backer, L.C.; Dickey, R.W.; Bernstein, J.; Schrank, K.; Kibler, S.; Stephan, W.; Gribble, M.O.; Bienfang, P.; et al. An updated review of ciguatera fish poisoning: Clinical, epidemiological, environmental, and public health management. *Mar. Drugs* **2017**, *15*, 72. [CrossRef]
9. Skinner, M.P.; Brewer, T.D.; Johnstone, R.; Fleming, L.E.; Lewis, R.J. Ciguatera fish poisoning in the pacific islands (1998 to 2008). *PLoS Negl. Trop. Dis.* **2011**, *5*, 1–7. [CrossRef]
10. Llewellyn, L.E. Revisiting the association between sea surface temperature and the epidemiology of fish poisoning in the South Pacific: Reassessing the link between ciguatera and climate change. *Toxicon* **2010**, *56*, 691–697. [CrossRef]
11. Tester, P.A.; Feldman, R.L.; Nau, A.W.; Kibler, S.R.; Wayne Litaker, R. Ciguatera fish poisoning and sea surface temperatures in the Caribbean Sea and the West Indies. *Toxicon* **2010**, *56*, 698–710. [CrossRef]
12. Farrell, H.; Edwards, A.; Zammit, A. Four recent ciguatera fish poisoning incidents in New South Wales, Australia linked to imported fish. *Commun. Dis. Intell.* **2019**, *43*. [CrossRef]
13. Bravo, J.; Suarez, F.C.; Ramirez, A.S.; Acosta, F. Ciguatera, an Emerging Human Poisoning in Europe. *J. Aquac. Mar. Biol.* **2015**, *3*, 1–6.
14. Chinain, M.; Gatti, C.M.; Roué, M.; Darius, H.T. Ciguatera poisoning in French Polynesia: Insights into the novel trends of an ancient disease. *New Microbes New Infect.* **2019**, *31*, 100565. [CrossRef] [PubMed]
15. Nishimura, T.; Sato, S.; Tawong, W.; Sakanari, H.; Uehara, K.; Shah, M.M.R.; Suda, S.; Yasumoto, T.; Taira, Y.; Yamaguchi, H.; et al. Genetic Diversity and Distribution of the Ciguatera-Causing Dinoflagellate *Gambierdiscus* spp. (Dinophyceae) in Coastal Areas of Japan. *PLoS ONE* **2013**, *8*, e60882. [CrossRef] [PubMed]
16. Tester, P.A.; Vandersea, M.W.; Buckel, C.A.; Kibler, S.R.; Holland, W.C.; Davenport, E.D.; Clark, R.D.; Edwards, K.F.; Taylor, J.C.; Pluym, J.L.V.; et al. *Gambierdiscus* (Dinophyceae) species diversity in the flower garden banks national marine sanctuary, Northern Gulf of Mexico, USA. *Harmful Algae* **2013**, *29*, 1–9. [CrossRef]
17. Pérez-Arellano, J.L.; Luzardo, O.P.; Brito, A.P.; Cabrera, M.H.; Zumbado, M.; Carranza, C.; Angel-Moreno, A.; Dickey, R.W.; Boada, L.D. Ciguatera fish poisoning, Canary Islands. *Emerg. Infect. Dis.* **2005**, *11*, 1981–1982. [CrossRef]
18. Gouveia, N.N.; Vale, P.; Gouveia, N.; Delgado, J. Primeiro Registo da Ocorrência de Episódios do Tipo Ciguatérico no Arquipélago da Madeira. In *Algas Toxicas e Biotoxinas nas Águas da Península Ibérica*; IPIMAR: Lisboa, Portugal, 2010; pp. 152–157.
19. Falcón García, I. *Epidemiología de la Intoxicación Alimentaria por Ciguatoxinas en Canarias*; Hospital Universitario Nuestra Señora de Candelaria: Santa Cruz de Tenerife, Spain, 2018.
20. Caillaud, A.; Eixarch, H.; de la Iglesia, P.; Rodriguez, M.; Dominguez, L.; Andree, K.B.; Diogène, J. Towards the standardisation of the neuroblastoma (neuro-2a) cell-based assay for ciguatoxin-like toxicity detection in fish: Application to fish caught in the Canary Islands. *Food Addit. Contam. Part A Chem. Anal. Control. Expo. Risk Assess.* **2012**, *29*, 1000–1010. [CrossRef]
21. Sanchez-Henao, J.A.; García-Álvarez, N.; Fernández, A.; Saavedra, P.; Silva Sergent, F.; Padilla, D.; Acosta-Hernández, B.; Martel Suárez, M.; Diogène, J.; Real, F. Predictive score and probability of CTX-like toxicity in fish samples from the official control of ciguatera in the Canary Islands. *Sci. Total Environ.* **2019**, *673*, 576–584. [CrossRef]
22. Munday, R.; Murray, S.; Rhodes, L.; Larsson, M.; Harwood, D. Ciguatoxins and Maitotoxins in Extracts of Sixteen *Gambierdiscus* Isolates and One *Fukuyoa* Isolate from the South Pacific and Their Toxicity to Mice by Intraperitoneal and Oral Administration. *Mar. Drugs* **2017**, *15*, 208. [CrossRef]



Annex 4

UNIVERSITAT ROVIRA I VIRGILI
DEVELOPMENT OF BIOANALYTICAL DEVICES FOR THE DETECTION OF CIGUATOXINS AND THE CIGUATOXIN PRODUCING GENERA GAMBIERDISCUS AND FUKUYOA
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CHAPTER SEVEN

The wide spectrum of methods available to study marine neurotoxins

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1. Introduction

Marine neurotoxins are present in numerous organisms and may play an important role in community structure and interspecies interactions within the ecosystems. Some marine neurotoxins are extremely potent and are responsible for serious seafood poisoning events. Marine neurotoxins are in the target of curiosity, originally due to their impact on the

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health of humans. Paralytic shellfish poisoning (PSP) toxins, among which saxitoxin (STX) is the most notorious, were probably the first toxins under the focus of scientists. Major advancement on PSP toxin research occurred after the early work of Sommer and Meyer (1937) focusing on their detection. Previously, some 2300 years ago, another group of marine neurotoxins, the tetrodotoxins (TTXs), were already in the scene. Consumption of puffer fish that contains TTXs, also known as *fugu* (https://en.wikipedia.org/wiki/Fugu), already occurred in Japan, with most probable occurrence of poisoning cases. In the 16th century, ciguatera poisoning, explained by the presence of ciguatoxins (CTXs) in fish, was probably the first case of seafood poisoning involving neurotoxins that has been described (Urdaneta, 1580).

Neurotoxins have specific mechanisms of action that will ultimately determine their toxicological effects. These rely on the structure of neurotoxins but also on the physiology of the living organism that they reach, and the available targets that are present at the cellular level. For several years, the role that neurotoxins play in the environment has also gained attention in order to better understand how these molecules may affect the complex relations among organisms and the effects that they cause in marine animals (Landsberg et al., 2014).

Hence, the assessment of neurotoxins in the environment is a need that responds to many different questions and interests. One of them is clearly the understanding of the toxicological processes in which neurotoxins are involved. For one side, a toxicologist will focus on the understanding of their mechanism of action at the cellular level, and eventually all the previous steps that occur within an organism, including the absorption of neurotoxins, their distribution, metabolization and excretion. Interestingly, the metabolization processes occurring in organisms are crucial to understand the complexity of neurotoxins, since the original neurotoxins produced in nature may suffer structural changes within organisms, resulting in new neurotoxic derivatives. Hence, a key issue is describing the neurotoxins responsible for the toxicological effects found in nature, with a particular interest to establish links between molecular structure and activity. It would also bring knowledge on the complex interrelationships among organisms to better understand life and cell mechanisms and interactions.

Another need arises when marine neurotoxins are involved in serious toxicological episodes, for example, those related to seafood consumption. In order to ensure that seafood reaching consumers is safe and to manage possible risks, effective and reliable monitoring programs need to be set in place. This means that control laboratories need to have rapid, sensitive

and cost-effective methods to determine the presence and amount of neurotoxins, sometimes with the requirement to fulfill legislative regulations.

A third example would be the need to have effective methodological strategies to identify novel neurotoxins in our oceans, for example, with the aim of discovering new molecules having original mechanisms of action. This would allow, for example, to identify new neurotoxic molecules that may have therapeutic applications.

As knowledge on marine neurotoxins evolved, so did the associated methodologies to assess neurotoxins in nature (Diogène and Campàs, 2017). In this chapter we will focus on the available methodological strategies to assess neurotoxins in the marine environment. Our aim is not to provide an exhaustive directory of methods described in the literature for each marine neurotoxin, but to provide the reader with a rationale to select, among many, the methodological approach, or combination of methods, that best fits her or his interest when addressing the detection or quantification of marine neurotoxins. We will illustrate the different sections with a few remarkable examples of selected scientific publications that have addressed marine neurotoxins assessment to different extents.

2. Animal bioassays for marine toxins

Animal bioassays for the evaluation of marine toxins, and more specifically neurotoxins, were used in the initial steps following poisoning events occurring after consumption of seafood. They were implemented well before scientists were aware of the nature of the toxic compound, and constituted a tool to understand and manage poisoning episodes and reduce their impact. An example was the development of mouse bioassays (MBAs) for recognition of PSP toxins (Sommer and Meyer, 1937) or CTXs (Banner et al., 1960), well before their structures were elucidated. This strategy was so efficient, that animal bioassays were routinely implemented for monitoring purposes as reliable methods to screen the presence of PSP, amnesic shellfish poisoning (ASP), diarrheic shellfish poisoning (DSP) and ciguatera toxins, among others. Over the years, instrumental analysis and other alternative methods to animal bioassays such as those based on mammalian cells, receptors or antibodies, for example, have been proposed and implemented, not only as tools to reduce animal bioassays, but also because of their reliability to confirm and/or quantify marine toxins or the toxicity of seafood. This approach has been applied in several international regulations such as the European Commission Regulation (EC) No 15/2011 focusing

on the recognized testing methods for detecting marine biotoxins in live bivalve mollusks. These regulations place instrumental analysis, among other methods, as a key strategy to evaluate neurotoxins in marine food. An example is the implementation of liquid chromatography coupled to tandem mass spectrometry (LC-MS/MS) as the reference method for the detection and quantification of the neurotoxin okadaic acid (OA). Although presently, animal bioassays for the screening of marine toxins, such as the MBA for PSP toxins and the one for ciguatera, are still in use in some countries, their use in routine monitoring programs has been banned or has seriously decreased in most countries. The application of animal bioassays for the evaluation of marine neurotoxins is thought of utility for specific toxicological objectives very clearly defined and always following current regulations on animal testing and the associated ethical justifications. Due to ethical restrictions, these should be used strictly only when complementary methods do not provide the adequate information to describe neurotoxins and their effects. Hence, the scientific community should always take into consideration that the risk assessment of marine toxins should always prioritize alternative methods to animal bioassays when evaluating the presence of marine neurotoxins in the food or the environment.

Toxicology is a complex discipline that, among others, is focused on the understanding of toxicological processes in nature, particularly those affecting humans. Several strategies exist to study the mechanism of action of marine neurotoxins, and this includes several toxicological approaches such as *in vivo* or *in vitro* assays, but also other approaches such as biochemical evaluation or prediction using *in silico* approaches (Soto-Liebe et al., 2013). *In vivo* models integrate the different factors that will determine a toxicological response, including absorption, distribution, metabolism and excretion of neurotoxins. Hence, as wholistic models, they offer a unique description of the biological potency of neurotoxins. Animal bioassays may be used to illustrate the particular symptomatology of toxins when no alternative exists. Mice have been used to describe the symptoms of intoxication after exposure to CTXs, including hypersalivation, diarrhea, lachrymation, limb paralysis and respiratory distress (Munday, 2014). Animal bioassays may also be implemented to better describe structural pathology, toxin kinetics and organ toxicity. Tubaro et al. (2011), for example, described the acute oral toxicity of a new analog of palytoxin (PlTX) that caused inflammatory lesions in the non-glandular area of the stomach. Animal bioassays may also be essential to identify therapies or antagonists, although *in silico* and *in vitro* approaches also provide very valuable information in these fields.

Mammalian models offer a toxicological approach that includes the final toxicological target (e.g., toxin receptors), which may be lacking in some reductionist models such as cell-based assays (CBAs). As an example, studies in rats showed the age-related supersensitivity to domoic acid (DA) (Hesp et al., 2007). Munday (2017) summarized very well the different fields in which studies in animals could provide unique and valuable information on marine toxins from the risk assessment perspective, and especially on the role of animal bioassays in the hazard characterization stage. A major contribution of his work was to prioritize the mode of administration of toxins through oral route, in order to better reproduce the context of human exposure, in detriment of intra-peritoneal injection of toxins in animals.

Finally, an important contribution that animal bioassays have on the study of marine neurotoxins is the establishment of toxicity equivalency factors (TEFs), which consist in the relative toxicity of different toxin analogs in relation to a toxin of reference. Instrumental analysis methods such as ultra-performance liquid chromatography coupled to fluorescence detectors (UPLC-FLD), LC-MS/MS or liquid chromatography coupled to high-resolution mass spectrometry (LC-HRMS), if sensitive enough, can confirm the presence of the different neurotoxin analogs and provide quite precisely their amounts. This is the case for PSP toxins, CTXs, PlTXs, and many other toxins that are evaluated using instrumental analysis. Taking into consideration that the toxicity of the different analogs varies, the TEF of each specific analog is required to estimate the toxicity of a sample, according to the concentrations of the different analogs. Hence, when using instrumental analysis, it is necessary to have precise TEFs for all analogs to conclude on the overall toxicity of the "cocktail" of toxins present in the sample. A concise well-designed toxicological study with animals for the different toxin analogs can provide the required TEFs, which will ultimately be used for routine monitoring of neurotoxins using instrumental analysis. Interestingly, if possible, other assays such as CBAs or functional assays may also provide complementary information regarding TEFs, and the use of animals might not be necessary.

3. Cell-based assays for marine toxins

When evaluating the toxicity of marine neurotoxins and their mechanisms of action, cell models are unique since they provide the most basic response of a living structure to the stimulus that these neurotoxins may enhance (Fessard, 2017). The membrane of the cell unit, which delimits

the cell, has the receptors that will interact with the neurotoxins. Hence, the toxicological action of neurotoxins starts with the interaction of the toxin with the cell membrane receptors, a disturbance that is followed by consequent physiological changes within the cell membrane and/or the cytoplasm. A cascade of effects may follow, altering several biochemical routes and the functioning of organelles, and enhancing a cellular homeostasis. By describing and understanding these processes, one will ultimately determine the basis of the mechanism of action of these toxins at the cellular level.

Cell-based assays (CBAs) for neurotoxins consist in the exposure of cells in culture to the toxins and the consequent evaluation of their effect(s) (Reverté et al., 2014). Examples of these are the early works focusing on the use of neuroblastoma Neuro-2a cells sensitive to STXs and TTXs as a way of evaluating the presence and toxicity of these toxins as an alternative to the MBAs (Kogure et al., 1988). In that sense, and in reference to the more global toxicological response that an *in vivo* assay or a poisoning event in humans or animals may provide, CBAs are reductionist approaches since they help to evidence effects of neurotoxins on particular cells, but do not provide a holistic response. For example, the effect of OA has been extensively evaluated on mammalian cells (Fernández et al., 1991; Rubiolo et al., 2011).

In addition, the use of cells in the laboratory may favor the refinement of the evaluation of the toxicity by multiple strategies such as the use of agonists and antagonists. This is the case of the Neuro-2a CBA for CTXs developed by Manger et al. (1993), which includes the use of ouabain and veratridine. Cell-based assays for neurotoxins also include the observation of structural changes, like the cytoskeleton (Fernández et al., 1991; Louzao et al., 2008), or the identification of physiological changes such as ion conductance (Meunier et al., 2009).

The number of mammalian CBAs that may be designed and implemented is unlimited due to the multiple combinations that one may define. First, the cell type or cell strain, whether primary cultures freshly initiated from the organs of animals or established immortal cell strains, has to be selected. This step will determine the properties and limitations of the cellular model, and hence those of the CBA. Primary cultures require previous steps to disaggregate tissues and isolate cells, and are more difficult to handle, especially when there is a need to maintain cells over long times. Nonetheless, they provide cellular models whose toxicological response may be more indicative of the cellular response *in vivo* than that obtained

with immortalized cell strains, since the former may express the existing membrane receptors in the organism while immortalized cells may not. The study of DA has relied on primary cell cultures. The use of primary cultures of cerebellar neurons is an example, since glutamatergic neurons express all types of excitatory amino acid receptors in culture, as well as voltage-sensitive ion channels including calcium and sodium channels (Novelli et al., 1990, 1992; Pérez-Gómez et al., 2018). Apart from being implemented for ASP toxin studies, primary cultures of cerebellar neurons have also been used to develop CBAs for PSP and DSP toxins (García-Rodríguez et al., 1998). Other decisions taken when conceiving the CBA, and that will determine the toxicological responses obtained, include the bottom-line toxicological parameter that is evaluated, for example, cell viability, ion concentration, cytoskeleton alteration and more. Other factors that determine the type of assay include the time of evaluation and kinetics, the use of agonist or antagonist drugs or, for example, the instrumentation used to evidence the cellular responses. For example, a very recent work has measured the electrical activity of cortical neurons grown on microelectrode arrays (MEAs) in the presence of a *Prorocentrum hoffmannianum* extract containing prorocentroic acid in a non-invasive way (extracellularly) (Domínguez et al., 2021).

The toxicological evaluation of the potential effects of new emerging neurotoxins in humans is unconceivable without the use of mammalian cells. Cell-based assays are unique tools to describe and understand the toxicity and mechanism of action of marine neurotoxins. Cell-based assays have not only significantly contributed to reduce the use of animal models in toxicological studies for all marine neurotoxins, but also in the monitoring of marine neurotoxins in marine food for management purposes, such as CTXs.

3.1 The cyclic imines case

Several tests have been developed to determine the basis of the neurotoxicity of cyclic imines (CIs). Initial works on the mechanism of action of CIs toxins suggested the possible implication of muscarinic acetylcholine receptors (mAChRs) in the toxicity of these compounds (Gill et al., 2003).

Another important study performed by Dragunow et al. (2005) investigated the effects of three gymnodimine (GYM) analogs on the viability of mouse Neuro-2a cells. The authors showed that these molecules had a remarkably similar mode of action on Neuro-2a cells and the toxic effect

on the viability was very low. In contrast, pre-exposure of cells to these four molecules sensitized them to the toxic effects of OA.

Bourne et al. (2010) confirmed the interaction of 13-desmethyl spirolide C (13-desMeC SPX) and gymnodimine-A (GYM-A) with several muscle and neuronal types of nicotinic acetylcholine receptors (nAChRs) expressed in mammalian cells. They also performed functional analysis of both toxins using voltage-clamp recordings with *Xenopus* oocytes expressing muscular and neuronal types of receptors.

Wandscheer et al. (2010) studied the effect of spirolides (SPXs) on mAChRs in the human neuroblastoma cell line BE(2)-M17. They demonstrated that 13-desMeC SPX inhibited ACh-induced Ca^{2+} signals, while the reversible competitive antagonist atropine diminished the inhibitory effect of the 13-desMeC SPX.

Additional works, using frog and mouse nerve muscle preparations, studied the effect of GYMs (Kharrat et al., 2008), pinnatoxins (PnTxs) (Hellyer et al., 2014) and SPXs (Aráoz et al., 2015; Couesnon et al., 2016) on neuromuscular transmission and their effect on muscular and neuronal types of nAChRs. These studies demonstrated that these toxins blocked, in a time and concentration-dependent manner, the twitch response when the nerve was stimulated, suggesting that these toxins should be a nAChRs blocker in muscles. In order to confirm this blockage, the researchers performed patch clamp experiments with *Xenopus* embryonary myocytes, which have been known to express nAChRs on their membrane surface. Hence, CBAs, in this case most approaching primary cultures, have been crucial to identify the mechanism of action of CIs, and identify their mechanism of action associated to nAChRs.

4. Receptor-binding assays and biosensors for marine toxins

Receptor-binding assays (RBAs) are functional assays based on the binding interaction of a compound, in this case a marine toxin, to a specific cellular receptor (Bottein Dechraoui et al., 2005). In the assay, a labeled ligand (e.g., with radioactivity or fluorescence) is usually required to act as a tracer and competes with the toxin for the receptor sites. This competition allows the quantification of the toxins in natural samples, since the specific signal decreases with increasing amounts of toxins. Like in CBAs, RBAs provide measurements that are the result of a composite toxicity, i.e., the combined toxicity of several toxin derivatives sharing a common

mechanism of action and that are present altogether in a sample. Although they may share the same receptor, the toxin analogs may bind with different affinities to the receptor. As for the animal assays, equivalence factors, in this case inhibition equivalence factors (IEFs), among the toxin derivatives relative to a reference toxin can be obtained. Thus, elucidation of IEFs of these different analogs allows to better compare the RBA results with those obtained with other analysis techniques.

Receptor-binding assays were initially developed using ligands labeled with radioactive isotopes (Lewis et al., 1991; Servent et al., 1997). Nevertheless, in order to avoid the use of hazardous compounds, fluorescent (Alfonso et al., 2012; McCall et al., 2014; Otero et al., 2011), chemiluminescent (Rodríguez et al., 2011, 2013a) and colorimetric techniques (Aráoz et al., 2012; Rubio et al., 2014) have been recently proposed and used for the detection.

Regarding the format, RBAs were originally developed using synaptosomes (which are cell fractions containing the receptors) in suspension in a test tube with separation processes performed by filtration or centrifugation (Dechraoui et al., 1999; Lewis and Holmes, 1993; Lombet et al., 1987). Later, 96-well filter-plates have been used (Doucette et al., 1997; Powell and Doucette, 1999; Van Dolah et al., 1994). More recently, in order to avoid filtration or centrifugation, new solid-phase formats, where some of the RBA components are immobilized, have been proposed (Rodríguez et al., 2011, 2013a; Rubio et al., 2014).

Like in other biological or biochemical methods, the analysis of toxins in natural samples, such as microalgae, fish or shellfish, requires the prior investigation of the potential matrix effects that may interfere on the assay. Sometimes, sample clean-up and purification processes solve this issue.

Several RBAs have been developed for marine neurotoxins, which differ according to their mode of action. Paralytic shellfish poisoning toxins act on site-1 of voltage-gated sodium channels (VGSCs) (Catterall, 2000), CTXs and brevetoxins (PbTXs) act on site-5 of VGSCs (Benoit et al., 1996; Hidalgo et al., 2002; Mattei et al., 1999; Molgó et al., 1993), azaspiracids (AZAs) act on voltage-gated potassium channels (VGKCs) (Twiner et al., 2012), ASP toxins act on glutamate receptors (Berman et al., 2002; Novelli et al., 1990, 1992; Ramsdell, 2007), CIs act on nAChRs (Bourne et al., 2010; Kharrat et al., 2008), and PlTXs act on the Na^+/K^+-ATPase pump (Hilgemann, 2003).

Biosensors are bioanalytical devices that consist of an immobilized biorecognition molecule in intimate contact with a transducer that will

provide a signal that is proportional to the concentration of a specific analyte. This approach has been applied, among many other recognition molecules, to cellular receptors that interact with neurotoxins. To date, only two receptor-binding biosensors, both optical, have been developed, for the detection of PlTX (Alfonso et al., 2014; Vale-Gonzalez et al., 2007).

4.1 The cyclic imines case

Cyclic imines are very-fast acting neurotoxins (Bourne et al., 2010) that act as potent antagonists of nAChRs (Aráoz et al., 2011; Stivala et al., 2015). Although these neurotoxins are not presently under legal regulations, the potential exposure to CIs is of concern given their capacity to cross the intestinal as well as the brain blood barrier (Alonso et al., 2013; Munday et al., 2012).

Several RBAs for CIs have been developed, based on their affinity toward nAChRs, using α-bungarotoxin (α-BTX) coupled to either biotin or fluorophore, as a competitive antagonist of nAChR and as a toxin tracer. The first works were fluorescence polarization (FP) indirect assays in solution, proposed for the detection of GYM-A, 13-desMeC SPX (Vilariño et al., 2009) and 13,19-didesmethyl spirolide (13,19-didesMeC SPX) (Fonfría et al., 2010a), using fluorescently labeled α-BTX as a competitive tracer. The presence of CIs in the reaction mixture inhibited the binding of the fluorescent tracer to nAChRs previously obtained from the fish *Torpedo marmorata*, in a concentration-dependent manner. The assays provided IC_{50} values as low as 391.0, 98.2 and 63.4 nM for GYM-A, 13-desMeC SPX and 13,19-didesMeC SPX, respectively. Matrix effects for mussels, cockles, clams and scallops were evaluated, and observed not to be significant (Fonfría et al., 2010b). Otero et al. (2011) also developed a FP assay in solution for 13-desMeC SPX and 13,19-didesMeC SPX, but in this case, it was direct and did not require α-BTX. The binding of CIs to fluorescently labeled nAChRs caused a decrease of the fluorescence measurement, proportional to the toxin concentration. The assay provided IC_{50} values of 25 and 150 nM for 13-desMeC SPX and 13,19-didesMeC SPX, respectively.

Solid-phase RBAs have also been developed, avoiding the requirement of the filtration or centrifugation steps previously mentioned. In the first of these works, biotinylated α-BTX was immobilized on streptavidin-coated 96-well microtiter plates (Rodríguez et al., 2011). The method consisted in

the competition between 13-desMeC SPX free in solution and immobilized α-BTX for binding to nAChRs. Then, anti-nAChR antibodies were added to interact with the nAChRs and detected using a secondary horse-radish peroxidase (HRP)-labeled antibody. The detection was performed by chemiluminescence, fluorescence or colorimetry, using appropriate substrates. The lowest IC_{50} values, obtained with chemiluminescence, were 31.4 and 526.0 nM for 13-desMeC SPX and GYM-A, respectively. The assay was applied to cockles, showing similar calibration curves. In order to get high-throughput screening, the same research group (Rodríguez et al., 2013a) developed the assay in 384-well microtiter plates. In this case, they pre-incubated the CIs with the nAChRs and subsequently added the biotinylated α-BTX. The complex was then immobilized on the streptavidin-coated plates for subsequent detection with the antibodies. This format allowed a reduction of the volume of reagents per sample and an increase of the number of samples per plate (simultaneous testing of a maximum of 120 samples).

Immobilization of nAChRs instead of α-BTX has also been exploited (Aráoz et al., 2015; Rubio et al., 2014). In this case, biotinylated α-BTX was used as a tracer, combined with streptavidin-HRP for the colorimetric detection. The assay showed IC_{50} values of 24.3, 4.0, 5.2 and 184.0 μg/L for pinnatoxin A (PnTx-A), pinnatoxin G (PnTx-G), 13-desMeC SPX and 12-methyl gymnodimine-A (12-Me GYM-A), respectively. In Aráoz et al. (2012), the same authors eluted the CIs from the immobilized nAChRs to analyze the eluate with mass spectrometry in order to be able to identify the different compounds of the CI family.

Another interesting RBA is the one developed using microspheres in suspension and flow cytometry as a detection technique (Rodríguez et al., 2013b). In this assay, *Torpedo marmorata* nAChRs or *Lymnaea stagnalis* ACh-binding proteins were immobilized on carboxylated microspheres, and CIs competed with biotinylated α-BTX for these biorecognition molecules. The competitive binding was recorded using the fluorescent phycoerythrin-labeled (PE-labeled) streptavidin. Both receptors allowed the nanomolar detection of 13-desMeC SPX, 13,19-desMeC SPX, 20-methyl SPX-G (20-Me SPX-G) and GYM, with different sensitivities. The application of the assay to the analysis of mussels, clams and scallops was demonstrated.

Finally, it is important to mention that Hellyer et al. (Hellyer et al., 2014) synthesized the first fluorescently labeled pinnatoxin F (PnTx-F), with a promising use as a probe in RBAs.

5. Immunoassays and immunosensors for marine toxins

Antibodies (Abs) are proteins produced by the immune system in response to foreign molecules that enter the body. Antibodies can be used as probes for the detection of molecules of interest with high sensitivity and specificity. Both monoclonal (homogenous isotype and antigen specificity) and polyclonal (heterogeneous isotype and antigen specificity) Abs are available for a large range of analytes in the field of food safety and clinical diagnosis. Polyclonal Abs (pAbs) are isolated from the sera of animals that have been immunized against a target antigen, and are composed of a mix of different Abs against different epitopes. Additionally, a pAb supply is dependent on the animal source, and thus differences exist between batches. In contrast, monoclonal antibodies (mAbs) are obtained from hybridomas or recombinantly from expression vectors, which ensure continuous supply of homogenous antibodies.

When developing immunoassays and immunosensors, the size and structure of the antigen play an important role in the format to be adopted (Leonardo et al., 2017a). While in competitive assays, free and immobilized or labeled antigens compete for an Ab, in sandwich assays the target analyte is sandwiched between two Abs: a capture Ab, which is immobilized and recognizes the analyte of interest, and a detector Ab, which also recognizes the antigen through another antigenic site. Consequently, in the development of sandwich immunoassays, only large molecules with different antigenic epitopes can be targeted. The way an antigen is tethered on a solid support to develop an immunoassay is also relevant: the antigenic site must remain accessible after immobilization.

Although other biomolecules such as receptors, enzymes or aptamers (oligonucleotides or oligopeptide molecules) can be used as biorecognition elements, most of the biochemical assays and biosensors for marine toxins are based on Abs due to their major availability in relation to other molecules. When Abs are used as recognition molecules, we talk about immunoassays or immunosensors. Antibodies may have the ability to detect different toxin congeners if they share a structurally similar fragment. This cross-reactivity may be advantageous or not, depending on whether the purpose is to detect the whole family of toxins (not all of them necessarily having the same toxicological potency) or just a specific congener. Most immunoassays and immunosensors for marine toxins were initially focused on OA and dinophysistoxins (DTXs) (Campàs et al., 2008; Leonardo et al., 2018a). Nonetheless, some immunoassays and immunosensors for other

neurotoxins, such as DA (Kania et al., 2003; Kreuzer et al., 2002), STX (Carter et al., 1993), PbTXs (Carter et al., 1993; Kreuzer et al., 2002; Tang et al., 2011), PlTXs (Zamolo et al., 2012), AZAs (Leonardo et al., 2017b, 2018b), TTXs (Campàs et al., 2020; Leonardo et al., 2019; Rambla-Alegre et al., 2018; Reverté et al., 2015, 2017a,b, 2018) and CTXs (Gaiani et al., 2020; Hokama et al., 1987; Leonardo et al., 2020; Nagumo et al., 2004; Oguri et al., 2003; Park, 1995; Tsumuraya et al., 2006, 2010, 2014, 2018) have also been reported.

5.1 The tetrodotoxins case

Tetrodotoxin has been widely known as the puffer fish toxin, since it was originally discovered in this fish. In Japanese waters, the presence of puffer fish belonging to the Tetraodontidae family is very common and *fugu* is considered a gastronomic delicacy. This dish is so popular, regardless of its potential hazard, that regulations have been put in place. A value of 2 mg of TTX equiv./kg of edible portion has been used as a criterion to judge the acceptability of puffer fish as food in this country (Mahmud et al., 1999). Moreover, the Ministry of Health, Labor and Welfare of Japan published a guide with the edible parts and species of puffer fish that are allowed for consumption (HP of Ministry of Health, Labor and Welfare of Japan, 2017). In Europe, the legislation establishes that poisonous fish of the family Tetraodontidae and products derived from them must not be placed on the European markets (Commission Regulation (EU) No 853/2004, 2004). Although puffer fish containing high levels of TTXs were not common in marine European waters, as a result of the opening of the Suez Canal, the puffer fish species *Lagocephalus sceleratus*, which contains high levels of TTXs, has entered the Mediterranean Sea. It is possible that the increase of water temperatures in the last 20 years may have favored the establishment of this species in the Mediterranean. Several poisoning cases related with this species along the Eastern Mediterranean coastal countries have been reported (Bentur et al., 2008; Kheifets et al., 2012).

The production of Abs for the detection of TTX has been hindered by the relatively small size of this toxin (319 Da) and the need to conjugate it to a carrier protein to get animal immunization. At Queen's University of Belfast, a mAb against TTX was produced using mice immunized with TTX-bovine serum album (BSA) conjugate (Campbell et al., 2013; Kawatsu et al., 1997). Our group has developed some immunoassays and immunosensors for the detection of TTXs using that mAb.

Reverté et al. (2015) developed a colorimetric immunoassay based on the immobilization of TTX on maleimide-modified microtiter plates through carboxylated polyethylene glycol-dithiols. This immobilization strategy avoided the need to conjugate TTX to protein carriers, which results in random antigen immobilization and sometimes high non-specific adsorption. The use of dithiols resulted in an ordered and oriented TTX immobilization, spaced enough to favor interaction with the Ab. A competition step between immobilized and free TTX from the sample for the mAb binding sites was performed, and an HRP-labeled secondary Ab, able to recognize the primary mAb, was used for signal recording, the response being inversely proportional to the free TTX concentration.

It is important to have in mind that the reduction in the signal indicates competition, but it does not inform about which analytes bind to the Ab. For this reason, the response of the immunoassay to different TTX analogs (5,6,11-trideoxy-TTX, 5-deoxy-TTX and 11-deoxy-TTX, 11-nor-TTX-6-ol, and 4,9-anhydro-TTX) was evaluated and the corresponding cross-reactivity factors (CRFs) were established. Cross-reactivity factors depend mainly on the affinity of the Ab for the different analogs, but also on the assay configuration and the strategy used for the immobilization of the antigens. The establishment of CRFs is important to better understand the comparison between the quantifications provided by immunochemical tools and other analytical methods based on different recognition principles, such as the LC-MS/MS.

Hence, the immunoassay for the detection of TTXs was applied to analyze puffer fish samples obtained from Greece and Spain (Rambla-Alegre et al., 2017; Reverté et al., 2015). Prior to the analysis of samples, puffer fish matrix effects were evaluated and correction factors (CFs) for intestinal tract, muscle, skin, liver and gonads were established (Reverté et al., 2015). A limit of detection (LOD) of 0.23 mg TTX/kg puffer fish was achieved, which is well below the Japanese regulation for food safety. Results were compared with the total sum of TTXs achieved by LC-MS/MS or LC-HRMS after applying the corresponding CRFs to the individual TTX contents, showing good correlations. Additionally, the presence of a toxic *L. sceleratus* individual containing high amounts of TTXs was also reported in the Western Mediterranean Sea (Rambla-Alegre et al., 2017).

With the aim of moving toward miniaturized and compact analytical devices, a biosensor for TTX was developed. The colorimetric immunoassay was adapted to gold electrode arrays for the development of an electrochemical immunosensor (Reverté et al., 2017a). In this case, dithiols were

self-assembled on gold, not only providing an oriented, stable and spaced immobilization of TTX, but also favoring electron transfer. Moreover, the use of a redox mediator that precipitated on the electrode after reaction with the HRP-labeled secondary Ab further increased the current intensities. No matrix effects were observed at 40 mg/mL of puffer fish muscle, skin, liver or gonads, achieving an effective LOD as low as 0.07 mg TTX/kg tissue. Three species of puffer fish were considered. Three individuals of *L. lagocephalus*, two *Sphoeroides pachygaster* and one *L. sceleratus* pufferfish from the Mediterranean coast of Spain were analyzed and results were compared with those obtained with the colorimetric immunoassay, a surface plasmon resonance (SPR) immunosensor (Campbell et al., 2013) and LC-HRMS analysis. Whereas no TTX was found in either *L. lagocephalus* or *S. pachygaster*, high TTX contents were determined in *L. sceleratus* tissues by all techniques.

On the other hand, an optical biosensor based on planar waveguide technology was also developed (Reverté et al., 2017b). In this case, a TTX conjugate was spotted on waveguide cartridges and the interaction upon addition of the mAb was measured using a fluorophore-labeled secondary Ab. *Lagocephalus sceleratus* puffer fish samples from Greece and *Lagocephalus lunaris* puffer fish muscle samples associated with several outbreaks in the US were analyzed by the biosensor, which provided an LOD of 0.4 mg TTX/kg.

The presence of TTX has not only been reported in pufferfish, but also in many other organisms, such as amphibians, echinoderms or cephalopods (Noguchi and Arakawa, 2008). However, presently high concern lies on the presence of TTX in marine bivalves and gastropods. In Europe, the first toxicity report related with TTX-contaminated shellfish occurred in Spain in 2007 by the consumption of contaminated trumpet shells from Portugal (Rodríguez et al., 2008). Since then, TTXs have been detected in bivalve shellfish in different parts of Europe, including the Mediterranean Sea, Greece (Vlamis et al., 2015), Spain (Leão et al., 2018), Italy (Dell'Aversano et al., 2019) and France (Hort et al., 2020), and the Atlantic Ocean, England (Turner et al., 2015a), the Netherlands (Gerssen et al., 2018) and France (Hort et al., 2020), although usually at low concentrations. The European Food Safety Authority (EFSA) has concluded that concentrations below 44 μg of TTX equiv./kg shellfish meat, based on a large portion size of 400 g, do not result in adverse effects in humans (EFSA, 2017). Therefore, the colorimetric immunoassay developed for puffer fish was adapted to oysters and mussels (Reverté et al., 2018). In this

case, dithiols were replaced by cysteamine, which simplified the protocol and shortened the assay time. The immunoassay experienced strong shellfish matrix effects, so a solid-phase extraction (SPE) clean-up step followed by solvent evaporation prior to the immunoassay was usually required. However, this step substantially decreased the TTX recovery. Effective LODs between 20 and 50 μg/kg shellfish matrix were obtained. Since lower LODs were pursued, a new configuration using magnetic beads (MBs) as immobilization supports instead of microtiter plates was explored (Campàs et al., 2020). The use of MBs improved the assay kinetics and the efficiency of the washing steps, resulting in lower matrix effects and LOD, which in this case was 0.5 ng/mL (instead of 2 ng/mL obtained using plates). This four-times lower LOD together with the higher robustness to shellfish matrix effects, resulted in one order of magnitude lower effective LODs (1 μg/kg for oyster and razor clams and 3.3 μg/kg for mussels), which were well below the EFSA guidance threshold of 44 μg/kg.

The same MB-based configuration was used in development of an electrochemical immunosensor (Leonardo et al., 2019). In this case, the MB-immunocomplex was immobilized on screen-printed electrodes by placing a magnet below the working electrode. This immobilization strategy avoided coating the electrode surface with immunoreagents, which may hinder electron transfer. The immunosensor was applied to the analysis of two juvenile *L. sceleratus* puffer fish individuals caught in the North Aegean Sea and revealed the presence of significant TTX contents in puffer fish at this early stage for the first time. This finding increases the risk that this species may represent for accidental consumers, as juvenile *L. sceleratus* may intermingle with small commercial species such as picarel or anchovy and are difficult to distinguish by non-professional and non-experienced people.

Finally, the cysteamine-based colorimetric immunoassay developed for shellfish was adapted to the analysis of urine to provide a screening tool for clinical diagnosis in case of a poisoning incidence (Rambla-Alegre et al., 2018). The assay was applied to the analysis of samples from two patients from the Territorial Hospital of New Caledonia that had suffered from a puffer fish intoxication. The presence of TTXs was detected in all samples, and the contents agreed with the degree of poisoning observed from clinical symptoms and with the expected TTX excretion rates. The complementary analysis by LC-MS/MS confirmed the presence of TTX and other TTX analogs, highlighting the complementarity between techniques.

5.2 The ciguatoxins case

Ciguatoxins are neurotoxic cyclic polyether compounds produced by marine epibenthic microalgae (dinoflagellates) of the genera *Gambierdiscus* and *Fukuyoa* (Chinain et al., 2010; Lewis, 2001; Yasumoto et al., 2000). Ciguatoxins enter the food webs after consumption of the microalgae by herbivorous fish and may be transferred to carnivorous fish (Ledreux et al., 2014). Once humans ingest fish contaminated with CTXs, they may develop serious digestive, cardiac and neurologic symptoms resulting in ciguatera, which is one of the most common food borne diseases affecting up to 50,000 people per year (EFSA, 2010). Currently, around 30 analogs of CTXs have been described (also known as Pacific Ocean CTXs (P-CTXs) (Lehane and Lewis, 2000), Caribbean Sea CTXs (C-CTXs) (Lewis et al., 1998) or Indian Ocean CTXs (I-CTXs) (Hamilton et al., 2002)). Up to date, there are no regulatory limits for the presence of CTXs in fish, and only some guidelines relative to the amounts of toxins not causing toxic effects, for example, 0.01 μg/kg of CTX1B equivalents, have been proposed by the United States Food and Drug Administration (US FDA, 2020).

The production of specific Abs for CTXs have been hindered by the scarcity, toxicity and chemical complexity of CTXs. The first ELISAs for CTXs were developed using pAbs and evolved into two immunostrip tests commercialized in the past as Cigua-Check™ (Hokama et al., 1987) and Ciguatect™ test kit (Park, 1995). Even though both tests were able to detect CTXs, they showed a high cross-reactivity with OA and discrepancies with the MBA, making them unsuitable for the screening of contaminated fish (Dickey et al., 1994). To overcome the problems related to the cross-reactivity with other marine toxins and the lack of pure CTXs, synthetic fragments from different parts of CTXs have been used to develop mAbs, conferring high specificity and sensitivity (Nagumo et al., 2004; Tsumuraya et al., 2010, 2014, 2018). Particularly, three different mAbs (3G8, 10C9, and 8H4) were produced to specifically bind one of the wings of the four principal congeners of CTXs (CTX1B, CTX3C, 51-hydroxyCTX3C, and 54-deoxyCTX1B), without cross-reactivity with the other main marine toxins (Tsumuraya et al., 2014). Specifically, the 3G8 mAb has affinity for the left wing of CTX1B and 54-deoxyCTX1B (Tsumuraya et al., 2012), the 10C9 mAb for the left wing of CTX3C and 51-hydroxyCTX3C (Oguri et al., 2003), and the 8H4 mAb for the right wing of the four congeners (Tsumuraya et al., 2006). The combination of mAbs against different fragments of CTXs allowed the development of sandwich immunoassays, in

which two of them were used as capture (3G8 and 10C9) and the other (8H4) was labeled with enzymes for the detection. The successful results obtained by Tsumuraya et al. inspired the development of electrochemical immunosensors. Hence, the first immunosensor for the screening of CTXs in fish has been developed by the immobilization of the two capture antibodies on MBs, and by labeling the detector antibody (8H4) with biotin for the subsequent interaction with poly-HRP-streptavidin (Leonardo et al., 2020). The applicability of the device was successfully demonstrated, enabling the discrimination between contaminated and non-contaminated fish samples and the detection of P-CTX1B contents at the FDA level of 0.01 μg/kg. Additionally, the electrochemical immunosensor was exploited for the analysis of extracts from several *Gambierdiscus* and *Fukuyoa* species, allowing the discrimination between the two series of congeners (P-CTX1B and P-CTX3C), and so giving new information about the toxin profile of both genera (Gaiani et al., 2020). These strategies represent a step forward in the ciguatera risk management, because they are cheap, fast and easy to use, even though they do not detect all the existing CTXs. Nevertheless, the research on CTXs structure is ongoing. In fact, Tsumuraya and its group are currently working on developing mAbs able to bind to the wings of C-CTX3C and its congeners. Once those mAbs will be available, they will probably be integrated in biotechnological devices, allowing to provide a more exhaustive sample screening.

6. Enzyme-based assays and biosensors for marine toxins

Enzyme-based assays and biosensors exploit the ability of enzymes to react with their substrates or to be inhibited/activated by some compounds. Since marine toxins are not enzyme substrates, their detection is usually based on their inhibitory effect on enzymes. This functional recognition may not be related with the toxicity of the toxin. Consequently, in enzyme-based assays and biosensors, elucidation of IEFs of the different analogs of a family of toxins allows to compare the results with those obtained with other analysis techniques. Inhibition equivalency factors are equivalent to TEFs obtained in CBAs and to CRFs in immunoassays and immunosensors.

The enzyme-based assays and biosensors for marine toxins are practically limited to those exploiting protein phosphatases for the detection of toxins of the DSP group (see below). Only a few works describe the use of

phosphodiesterases for the detection of yessotoxins (Alfonso et al., 2004, 2005; Campàs et al., 2010; Fonfría et al., 2008; Moury et al., 2009; Pazos et al., 2004).

6.1 The okadaic acid and dinophysistoxins case

The well-known inhibitory effect of OA and DTXs on the activity of protein phosphatases 2A and 1 (PP2A and PP1) has been exploited in the development of assays and biosensors based on enzymes for these toxins. Colorimetry (Della Loggia et al., 1999; Tubaro et al., 1996) and fluorescence (Mountfort et al., 1999, 2001; Vieytes et al., 1997) have been used as detection techniques in the assays, whereas biosensors are electrochemical (Campàs and Marty, 2007; Molinero-Abad et al., 2019; Zhou et al., 2016).

One of the main limitations of PPs is their inherent instability. Nevertheless, strategies have been undertaken to improve the enzyme stability, based on the conjugation of PPs to MBs (Garibo et al., 2012b) or their immobilization using polymers and gels on microtiter plates (Hayat et al., 2012; Sassolas et al., 2011) or electrodes (Campàs and Marty, 2007), this last leading to the corresponding biosensor.

As previously mentioned, the establishment of IEFs is important to better understand the results obtained with functional assays compared to instrumental analysis. Several works describe the establishment of such factors, which may differ depending on differences in enzyme source, enzyme concentration, toxin standard purity, enzyme substrate and buffer composition (Aune et al., 2007; Garibo et al., 2013; Ikehara et al., 2010; Mountfort et al., 2001; Rivas et al., 2000; Smienk et al., 2012; Takai et al., 1992). These differences indicate that a full characterization of the assay or biosensor must be performed before its implementation in monitoring programs.

One important issue when working with PPs is the fact that they are very susceptible to matrix effects during the analysis of natural samples, since the recognition of the target takes place at the same moment than the enzyme reacts with its substrate. The protocol for the toxin extraction from the sample certainly plays a role and requires optimization, which of course depends on the analysis technique. Besides, matrix loading concentrations must be carefully controlled. In this regard, our group established the loading limits for Mediterranean mussel (*Mytilus galloprovincialis*), wedge clam (*Donax trunculus*), Pacific oyster (*Crassostrea gigas*) and flat oyster (*Ostrea edulis*) in assays using wild and recombinant PP2As (Garibo et al., 2012a). Additionally, clean-up of samples using solid-phase extraction portioning

allows the use of higher matrix loading concentrations for both shellfish (Cañete et al., 2010) and microalgae (Caillaud et al., 2010) samples, which allows to detect even trace toxin amounts, like has been the case for OA in cultures of the marine microalgae *Prorocentrum rhathymum* (Caillaud et al., 2010).

Finally, it is important to mention the commercially available OkaTest kit from ZEULAB S.L. This kit has been validated and proved to fulfill the required international guidelines (Smienk et al., 2012, 2013). Researchers have had special care to guarantee the stability of the components and the robustness of the method. The LOD and LOQ are 44 and 56 μg OA equiv./kg, so far below the European limit of 160 μg/kg. The OkaTest can therefore be used as a supplementary test to the reference method for determination of the DSP toxins group in mollusks.

7. Aptamer-based assays and aptasensors for marine toxins

Aptamers are single-stranded DNA (less often RNA or peptides) that bind to a specific target, folding into a unique tridimensional conformation. They are produced by systematic evolution of ligands by exponential enrichment (SELEX). In this iterative process, the analyte of interest is exposed to a random oligonucleotide library, and after binding and separation of bound from unbound oligonucleotides, the cycle starts again. Negative or counter selections using the immobilization support (required for partition) or similar targets, respectively, are sometimes included to remove non-specific binding sequences and to enhance the specificity of the aptamers. Oligonucleotides maintained after several cycles, which are the ones that should better recognize the analyte of interest, are then PCR-amplified and sequenced. Although the SELEX process may be long and tedious (because of the iterative cycles), once the sequence is elucidated, the production of the aptamer is straightforward.

Aptamers have been proposed as recognition elements alternative to antibodies in assays and biosensors because of several advantages: their production does not involve animal experimentation and does not suffer from batch-to-batch variation, they are highly stable, they can be easily modified to include functionalities at their extremes, and their configuration can be tuned to be adapted to label-free and/or non-competition formats. The main limitation of the use of aptamers would be the specific and stringent working conditions (e.g., buffer pH, composition and temperature), which

may pose a problem when analyzing natural samples. As it happens with antibodies, aptamers may have higher or lower affinity for the toxin congeners of a same toxin group. Ideal aptamers would be those detecting toxic congeners, although this will of course depend on the chemical structure of the congeners. Nevertheless, although CRFs may not correlate with TEFs, aptamers are usually produced against the parent toxin of a family of toxins, which in most cases is the most toxic and most abundant.

Several aptamers have been produced against marine toxins and exploited in assay or biosensor formats: TTX (Gu et al., 2018; Shao et al., 2012), STX (Gu et al., 2018; Ha et al., 2019; Handy et al., 2013; Zheng et al., 2015), OA (Eissa et al., 2013; Gu et al., 2016), PbTX (Eissa et al., 2015; Tian et al., 2016), gonyautoxin 1&4 (GTX1&4) (Gao et al., 2016), PlTX (Gao et al., 2017a) and DA (Gu et al., 2018). The literature on this subject, although still scarce, is growing in the recent years.

7.1 The paralytic shellfish poisoning toxins case

In 2013, Handy et al. (2013) produced an aptamer that targets STX. Because of the small size of this toxin, they used a hapten-carrier complex conjugated to MBs for the SELEX. The aptamer was characterized by surface plasmon resonance using two different formats. In the former, the binding of the aptamer to a surface-bound STX was evaluated, but no competition was performed. In the latter, the characterization consisted of the pre-incubation of STX with the aptamer and the injection of the mixture over an anti-STX antibody surface. Therefore, the corresponding optical aptasensor was not developed.

This aptamer was exploited by Alfaro et al. (2015) in a label-free fluorescence assay, using a dsDNA-binding dye. Incubation of STX with the aptamer caused an increase of the dsDNA area and thus an increase in the fluorescence signal. The assay showed an LOD of 7.5 ng/mL and no cross-reactivity toward gonyautoxin 2&3 (GTX2&3). Unfortunately, the calibration curve in the presence of shellfish matrix was considerably affected by the compounds of the extract, which the authors attributed to a poor binding affinity of the aptamer.

This aptamer was also used in the development of a label-free electrochemical aptasensor. It is also very important to be aware that in the development of aptasensors, the aptamer immobilization is crucial. The sequence that recognizes the target must be accessible, and the aptamer must be able to adopt its tridimensional conformation upon recognition. In this work, the

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amino-terminated aptamer was covalently immobilized on multi-walled carbon nanotubes (MWCNTs), previously immobilized on a gold electrode through an octadecanethiol self-assembled monolayer (SAM). Methylene blue, accumulated in the MWCNTs/SAM film by electrostatic interactions, was used as a redox indicator. In the absence of STX, the indicator generated an oxidation signal. When the target was present, the folded aptamer caused mediator releasing and its rigid structure inhibited the electron transfer, resulting in a lower electrochemical signal. The biosensor attained an LOD of 0.11 ng/mL, much lower than the previously described fluorescence assay, which indicated that not only the aptamer, but also the format and the detection technique are important for the final performance of the system. The biosensor did not show cross-reactivity toward other marine toxins, such as OA, neosaxitoxin (NEO) and GTX1&4. When applied to the analysis of mussel samples, results showed recovery values between 63% and 121%.

Further rational site-directed mutations (to better stabilize the tridimensional conformation and to increase the interaction with the targe) and truncations (to remove unnecessary nucleotides) of the previously reported aptamer resulted in a new aptamer with a 30-fold higher affinity (Zheng et al., 2015). This new aptamer was used to develop a label-free biolayer interferometry aptasensor, where the interaction between the target and the immobilized aptamer results in a change in the optical thickness and mass density of the aptamer layer and a shift in the interference pattern, generating the optical signal (Gao et al., 2017b). The optical biosensor attained an LOD of 0.5 ng/mL and did not cross-react with NEO, GTX1&4 and GTX2&3. The applicability to spiked shellfish, ribbon fish and water components was demonstrated, with recoveries of 101–107%.

Another interesting fluoresce-based assay for STX is that proposed by Gu et al. (2018) with their own aptamer. The detection principle was based on the interaction between the aptamer with magnetic reduced graphene oxide (MRGO), also exploited in the previous SELEX process. Upon target incubation, the folding of the aptamer, which had been fluorescently labeled, cause its release from the MRGO and, after magnetic separation, the fluorescence intensity from the supernatant was measured. The assay attained an LOD of 0.39 ng/mL and showed good recovery values (85–96%) when applied to clam samples. Graphene oxide (GO) was also used by Ha et al. (2019) to produce their aptamer, although in that case they used centrifugation to separate bound and unbound aptamers, instead of magnetism. The subsequent label-free biosensor, which was based on the immobilization of the

aptamer on a gold nanorod-modified chip and used localized surface plasmon resonance as a detection technique, showed an LOD of 2.46ng/mL and good recovery values (96–116%) in the analysis of spiked mussel samples.

8. Instrumental analysis techniques for marine toxins

Chromatography-based methods are widely used techniques for the determination and quantification of marine neurotoxins as well as for confirmatory purposes. Most of them are sensitive enough to detect levels that are considered as hazardous for humans. For example, ASP toxins present in seafood are quantified using high-performance liquid chromatography coupled to ultraviolet detection (HPLC-UV) (EURLMB, 2008). In the EU, an LC-MS/MS method (EURLMB, 2015) is currently the reference method for the quantification of lipophilic marine toxins. In 2015, this method replaced the MBA (Commission Regulation (EU) No 15/2011, 2011).

Several factors limit the use of the instrumental approach for marine neurotoxins analysis in routine. One is the lack of certified toxins and reference material, which are required both for unequivocal characterization and quantitative analysis. When addressing extensive families of toxins, identification needs to contemplate the structural complexity of the neurotoxin groups with numerous toxin derivatives. Finally, some methods may lack sensitivity since some of these toxins present high toxicity and may be hazardous at concentrations in fish or shellfish that are difficult to detect. It is important to highlight that previous to LC analysis, purification procedures using solid-phase extraction (SPE) and quenchers among other strategies have to be considered to improve the elimination of the matrix effect and interfering compounds. Other possible disadvantages are that it is an expensive technique and highly trained personnel is needed. Nonetheless, this approach is the unique solution for unequivocal identification of the neurotoxins present in a sample.

The LC-MS/MS approach is usually chosen for robust quantitative analyses in routine, offering high sensitivity. On the other hand, LC-HRMS is usually chosen for the confirmation of the presence of marine neurotoxin analogs and also unknown compounds that are present in the samples but that are not specifically targeted (non-target approach). It is an emerging and very attractive approach that combines qualitative and quantitative analyses, minimizing the matrix effects and reducing the inaccuracies (false positives and negatives) associated with other LC-MS techniques. It offers

mass assignment with an accuracy of 0.001 amu compared with 1 amu in LC-MS. Additionally, the full mass spectrum and acquired data can be used for retrospective analysis of new neurotoxins without the need to re-analyze the samples. Confirmation of structural diagnosis may have to come through additional techniques such as nuclear magnetic resonance (NMR).

Several instrumental analysis methods have been developed for marine toxins: ASP toxins (De la Iglesia et al., 2011; McCarron et al., 2014), lipophilic toxins (García-Altares et al., 2013; Gerssen et al., 2009), PSP toxins (Boundy et al., 2015; Lawrence et al., 2005;Turner et al., 2015b), which are EU-regulated marine toxins (Rodríguez et al., 2018), and also the non-regulated EU TTXs (Gerssen et al., 2018; Rambla-Alegre et al., 2018; Turner et al., 2017a,b), PbTXs (Abraham et al., 2012), PlTXs (Brissard et al., 2015; Ciminiello et al., 2011) and CTXs (Diogène et al., 2017; Estevez et al., 2019, 2020).

Analytical methodologies need to be re-evaluated and updated if necessary when new toxins are identified. In addition, to perform the risk assessment associated with the consumption of contaminated seafood, the inclusion of all toxins and their TEFs is needed and the analysis method needs to be formally validated with intra- and inter-laboratory exercises.

8.1 The paralytic shellfish poisoning toxins case

Paralytic shellfish poisoning is caused by the consumption of shellfish contaminated with STX and analogs, produced by certain species of harmful algae such as microalgae of the genera *Alexandrium* and *Gymnodinium* (marine dinoflagellates) and some freshwater cyanobacteria (Diener et al., 2006). The earliest documented report of intoxication by PSP toxins occurred in Canada in 1798 (Kao, 1993). Since then, numerous intoxications and incidences have been reported around the world (Burrell et al., 2013). There are at least 57 analogs of STX reported (Wiese et al., 2010), and they may vary in toxicity. However, monitoring laboratories typically analyze the parent compound STX and nine other analogs. The regulatory limit for PSP toxins in shellfish is 800 μg equiv. STXdiHCl/kg (Commission Regulation (EU) No 853/2004, 2004; US FDA, 2020).

The MBA is the most widely used detection method for PSP toxins around the world (US FDA, 2020), but it is no longer the reference method in Europe since the January 1, 2019. Presently, an instrumental method based on HPLC-FLD (AOAC official method 2005.06), also known as the "Lawrence method," is the reference method in Europe (Commission Implementing Regulation (EU) No 2019/627, 2019). This method was

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incorporated into the European legislation in 2006 (Commission Regulation (EC) No 1664/2006, 2006) and since then, it has been refined, validated and implemented (Ben-Gigirey et al., 2012; Harwood et al., 2013; Hatfield and Turner, 2012; Reis Costa et al., 2014; Turner et al., 2009, 2012, 2014a; Turner and Hatfield, 2012) in several European official control monitoring programs. In 2019, a simplified version for the screening and semi-quantitation of PSP toxins was presented by the EU Reference Laboratory on Marine Biotoxins (EURLMB, 2019), followed by the EURLMB SOP for the analysis of PSP published in 2020 (EURLMB, 2020). The Commission Implementing Regulation (EU) No 2019/627 (2019) will be soon modified, and it is expected that the reference method will be changed to the UNE-EN 14526 (2017) based on Lawrence et al. (2005). This new method includes a greater applicability, revised extraction procedure and chromatographic conditions, and several guidelines to improve calculations when several toxins are present.

The EU official method for PSP toxins requires an extraction and two sample clean-up steps, followed with a manual oxidation of shellfish extracts to generate fluorescent derivatives. Then, these oxidation products are chromatographed and detected by a FLD.

Shellfish can firstly be qualitatively "screened" using the periodate oxidation of C18 SPE-cleaned extracts for the determination of the presence of all known PSP toxin analogs. This "screening" approach facilitates the assessment of PSP toxins in shellfish. Samples where no chromatographic peaks are found are deemed negative and no further analysis is required. Samples that show chromatographic peaks at the same retention times as known PSP standards are analyzed by the full quantitation method.

The full quantitation method requires typically four analyses for each sample: (1) peroxide-oxidized C18 SPE-cleaned extracts for non-N-hydroxylated PSP toxins for STX, GTX2&3, *N*-sulfocarbamoyl gonyautoxin 2&3 (C1&2), gonyautoxin 5 (GTX5) (B1), decarbamoyl saxitoxin (dcSTX) and decarbamoyl gonyautoxin 2&3 dcGTX2&3, (2) periodate-oxidized post-ion exchange fraction (COOH) SPE clean-up F2 for GTX1&4 and gonyautoxin 6 (GTX6) (B2), (3) periodate-oxidized F3 for NEO and decarbamoyl neosaxitoxin (dcNEO), and (4) unoxidized extract for naturally-fluorescent matrix co-extractive components (Turner et al., 2009). In addition, for the determination of C3&4, periodate-oxidized F1 would also be required after a hydrolysis step (Reis Costa et al., 2014).

Currently, only 10 hydrophilic PSP toxin analogs/standards are commercially available. Recently, the certified standard of GTX6 has been made

commercially available in Europe and should be included in the analytical method for monitoring purposes.

In all cases, quantitation of individual toxins is conducted using direct comparison of peak areas from samples against those obtained from pure standard mixes of known concentrations. There are several cases where mixtures of analogs that contain a shared oxidation product are present (dcSTX with NEO and/or dcNEO; dcGTX2&3 with GTX1&4). In that case, additional calculations are required to estimate and subtract the contributions to toxin concentrations from the interfering analogs (Lawrence et al., 2005; UNE-EN 14526, 2017). In this method, the choice and use of the matrix modifier in the periodate oxidation is very important. The control of post-oxidation reaction temperatures and times is also important to get reproducible results. Obviously, it is critical to consider all shellfish species that are being analyzed and perform an in-house validation.

In recent years, an LC-MS/MS method for PSP toxins detection and quantitation has been described. Boundy et al. (2015) developed and validated a method that uses carbon SPE clean-up to remove salt-based interferences. Turner et al. (2015b) performed a full single laboratory validation, and this method is currently undergoing an international collaborative study. Several advantages, such as rapid, single-step extraction and single-shot analysis, are provided. In addition, this LC-MS/MS method also includes TTX, which will be co-extracted with PSP toxins (Turner et al., 2017a).

Finally, instrumental analysis techniques have contributed to the assessment of marine neurotoxins in the environment with different purposes. Monitoring programs using these methodologies are implemented worldwide in order to protect public health and ensure the quality of seafood. Turner et al. (2014b) showed the variability of PSP toxin occurrence and profiles in bivalve mollusks from Great Britain. Instrumental analysis has also been used in serious toxicological episodes where high concentrations of PSP toxins together with TTX were detected in Sicily (Dell'Aversano et al., 2019) and high PSP levels in Portugal have caused different case repots such as the one described by Lopes de Carvalho et al. (2019), which describes toxin elimination and metabolization in the fluids of two patients who ingested these mussels. These analytical methodologies have allowed to describe changes in the profile and behavior of PSP toxic episodes (Braga et al., 2018) and the assessment of the bioaccessibility of different marine biotoxins in naturally contaminated shellfish, including PSP toxins (Alves et al., 2019). Additionally, these methodologies have also been used in studies of detoxification procedures to ensure that shellfish reaching consumers is safe.

Several reports described that the concentration of some PSP analogs in bivalves can be reduced (Reis Costa et al., 2018). However, studies on shellfish detoxification to mitigate this problem are still very scarce (Cabado et al., 2020; García et al., 2010).

9. Conclusions

The development of detection methods for marine neurotoxins is a field in continuous growth because of the impact they have in human and animal health. Great progress has been achieved in the last years, which has given as a result a plethora of methods, with different extent of development and implementation. However, some challenges still need to be resolved. Despite the ethical concerns, animal bioassays may provide, from a toxicological perspective, key information on the pathologies that neurotoxins cause in a whole organism. Nonetheless, *in silico* and *in vitro* approaches may definitively provide very interesting information to be considered when predicting the toxicological effects of new neurotoxins, and hence contribute to reduce the number of animal assays. The particular approach of LC-HRMS and NMR that allow to elucidate the structure of new neurotoxins, in combination with the toxicological data of known compounds, contribute to better assess the structure/activity relations linking molecular structure and toxic potential. Although CBAs have demonstrated to be crucial to describe the mechanism of action of toxins and can also be applied for routine monitoring, they have a certain degree of variability in the response, as any toxicological model, and efforts should still be made to standardize, harmonize and validate the methods. In receptor-binding assays and biosensors, availability of commercial receptors and tracers is still a pending issue, which certainly limits their implementation. In immunoassays and immunosensors, the availability of antibodies able to recognize the maximum number of toxic congeners into a same family is a must. Enzyme-based assays and sensors for marine toxins are practically limited to those based on PP inhibition by OA and DTXs. The identification of enzymes able to recognize other marine toxins is desired, which leads to the necessity to further investigate the implications of marine toxins in cellular processes. More aptamers for marine toxins need to be produced, as well as aptamer-based assays and aptasensors to be developed. Additionally, the analysis of naturally contaminated samples is still an issue to tackle, since most systems have been only applied to the analysis of spiked samples. Instrumental analysis depends on the availability of certified toxins

and reference material, which has been and still is being addressed by many research groups and projects. Other limitations are the high cost and the requirement of skilled personnel.

Our experience has taught us that many times the best strategy to assess marine neurotoxins in the marine environment is the combination of methods. Animal bioassays will always remain the wild card when no other detection strategies work. Methods such as CBAs, RBAs, immunoassays, enzyme-based assays, aptamer-based assays and the corresponding biosensors can be used for rapid screening of neurotoxins in samples, although precise quantification is also possible. Instrumental analysis can provide a full characterization of the multi-toxin profile of neurotoxins in a sample and confirm responses obtained with biological, biochemical and biotechnological methods. The complementarity of methods will merge all the individual advantages into a global strategy to properly tackle the assessment of marine neurotoxins. The analysis of samples with different strategies will certainly provide complementary information to fully characterize them.

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References

Abraham, A., Wang, Y., El Said, K.R., Plakas, S.M., 2012. Characerization of brevetoxin metabolism in *Karenia brevis* bloom-exposed clams (*Mercenaria* sp.) by LC-MS/MS. Toxicon 60, 1030–1040.

Alfaro, K., Bustos, P., O'Sullivan, C., Conejeros, P., 2015. Facile and cost-effective detection of saxitoxin exploiting aptamer structural switching. Food Technol. Biotechnol. 53, 337–341.

Alfonso, A., Vieytes, M.R., Yasumoto, T., Botana, L.M., 2004. A rapid microplate fluorescence method to detect yessotoxins based on their capacity to activate phosphodiesterases. Anal. Biochem. 326, 93–99.

Alfonso, C., Alfonso, A., Vieytes, M.R., Yasumoto, T., Botana, L.M., 2005. Quantification of yessotoxin using the fluorescence polarization technique and study of the adequate extraction procedure. Anal. Biochem. 344, 266–274.

Alfonso, A., Fernández-Araujo, A., Alfonso, C., Caramés, B., Tobio, A., Louzao, M.C., Vieytes, M.R., Botana, L.M., 2012. Palytoxin detection and quantification using the fluorescence polarization technique. Anal. Biochem. 424, 64–70.

Alfonso, A., Pazos, M.J., Fernández-Araujo, A., Tobio, A., Alfonso, C., Vieytes, M.R., Botana, L.M., 2014. Surface plasmon resonance biosensor method for palytoxin detection based on Na^+, K^+-ATPase affinity. Toxins 6, 96–107.

Alonso, E., Otero, P., Vale, C., Alfonso, A., Antelo, A., Giménez-Llort, L., Chabaud, L., Guillou, C., Botana, L.M., 2013. Benefit of 13-desmetyl spirolide C treatment in triple transgenic mouse model of Alzheimer disease: beta-amyloid and neuronal markers improvement. Curr. Alzheimer Res. 10, 279–289.

Alves, R.N., Rambla-Alegre, M., Braga, A.C., Maulvault, A.L., Barbosa, V., Campàs, M., Reverté, L., Flores, C., Caixach, J., Kilcoyne, J., Costa, P.R., Diogène, J., Marques, A., 2019. Bioaccessibility of lipophilic and hydrophilic marine biotoxins in seafood: an in vitro digestion approach. Food Chem. Toxicol. 129, 153–161.

Aráoz, R., Servent, D., Molgó, J., Iorga, B.I., Fruchart-Gaillard, C., Benoit, E., Gu, Z., Stivala, C., Zakarian, A., 2011. Total synthesis of pinnatoxins A and G and revision of the mode of action of pinnatoxin A. J. Am. Chem. Soc. 133, 10499–10511.

Aráoz, R., Ramos, S., Pelissier, F., Guerineau, V., Benoit, E., Vilariño, N., Botana, L.M., Zakarian, A., Molgó, J., 2012. Coupling the *Torpedo* microplate-receptor binding assay with mass spectrometry to detect cyclic imine neurotoxins. Anal. Chem. 84, 10445–10453.

Aráoz, R., Ouanounou, G., Iorga, B.I., Goudet, A., Alili, D., Amar, M., Benoit, E., Molgó, J., Servent, 2015. The neurotoxic effect of 13,19-didesmethyl and 13-desmethyl spirolide C phycotoxins is mainly mediated by nicotinic rather than muscarinic acetylcholine receptors. Toxicol. Sci. 147, 156–167.

Aune, T., Larsen, S., Aasen, J.A.B., Rrehmann, N., Satake, M., Hess, P., 2007. Relative toxicity of dinophysistoxin-2 (DTX-2) compared with okadaic acid based on acute intraperitoneal toxicity in mice. Toxicon 49, 1–7.

Banner, A., Scheuer, P., Sasaki, S., Helfrich, P., Alender, C., 1960. Observations on ciguatera-type etoxin in fish. Ann. N.Y. Acad. Sci. 90, 770–787.

Ben-Gigirey, B., Rodríguez-Velasco, M.L., Gago-Martínez, A., 2012. Extension of the validation of AOAC official method 2005.06 for dc-GTX2,3: interlaboratory study. J. AOAC Int. 95, 111–121.

Benoit, E., Juzans, P., Legrand, A.M., Molgó, J., 1996. Nodal swelling produced by ciguatoxin-induced selective activation of sodium channels in myelinated nerve fibers. Neuroscience 71, 1121–1131.

Bentur, Y., Ashkar, J., Lurie, Y., Levy, Y., Azzam, Z.S., Litmanovich, M., Golik, M., Gurevych, B., Golani, D., Eisenman, A., 2008. Lessepsian migration and tetrodotoxin poisoning due to *Lagocephalus sceleratus* (Gmelin, 1789) in the Mediterranean Sea. Toxicon 52, 964–968.

Berman, F.W., LePage, K.T., Murray, T.F., 2002. Domoic acid neurotoxicity in cultured cerebellar granule neurons is controlled preferentially by the NMDA receptor Ca^{2+} influx pathway. Brain Res. 924, 20–29.

Bottein Dechraoui, Y., Tiedeken, J.A., Persad, R., Wang, Z., Granade, H.R., Dickey, R.W., Ramsdell, J.S., 2005. Use of two detection methods to discriminate ciguatoxins from brevetoxins: application to great barracuda from Florida keys. Toxicon 46, 261–270.

Boundy, M.J., Selwood, A.I., Harwood, D.T., McNabb, P.S., Turner, A.D., 2015. Development of a sensitive and selective liquid chromatography-mass spectrometry method for high throughput analysis of paralytic shellfish toxins using graphitised carbon solid phase extraction. J. Chromatogr. A 1387, 1–12.

Bourne, Y., Radi, Z., Aráoz, R., Talley, T.T., Benoit, E., Servent, D., Taylor, P., Molgó, J., Marchot, P., 2010. Structural determinants in phycotoxins and AChBP conferring high affinity binding and nicotinic AChR antagonism. Proc. Natl. Acad. Sci. U. S. A. 107, 6076–6081.

Braga, A.C., Camacho, C., Marques, A., Gago-Martínez, A., Pacheco, M., Costa, P.R., 2018. Combined effects of warming and acidification on accumulation and elimination dynamics of paralytic shellfish toxins in mussels Mytilus galloprovincialis. Environ. Res. 164, 647–654.

Brissard, C., Hervé, F., Sibat, M., Séchet, V., Hess, P., Amzil, Z., Herrenknecht, C., 2015. Characterization of ovatoxin-h, a new ovatoxin analog, and evaluation of chromatographic columns for ovatoxin analysis and purification. J. Chromatogr. A 1388, 87–101.

Burrell, S., Gunnarsson, T., Gunnarsson, K., Clarke, D., Turner, A.D., 2013. First detection of paralytic shellfish poisoning (PSP) toxins in Icelandic mussels *(Mytilus edulis)*: links to causative phytoplankton species. Food Control 31, 295–301.

Cabado, A.G., Lago, J., González, V., Blanco, L., Paz, B., Diogène, J., Ferreres, L., Rambla-Alegre, M., 2020. Detoxification of paralytic shellfish poisoning toxins in naturally contaminated mussels, clams and scallops by an industrial procedure. Food Chem. Toxicol. 141, 111386.

Caillaud, A., de la Iglesia, P., Campàs, M., Elandaloussi, L., Fernández-Tejedor, M., Mohammad-Noor, N., Andree, K., Diogène, J., 2010. Evidence of okadaic acid production in a cultured strain of the marine dinoflagellate *Prorocentrum rhathymum* from Malaysia. Toxicon 55, 633–637.

Campàs, M., Marty, J.L., 2007. Enzyme sensor for the electrochemical detection of the marine toxin okadaic acid. Anal. Chim. Acta 605, 87–93.

Campàs, M., de la Iglesia, P., Le Berre, M., Kane, M., Diogène, J., Marty, J.L., 2008. Enzymatic recycling-based amperometric immunosensor for the ultrasensitive detection of okadaic acid in shellfih. Biosens. Bioelectron. 24, 716–722.

Campàs, M., de la Iglesia, P., Fernández-Tejedor, M., Diogène, J., 2010. Colorimetric and electrochemical phosphodiesterase inhibition assays for yessotoxin detection: development and comparison with LC-MS/MS. Anal. Bioanal. Chem. 396, 2321–2330.

Campàs, M., Reverté, J., Rambla-Alegre, M., Campbell, K., Gerssen, A., Diogène, J., 2020. A fast magnetic bead-based colorimetric immunoassay for the detection of tetrodotoxins in shellfish. Food Chem. Toxicol. 140, 111315.

Campbell, K., Barnes, P., Haughey, S.A., Higgins, C., Kawatsu, K., Vasconcelos, V., Elliott, C.T., 2013. Development and single laboratory validation of an optical biosensor assay for tetrodotoxin detection as a tool to combat emerging risks in European seafood. Anal. Bioanal. Chem. 405, 7753–7763.

Cañete, E., Campàs, M., de la Iglesia, P., Diogène, J., 2010. NG108-15 cell-based and protein phosphatase inhibition assays as alternative semiquantitative tools for the screening of lipophilic toxins in mussels. Okadaic acid detection. Toxicol. In Vitro 24, 611–619.

Carter, R.M., Poli, M.A., Pesavento, M., Sibley, D.E.T., Lubrano, G.J., Guilbault, G.G., 1993. Immunoelectrochemical biosensors for detection of saxitoxin and brevetoxin. Immunomethods 3, 128–133.

Catterall, W., 2000. From ionic curernts to molecular mechanisms: the structure and function of voltage-gated sodium channels. Neuron 26, 13–25.

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

Ciminiello, P., Dell'Aversano, C., Dello Iacovo, E., Fattorusso, E., Forino, M., Tartaglione, L., 2011. LC-MS of palytoxin and its analogues: state of the art and future perspectives. Toxicon 57, 376–389.

Commission Implementing Regulation (EU) No 2019/627, 2019. Laying down uniform practical arrangements for the performance of official controls on products of animal origin for human consumption in accordance with regulation (EU) 2017/625 of the European Parliament and of the council and amending commission regulation (EC) 2074/2005 as regards official controls. Off. J. Eur. Union L131, 18–30.
Commission Regulation (EC) No 1664/2006, 2006. Amending regulation (EC) no 2074/2005 as regards implementing measures for certain products of animal origin intended for human consumption and repealing certain implementing measures. Off. J. Eur. Union L320, 13–45.
Commission Regulation (EU) No 15/2011, 2011. Amending regulation (EC) no 2074/2005 as regards recognised testing methods for detecting marine biotoxins in live bivalve molluscs. Off. J. Eur. Union L6, 3–6.
Commission Regulation (EU) No 853/2004, 2004. Laying down specific hygiene rules for on the hygiene of foodstuffs. Off. J. Eur. Union L139, 55.
Couesnon, A., Aráoz, R., Iorga, B.I., Benoit, E., Reynaud, M., Servent, D., Molgó, J., 2016. The dinoflagellate toxin 20-methyl spirolide-G potently blocks skeletal muscle and neuronal nicotinic acetylcholine receptors. Toxins 8, 249.
De la Iglesia, P., Barber, E., Giménez, G., Rodríguez-Velasco, M.L., Villar-González, A., Diogène, J., 2011. High-throughput analysis of amnesic shellfish poisoning toxins in shellfish by ultra-performance rapid resolution LC-MS/MS. J. AOAC Int. 94, 555–564.
Dechraoui, M.Y., Naar, J., Pauillac, S., Legrand, A.M., 1999. Ciguatoxins and brevetoxins, neurotoxic polyether compounds active on sodium channels. Toxicon 37, 125–143.
Della Loggia, R., Sosa, S., Tubaro, A., 1999. Methodological improvement of the protein phosphatase inhibition assay for the detection of okadaic acid in mussels. Nat. Toxins 7, 387–391.
Dell'Aversano, C., Tartaglione, L., Polito, G., Dean, K., Giacobbe, M., Casablanca, S., Capellacci, S., Penna, A., Turner, A.D., 2019. First detection of tetrodoxin and high levels of paralytic shellfish poisoning toxins in shellfish from Sicily (Italy) by three different analytical methods. Chemosphere 215, 881–892.
Dickey, R.W., Granade, H.R., McClure, F.D., 1994. Evaluation of the ciguatect solid-phase immunobead assay for the detection of ciguatera-related biotoxins in Caribbean finfish. In: Proceedings of the International Workshop on Ciguatera Management, Brisbane, Australia, 13–16 April 1994. Memoirs of the Queensland Museum, Brisbane, pp. 481–488.
Diener, M., Erler, K., Hiller, S., Christian, B., Luckas, B., 2006. Determination of paralytic shellfish poisoning (PSP) toxins in dietary supplements by application of a new HPLC/FD method. Eur. Food Res. Technol. 224, 147–151.
Diogène, J., Campàs, M., 2017. Comprehensive Analytical Chemistry. In Recent Advances in the Analysis of Marine Toxins, vol. 78, Elsevier, Amsterdam.
Diogène, J., Reverté, L., Rambla-Alegre, M., Del Río, V., de la Iglesia, P., Campàs, M., Palacios, O., Flores, C., Caixach, J., Ralijaona, C., Razanajatovo, I., Pirog, A., Magalon, H., Arnich, N., Turquet, J., 2017. Identification of ciguatoxins in a shark involved in a fatal food poisoning in the Indian Ocean. Sci. Rep. 7, 8240.
Domínguez, H.J., Cabrera-García, D., Cuadrado, C., Novelli, A., Fernández-Sánchez, M.Y., Fernández, J.J., Hernández Daranas, A., 2021. Prorocentroic acid, a neuroactive super-carbon-chain compound from the dinoflagellate *Prorocentrum hoffmannianum*. Org. Lett. 23, 13–18.
Doucette, G.J., Logan, M.M., Ramsdell, J.S., Van Dolah, F.M., 1997. Development and preliminary validation of a microtiter plate-based receptor binding assay for paralytic shellfish poisoning toxins. Toxicon 35, 625–636.

Dragunow, M., Trzoss, M., Brimble, M.A., Cameron, R., Beuzenberg, V., Holland, P., Mountfort, D., 2005. Investigations into the cellular actions of the shellfish toxin gymnodimine and analogues. Environ. Toxicol. Pharmacol. 20, 305–312.
EFSA, 2010. Scientific opinion on marine biotoxins in shellfish—emerging toxins: ciguatoxin group. EFSA J. 8 (6), 1627.
EFSA, 2017. Risks for public health related to the presence of tetrodotoxin (TTX) and TTX analgoues in marine bivalves and gastropods. EFSA J. 15 (4), 4572.
Eissa, S., Ng, A., Siaj, M., Tavares, A.C., Zourob, M., 2013. Selection and identification of DNA aptamers against okadaic acid for biosensing application. Anal. Chem. 85, 11794–11801.
Eissa, S., Siaj, M., Zourob, M., 2015. Aptamer-based competitive electrochemical biosensor for brevetoxin-2. Biosens. Bioelectron. 69, 148–154.
Estevez, P., Castro, D., Leão, J.M., Yasumoto, T., Dickey, R., Gago-Martinez, A., 2019. Implementation of liquid chromatography tandem mass spectrometry for the analysis of ciguatera fish poisoning in contaminated fish samples from Atlantic coasts. Food Chem. 280, 8–14.
Estevez, P., Sibat, M., Leão, J.M., Reis Costa, P., Gago-Martínez, A., Hess, P., 2020. Liquid chromatography coupled to high-resolution mass spectrometry for the confirmation of Caribbean ciguatoxin-1 as the main toxin responsible for ciguatera poisoning caused by fish from European Atlantic coasts. Toxins 12, 267.
EURLMB, 2008. Interlaboratory Validation Study of the EU-Harmonised SOP-ASP-LC–UV. https://www.aesan.gob.es/CRLMB/docs/docs/procedimientos/EU-Harmonised-SOP-ASP-HPLC-UV_Version1.pdf.
EURLMB, 2015. Interlaboratory Validation Study of the EU-Harmonised SOP-LIPO-LC–MS/MS. https://www.aesan.gob.es/CRLMB/docs/docs/metodos_analiticos_de_desarrollo/EU-Harmonised-SOP-LIPO-LCMSMS_Version5.pdf.
EURLMB, 2019. AOAC 2005.06 Standard Operating Procedure. Simplified Version for the Screening and Semi-Quantitation of PSP Toxins. https://www.aesan.gob.es/en/CRLMB/docs/docs/metodos_analiticos_de_desarrollo/SOP_AOAC_2005_06_semi.pdf.
EURLMB, 2020. Standard Operating Procedure of Paralytic Shellfish Toxins (PST) by Precolumn HPLC-FLD According to OMA AOAC 2005.06. https://www.aesan.gob.es/en/CRLMB/docs/docs/metodos_analiticos_de_desarrollo/PST_OMA-AOAC2005-06_HPLC-FLD_EURLMB.pdf.
Fernández, M.T., Zitko, V., Gascón, S., Novelli, A., 1991. The marine toxin okadaic acid is a potent neurotoxin for cultured cerebellar neurons. Life Sci. 49, 157–162.
Fessard, V., 2017. Cytotoxicity assays: identification of toxins and mechanism of action. In: Diogène, J., Campàs, M. (Eds.), Comprehensive Analytical Chemistry. Recent Advances in the Analysis of Marine Toxins, vol. 78. Elsevier, Amsterdam, pp. 231–275.
Fonfría, E.S., Vilariño, N., Vieytes, M.R., Yasumoto, T., Botana, L.M., 2008. Feasibility of using a surface plasmon resonance-based biosensor to detect and quantify yessotoxin. Anal. Chim. Acta 617, 167–170.
Fonfría, E.S., Vilariño, N., Molgó, J., Aráoz, R., Otero, P., Espiña, B., Louzao, M.C., Alvarez, M., Botana, L.M., 2010a. Detection of 13,19-didesmethyl C spirolide by fluorescence polarization using *Torpedo* electrocyte membranes. Anal. Biochem. 403, 102–107.
Fonfría, S., Vilariño, N., Espiña, B., Louzao, M.C., Álvarez, M., Molgó, J., Aráoz, R., Botana, L.M., 2010b. Feasibility of gymnodimine and 13-desmethyl C spirolide detection by fluorescence polarization using a receptor-based assay in shellfish matrixes. Anal. Chim. Acta 657, 75–82.

Gaiani, G., Leonardo, S., Tudó, A., Toldrà, A., Rey, M., Andree, K.B., Tsumuraya, T., Hirama, M., Diogène, J., O'Sullivan, C.K., Alcaraz, C., Campàs, M., 2020. Rapid detection of ciguatoxins in *Gambierdiscus* and *Fukuyoa* with immunosensing tools. Ecotoxicol. Environ. Saf. 204, 11104.

Gao, S., Hu, B., Zheng, X., Cao, Y., Liu, D., Sun, M., Jiao, B., Wang, L., 2016. Gonyautoxin 1/4 aptamers with high-affinity and high-specificity: from efficient selection to aptasensor application. Biosens. Bioelectron. 79, 938–944.

Gao, S., Zheng, X., Hu, B., Sun, M., Wu, J., Jiao, B., Wang, L., 2017a. Enzyme-linked aptamer-based competitive biolayer interferometry biosensor for palytoxin. Biosens. Bioelectron. 89, 952–958.

Gao, S., Zheng, X., Wu, J., 2017b. A biolayer interferometry-based competitive biosensor for rapid and sensitive detection of saxitoxin. Sensors Actuators B Chem. 246, 169–174.

García, C., Barriga, A., Díaz, J.C., Lagos, M., Lagos, N., 2010. Route of metabolization and detoxification of paralytic shellfish toxins in humans. Toxicon 55, 135–144.

García-Altares, M., Diogène, J., de la Iglesia, P., 2013. The implementation of liquid chromatography tandem mass spectrometry for the official control of lipophilic toxins in seafood: single-laboratory validation under four chromatographic conditions. J. Chromatogr. A 1275, 48–60.

García-Rodríguez, A., Fernández-Sánchez, M.T., Reyero, M.I., Franco, J.M., Haya, K., Martin, J., Zitko, V., Salgado, C., Arévalo, F., Bermúdez, M., Fernández, M.L., Míguez, A., Novelli, A., 1998. Detection of PSP, ASP and DSP toxins by neuronal bioassay: comparison with HPLC and mouse bioassay. In: Reguera, B., Blanco, J., Fernández, M.L., Wyatt, T. (Eds.), Harmful Microalgae. Intergovernmental Oceanographic Commission of UNESCO and Xunta de Galicia Publishers, pp. 554–557.

Garibo, D., Dàmaso, E., Eixarch, H., de la Iglesia, P., Fernández-Tejedor, M., Diogène, J., Pazos, Y., Campàs, M., 2012a. Protein phosphatase inhibition assay for okadaic acid detection in shellfish: matrix effects, applicability and comparison with LC-MS/MS analysis. Harmful Algae 19, 68–75.

Garibo, D., Devic, E., Marty, J.L., Diogène, J., Unzueta, I., Blázquez, M., Campàs, M., 2012b. Conjugation of genetically engineered protein phosphatases to magnetic particles for okadaic acid detection. J. Biotechnol. 157, 89–95.

Garibo, D., de la Iglesia, P., Diogène, J., Campàs, M., 2013. Inhibition equivalency factors for dinophysistoxin-1 and dinophysistoxin-2 in protein phosphatase assays, applicability to the analysis of shellfish samples and comparison with LC-MS/MS. J. Agric. Food Chem. 61, 2572–2579.

Gerssen, A., Mulder, P.P., McElhinney, M.A., de Boer, J., 2009. Liquid chromatography-tandem mass spectrometry method for the detection of marine lipophilic toxins under alkaline conditions. J. Chromatogr. A 1216, 1421–1430.

Gerssen, A., Bovee, T., Klijnstra, M., Poelman, M., Portier, L., Hoogenboom, R., 2018. First report on the occurrence of tetrodotoxins in bivalve molluks in the Netherlands. Toxins 10, 450.

Gill, S., Murphy, M., Claussen, J., Richard, D., Quilliam, M., MacKinnon, S., LaBlanc, P., Mueller, R., Pulido, O., 2003. Neural injury biomarkers of novel shellfish toxins, spirolides: a pilot study using immunochemical and transcriptional analysis. Neurotoxicology 24, 593–604.

Gu, H., Duan, N., Wu, S., Hao, L., Xia, Y., Ma, X., Wang, Z., 2016. Graphene oxide-assisted non-immobilized SELEX of okadaic acid aptamer and the analytical application of aptasensor. Sci. Rep. 6, 21665.

Gu, H., Duan, N., Xia, Y., Hun, X., Wang, H., Wang, Z., 2018. Magnetic separation-based multiple SELEX for effectively selecting aptamers against saxitoxin, domoic acid, and tetrodotoxin. J. Agric. Food Chem. 66, 9801–9809.

Ha, S.J., Park, J.H., Lee, B., Kim, M.G., 2019. Label-free direct detection of saxitoxin based on a localized surface plasmon resonance aptasensor. Toxins 11, 274.

Hamilton, B., Hurbungs, M., Vernoux, J.P., Jones, A., Lewis, R.J., 2002. Isolation and characterisation of Indian Ocean ciguatoxin. Toxicon 40, 685–693.

Handy, S.M., Yakes, B.J., DeGrasse, J.A., Campbell, K., Elliott, C.T., Kanyuck, K.M., DeGrasse, S.L., 2013. First report of the use of a saxitoxin-protein conjugate to develop a DNA aptamer to a small molecule toxin. Toxicon 61, 30–37.

Harwood, D.T., Boundy, M., Selwood, A.I., Ginkel, R., MacKenzie, L., McNabb, P.S., 2013. Refinement and implementation of the Lawrence method (AOAC 2005.06) in a commercial laboratory: assay performance during an *Alexandrium catenella* bloom event. Harmful Algae 24, 20–31.

Hatfield, R.G., Turner, A.D., 2012. Rapid liquid chromatography for paralytic shellfish toxin analysis using superficially porous chromatography with AOAC official method 2005.06. J. AOAC Int. 95, 1089–1096.

Hayat, A., Barthelmebs, L., Marty, J.L., 2012. A simple colorimetric enzymatic assay for okadaic acid detection based on the immobilization of protein phosphatase 2A in sol-gel. Appl. Biochem. Biotechnol. 166, 47–56.

Hellyer, S.D., Selwood, A.I., van Ginkel, R., Munday, R., Sheard, P., Miles, C.O., Rhodes, L., Kerr, D.S., 2014. *In vitro* labelling of muscle type nicotinic receptors using a fluorophore-conjugated pinnatoxin F derivative. Toxicon 87, 17–25.

Hesp, B.R., Clarkson, A.N., Sawant, P.M., Steven Kerr, D., 2007. Domoic acid preconditioning and seizure induction in young and aged rats. Epilepsy Res. 76, 103–112.

Hidalgo, J., Liberona, J.L., Molgó, J., Jaimovich, E., 2002. Pacific ciguatoxin 1-b effect over Na + and K + currents, inositol 1,4,5-triphosphate content and intracellular Ca2 + signals in cultured rat myotubes. Br. J. Pharmacol. 137, 1055–1062.

Hilgemann, D.W., 2003. From a pump to a pore: how palytoxin opens the gates. Proc. Natl. Acad. Sci. U. S. A. 100, 386–388.

Hokama, Y., Shirai, L.K., Iwamoto, L.M., Kobayashi, M.N., Goto, C.S., Nakagawa, L.K., 1987. Assessment of a rapid enzyme immunoassay stick test for the detection of ciguatoxin and related polyether toxins in fish tissues. Biol. Bull. 172, 144–153.

Hort, V., Arnich, N., Guérin, T., Lavison-Bompard, G., Nicolas, M., 2020. First detection of tetrodotoxin in bivalves and gastropods from the French mainland coasts. Toxins 12, 599.

HP of Ministry of Health, Labor and Welfare of Japan, 2017. Avaialbe at: http://www.mhlw.go.jp/topics/syokuchu/poison/animal_01.html. (Accessed on 15th February 2021).

Ikehara, T., Imamura, S., Yoshino, A., Yasumoto, T., 2010. PP2A inhibition assay using recombinant enzyme for rapid detection of okadaic acid and its analogs in shellfish. Toxins 2, 195–204.

Kania, M., Kreuzer, M., Moore, E., Pravda, M., Hock, G., Guilbault, G., 2003. Development of polyclonal antibodies against domoic acid for their use in electrochemical biosensors. Anal. Lett. 36, 1851–1863.

Kao, C.Y., 1993. Paralytic shellfish poisoning. In: Falconer, I.R. (Ed.), Algal Toxins in Seafood and Drinking Water. Academic Press, London, pp. 75–86.

Kawatsu, K., Hamano, Y., Yoda, T., Terrano, Y., Shibata, T., 1997. Rapid and highly sensitive enzyme immunoassay for quantitative determination of tetrodotoxin. Jpn. J. Med. Sci. Biol. 50, 133–150.

Kharrat, T., Servent, D., Girard, E., Ouanounou, G., Amar, M., Marrouchi, R., Benoit, E., Molgó, J., 2008. The marine phycotoxin gymnodimine targets muscular and neuronal nicotinic acetylcholine receptor subtypes with high affinity. J. Neurochem. 107, 952–963.
Kheifets, J., Rozhavsky, B., Solomonovich, Z.G., Marianna, R., Soroksky, A., 2012. Severe tetrodotoxin poisoning after consumption of *Lagocephalus sceleratus* (pufferfish, Fugu) fished in Mediterranean Sea, treated with cholinesterase inhibitor. Case Rep. Crit. Care 2012, 782507.
Kogure, K., Tamplin, M.L., Simidu, U., Colwell, R.R., 1988. A tissue culture assay for tetrodotoxin, saxitoxin and related toxins. Toxicon 26, 191–197.
Kreuzer, M.P., Pravad, M., O'Sullivan, C.K., Guilbault, G.G., 2002. Novel electrochemical immunosensors for seafood toxin analysis. Toxicon 40, 1267–1274.
Landsberg, J., Lefebvre, K.A., Flewelling, L., 2014. Effects of toxic microalgae on marine organisms. In: Rossini, G.P. (Ed.), Toxins and Biologically Active Compounds from Microalgae. vol. 2. CRC Press, Boca Raton, pp. 379–449.
Lawrence, J.F., Niedzwiadek, B., Menard, C., 2005. AOAC official method 2005.06 paralytic shellfish poisoning toxins in shellfish. Prechromatographic oxidation and liquid chromatography with fluorescence detection. J. AOAC Int. 88, 1714–1732.
Leão, J., Lozano-Leon, A., Giráldez, J., Vilariño, Ó., Gago-Martínez, A., 2018. Preliminary results on the evaluation of the occurrence of tetrodotoxin associated to marine *Vibrio* spp. in bivalves from the Galician rias (northwest of Spain). Mar. Drugs 16, 81.
Ledreux, A., Brand, H., Chinain, M., Bottein, M.-Y.D., Ramsdell, J.S., 2014. Dynamics of ciguatoxins from *Gambierdiscus polynesiensis* in the benthic herbivore *Mugil cephalus*: trophic transfer implications. Harmful Algae 39, 165–174.
Lehane, L., Lewis, R.J., 2000. Ciguatera: recent advances but the risk remains. Int. J. Food Microbiol. 61, 91–125.
Leonardo, S., Toldrà, A., Campàs, M., 2017a. Trends and prospects on electrochemical biosensors for the detection of marine toxins. In: Diogène, J., Campàs, M. (Eds.), Comprehensive Analytical Chemistry. Recent Advances in the Analysis of Marine Toxins, vol. 78. Elsevier, Amsterdam, pp. 303–341.
Leonardo, S., Rambla-Alegre, M., Samdal, I.A., Miles, C.O., Kilcoyne, J., Diogène, J., O'Sullivan, C.K., Campàs, M., 2017b. Immunorecognition magnetic supports for the development of an electrochemical immunoassay for azaspiracid detection in mussels. Biosens. Bioelectron. 92, 200–206.
Leonardo, S., Toldrà, A., Rambla-Alegre, M., Fernández-Tejedor, M., Andree, K.B., Ferreres, L., Campbell, K., Elliott, C.T., O'Sullivan, C.K., Pazos, Y., Diogène, J., Campàs, M., 2018a. Self-assembled monolayer-based immunoassays for okadaic acid detection in seawater as monitoring tools. Mar. Environ. Res. 133, 6–14.
Leonardo, S., Kilcoyne, J., Samdal, I.A., Miles, C.O., O'Sullivan, C.K., Diogène, J., Campàs, M., 2018b. Detection of azaspiracids in mussels using electrochemical immunosensors for fast screening in monitoring programs. Sensors Actuators B Chem. 262, 818–827.
Leonardo, S., Kiparissis, S., Rambla-Alegre, M., Almarza, S., Roque, A., Andree, K.B., Christidis, A., Flores, C., Caixach, K., Campbell, K., Elliott, C.T., Aligizaki, K., Diogène, J., Campàs, M., 2019. Detection of tetrodotoxins in juvenile pufferfish *Lagocephalus sceleratus* (Gmelin, 1789) from the North Aegean Sea (Greece) by an electrochemical magnetic bead-based immunosensing tool. Food Chem. 290, 255–262.
Leonardo, S., Gaiani, G., Tsumuraya, T., Hirama, M., Turquet, J., Sagristà, N., Rambla-Alegre, M., Flores, C., Caixach, J., Diogène, J., O'Sullivan, C.K., Alcaraz, C., Campàs, M., 2020. Addressing the analytical challenges for the detection of ciguatoxins using an electrochemical biosensor. Anal. Chem. 92, 4858–4865.

Lewis, R.J., 2001. The changing face of ciguatera. Toxicon 39, 97–106.
Lewis, R.J., Holmes, M.J., 1993. Origin and transfer of toxins involved in ciguatera. Comp. Biochem. Physiol. Part C Toxicol. Pharmacol. 106, 615–628.
Lewis, R.J., Sellin, M., Poli, M.A., Norton, R.S., MacLeod, J.K., Sheil, M.M., 1991. Purification and characterization of ciguatoxins from moray eel (*Lycodontis Javanicus*, Muraenidae). Toxicon 29, 1115–1127.
Lewis, R.J., Vernoux, J.P., Brereton, I.M., 1998. Structure of Caribbean ciguatoxin isolated from *Caranx Iatus*. J. Am. Chem. Soc. 120, 5914–5920.
Lombet, A., Bidard, J.N., Lazdunski, M., 1987. Ciguatoxin and brevetoxins share a common receptor site on the neuronal voltage-dependent Na+ channel. FEBS Lett. 219, 355–359.
Lopes de Carvalho, I., Pelerito, A., Ribeiro, I., Cordeiro, R., Núncio, M.S., Vale, P., 2019. Paralytic shellfish poisoning due to ingestion of contaminated mussel: a 2018 case report in Caparica (Portugal). Toxicon X 4, 100017.
Louzao, M.C., Ares, I.R., Cagide, E., 2008. Marine toxins and the cytoskeleton: anew view of palytoxin toxicity. FEBS J. 275, 6067–6074.
Mahmud, Y., Yamamori, K., Noguchi, T., 1999. Occurrence of TTX in a brackfish wáter puffer "Midorifugu", *Tetraodon nigrovidiris*, collected from Thailand. J. Food Hygiene Soc. Japan 40, 363–367.
Manger, R., Leja, L., Lee, S., Hungerford, J., Wekell, M., 1993. Tetrazolium-based cell bioassay for neurotoxins active on voltage-sensitive sodium channels: semiautomated assay for saxitoxins, brevetoxins, and ciguatoxins. Anal. Biochem. 2014, 190–194.
Mattei, C., Dechraoui, M.Y., Molgó, J., Meunier, F.A., Legrand, A.M., Benoit, E., 1999. Neurotoxins targeting receptor site 5 of voltage-dependent sodium channels increase the nodal volume of myelinated axons. J. Neurosci. Res. 55, 666–673.
McCall, J.R., Jacocks, H.M., Niven, S.C., Poli, M.A., Baden, D.G., Bourdelais, A.J., 2014. Development and utilitzation of a fluorescence-based receptor-binding assay for the site 5 voltage-sensitive sodium channel ligands brevetoxin and ciguatoxin. J. AOAC Int. 97, 307–315.
McCarron, P., Wright, E., Quilliam, M.A., 2014. Liquid chromatography/mass spectrometry of domoic acid and lipophilic shellfish toxins with selected reaction monitoring and optional confirmation by library searching of product ion spectra. J. AOAC Int. 97, 316–324.
Meunier, F.A., Mattei, C., Molgó, J., 2009. Marine toxins potently affecting neurotransmitter release. In: Fusetani, N., Kem, W. (Eds.), Marine Toxins as Research Tools. Progress in Molecular and Subcellular Biology. vol. 46. Springer, Berlin, pp. 159–186.
Molgó, J., Gaudry-Talarmain, Y.M., Legrand, A.M., Moulian, N., 1993. Ciguatoxin extracted from poisonous moray eels *Gymnothorax javanicus* triggers acetylcholine release from *Torpedo* cholinergic synaptosomes via reserved Na(+)-Ca2+ exchange. Neurosci. Lett. 160, 65–68.
Molinero-Abad, B., Perez, L., Izquierdo, D., Escudero, I., Arcos-Martinez, M.J., 2019. Sensor system based on flexible screen-printed electrodes for electrochemical detection of okadaic acid in seawater. Talanta 192, 347–352.
Mountfort, D.O., Kennedy, G., Garthwaite, I., Quilliam, M.A., Truman, P., Hannah, D.J., 1999. Evaluation of the fluorometric protein phosphatase inhibition assay in the determination of okadaic acid in the mussels. Toxicon 37, 909–922.
Mountfort, D.O., Suzuki, T., Truman, P., 2001. Protein phosphatase inhibition assay adapted for determination of total DSP in contaminated mussels. Toxicon 39, 383–390.
Moury, R., Oishi, T., Torikai, K., Ujihara, S., Matsumori, N., Murata, M., Oshima, Y., 2009. Surface plasmon resonance-based detection of ladder-shaped polyethers by inhibition detection method. Bioorg. Med. Chem. Lett. 19, 2824–2828.

Munday, R., 2014. Toxicology of seafood toxins: a critical review. In: Botana, L. (Ed.), Seafood and Freshwater Toxins Pharmacology, Physiology, and Detection, third ed. CRC Press, Boca Raton, pp. 197–290.

Munday, R., 2017. Toxicology of seafood toxins: animal studies and mechanisms of action. In: Diogène, J., Campàs, M. (Eds.), Comprehensive Analytical Chemistry. Recent Advances in the Analysis of Marine Toxins, vol. 78. Elsevier, Amsterdam, pp. 211–229.

Munday, R., Selwood, A.I., Rhodes, L., 2012. Acute toxicity of pinnatoxins E, F and G to mice. Toxicon 60, 995–999.

Nagumo, Y., Oguri, H., Tsumoto, K., Shindo, Y., Hirama, M., Tsumuraya, T., Fujii, I., Tomioka, Y., Mizugaki, M., Kumagai, I., 2004. Phage-display selection of antibodies to the left end of CTX3C using synthetic fragments. J. Immunol. Methods 289, 137–146.

Noguchi, T., Arakawa, O., 2008. Tetrodotoxin—distribution and accumulation in aquatic organisms, and cases of human intoxication. Mar. Drugs 6, 220–242.

Novelli, A., Kispert, J., Reilly, A., Zitko, V., 1990. Excitatory amino acids toxicity in cerebellar granule cells in primary culture. Can. Dis. Wkly. Rep. 16 (Suppl. 1E), 83–88. discussion 88-9.

Novelli, A., Kispert, J., Fernández-Sánchez, M.T., Torreblanca, A., Zitko, V., 1992. Domoic acid-containing toxic mussels produce neurotoxicity in neuronal cultures through a synergism between excitatory amino acids. Brain Res. 577, 41–48.

Oguri, H., Hirama, M., Tsumuraya, T., Fujii, I., Maruyama, M., Uehara, H., Nagumo, Y., 2003. Synthesis-based approach toward direct sandwich immunoassay for ciguatoxin CTX3C. J. Am. Chem. Soc. 125, 7608–7612.

Otero, P., Alfonso, A., Alfonso, C., Aráoz, R., Molgó, J., Vieytes, M.R., Botana, L.M., 2011. First direct fluorescence polarization assay for the detection and quantification of spirolides in mussel samples. Anal. Chim. Acta 701, 200–208.

Park, D.L., 1995. Detection of ciguatera and diarrheic shellfish toxins in finfish and shellfish with ciguatect kit. J. AOAC Int. 78, 533–537.

Pazos, M.J., Alfonso, A., Vieytes, M.R., Yasumoto, T., Vieites, J.M., Botana, L.M., 2004. Resonant mirror biosensor detection method based on yessotoxin-phosphodiesterase interactions. Anal. Biochem. 335, 112–118.

Pérez-Gómez, A., Cabrera-García, D., Warm, D., Marini, A.M., Salas Puig, J., Fernández-Sánchez, M.T., Novelli, A., 2018. From the cover: selective enhancement of domoic acid toxicity in primary cultures of cerebellar granule cells by lowering extracellular Na^+ concentration. Toxicol. Sci. 161, 103–114.

Powell, C.L., Doucette, G.J., 1999. A receptor binding assay for paralytic shellfish poisoning toxins: recent advances and applications. Nat. Toxins 7, 393–400.

Rambla-Alegre, M., Reverté, L., Del Río, V., De la Iglesia, P., Palacios, O., Flores, C., Caixach, J., Campbell, J., Elliott, C.T., Izquierdo-Muñoz, A., Campàs, M., Diogène, J., 2017. Evaluation of tetrodotoxins in puffer fish caught along the Mediterranean coast of Spain. Toxin profile of *Lagocephalus sceleratus*. Environ. Res. 158, 1–6.

Rambla-Alegre, M., Leonardo, S., Barguil, Y., Flores, C., Caixach, J., Campbell, K., Elliott, C.T., Maillaud, C., Boundy, M.K., Harwood, D.T., Campàs, M., Diogène, J., 2018. Rapid screening and multi-toxin profile confirmation of tetrodotoxins and analogues in human body fluids derived from a puffer fish poisoning incident in New Caledonia. Food Chem. Toxicol. 112, 188–193.

Ramsdell, J.S., 2007. The molecular and integrative basis to domoic acid toxicity. In: Botana, L.M. (Ed.), Phycotoxins: Chemistry and Biochemistry. Blackwell Publishing, Oxford, pp. 223–250.

Reis Costa, P., Moita, T., Rodrigues, S.M., 2014. Estimating the contribution of N-sulfocarbamoyl paralytic shellfish toxin analogs GTX6 and C3+4 to the toxicity of mussels (*Mytilus galloprovincialis*) over a bloom of *Gymnodinium catenatum*. Harmful Algae 31, 35–40.

Reis Costa, P., Braga, A.C., Turner, A.D., 2018. Accumulation and elimination dynamics of the hydroxybenzoate saxitoxin analogues in mussels *Mytilus galloprovincialis* exposed to the toxic marine dinoflagellate *Gymnodinium catenatum*. Toxins 10, 428.

Reverté, L., Soliño, L., Carnicer, O., Diogène, J., Campàs, M., 2014. Alternative methods for the detection of emerging marine toxins: biosensors, biochemical assays and cell-based assays. Mar. Drugs 12, 5719–5763.

Reverté, L., de la Iglesia, P., del Río, V., Campbell, K., Elliott, C.T., Kawatsu, K., Katikou, P., Diogène, J., Campàs, M., 2015. Detection of tetrodotoxins in puffer fish by a self-assembled monolayer-based immunoassay and comparison with surface plasmon resonance, LC-MS/MS, and mouse bioassay. Anal. Chem. 87, 10839–10847.

Reverté, L., Campbell, K., Rambla-Alegre, M., Elliott, C.T., Diogène, J., Campàs, M., 2017a. Immunosensor array platforms based on self-assembled dithiols for the electrochemical detection of tetrodotoxins in puffer fish. Anal. Chim. Acta 989, 95–103.

Reverté, L., Campàs, M., Yakes, B.Y., Deeds, J.R., Katikou, P., Kawatsu, K., Lochhead, M., Elliott, C.T., Campbell, K., 2017b. Tetrodotoxin detection in puffer fish by a sensitive planar waveguide immunosensor. Sensors Actuators B Chem. 253, 967–976.

Reverté, L., Rambla-Alegre, M., Leonardo, S., Bellés, C., Campbell, K., Elliott, C.K., Gerssen, A., Klijnstra, M.D., Diogène, J., Campàs, M., 2018. Development and validation of a maleimide-based enzyme-linked immunosorbent assay for the detection of tetrodotoxin in oysters and mussels. Talanta 176, 659–666.

Rivas, M., Garcia, C., Liberona, J.L., Lagos, N., 2000. Biochemical characterization and inhibitory effects of dinophysistoxin-1, okadaic acid and microcystin 1-r on protein phosphatase 2a purified from the mussel *Mytilus chilensis*. Biol. Res. 33, 197–206.

Rodríguez, P., Alfonso, A., Vale, C., Alfonso, C., Vale, P., Tellez, A., Botana, L.M., 2008. Fist toxicity report of tetrodotoxin and 5,6,11-trideoxyTTX in the trumpet shell *Charonia lampas* in Europe. Anal. Chem. 80, 5622–5629.

Rodríguez, L.P., Vilariño, N., Molgó, J., Aráoz, R., Antelo, A., Vieytes, M.R., Botana, L.M., 2011. Solid-phase receptor-based assay for the detection of cyclic imines by chemiluminescence, fluorescence, or colorimetry. Anal. Chem. 83, 5857–5863.

Rodríguez, L.P., Vilariño, N., Molgó, J., Aráoz, R., Botana, L.M., 2013a. High-throughput receptor-based assay for the detection of spirolides by chemiluminescence. Toxicon 75, 35–43.

Rodríguez, L.P., Vilariño, N., Molgó, J., Aráoz, R., Louzao, M.C., Taylor, P., Talley, T., Botana, L.M., 2013b. Development of a solid-phase receptor-based assay for the detection of cyclic imines using a microsphere-flow cytometry system. Anal. Chem. 85, 2340–2347.

Rodríguez, I., Alfonso, A., González-Jartín, J.M., Vieytes, M.R., Botana, L.M., 2018. A single run UPLC-MS/MS method for detection of all EU-regulated marine toxins. Talanta 189, 622–628.

Rubio, F., Kamp, L., Carpino, J., Faltin, E., Loftin, K., Molgó, J., Aráoz, R., 2014. Colorimetric microtiter plate receptor-binding assay for the detection of freshwater and marine neurotoxins targeting the nicotinic acetylcholine receptors. Toxicon 91, 45–56.

Rubiolo, J.A., López-Alonso, H., Vega, F.V., Vieytes, M.R., Botana, L.M., 2011. Okadaic acid and dinophysis toxin 2 have differential toxicological effects in hepatic cell lines including cell cycle arrest, at G0/G1 or G2/M with aberrant mitosis depending on the cell line. Arch. Toxicol. 85, 1541–1550.

Sassolas, A., Catanante, G., Hayat, A., Marty, J.L., 2011. Development of an efficient protein phosphatase-based colorimetric test for okadaic acid detection. Anal. Chim. Acta 702, 262–278.

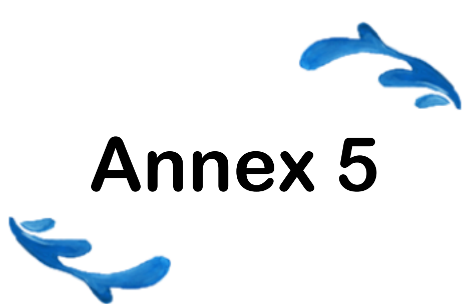
Servent, D., Winckler-Dietrich, V., Hu, H.Y., Keslller, P., Drevet, P., Betrand, D., Ménez, A., 1997. Only snake curaremimetic toxins with a fifth disulfide bond have high affinity for the neuronal alpha7 nicotinic receptor. J. Biol. Chem. 272, 24279–24286.

Shao, B., Gao, X., Yang, F., Chen, W., Miao, T., Peng, J., 2012. Screening and structure analysis of the aptamer against tetrodotoxin. Food Sci. Technol. 2, 347–351.
Smienk, H.G.F., Calvo, D., Razquin, P., Domínguez, E., Mata, L., 2012. Single laboratory validation of a ready-to-use phosphatase inhibition assay for detection of okadaic acid toxins. Toxins 5, 339–352.
Smienk, H., Domínguez, E., Rodríguez-Velasco, M.L., Clarke, D., Kapp, K., Katikou, P., Cabado, A.G., Otero, A., Vieites, J.M., Razquin, P., Mata, L., 2013. Quantitative determination of the okadaic acid toxins group by a colorimetric phosphatase inhibition assay: interlaboratory study. J. AOAC Int. 96, 77–85.
Sommer, H., Meyer, K.F., 1937. Paralytic shellfish poisoning. Arch. Pathol. 24, 560–598.
Soto-Liebe, K., López-Cortés, X.A., Fuentes-Valdes, J.J., Stucken, K., Gonzalez-Nilo, F., Vásquez, M., 2013. *In silico* analysis of putative paralytic shellfish poisoning toxins export proteins in cyanobacteria. PLoS One 8, e55664.
Stivala, C.E., Benoit, E., Aráoz, R., Servent, D., Novikov, A., Molgó, J., Zakarian, A., 2015. Synthesis and biology of cyclic imine toxins, an emerging class of potent, globally distributed marine toxins. Nat. Prod. Rep. 32, 411–435.
Takai, A., Murata, M., Isobe, M., Mieskes, G., Yasumoto, T., 1992. Inhibitory effect of okadaic acid derivatives on protein phosphatases. A study on structure-affinity relationship. Biochem. J. 284, 539–544.
Tang, D., Tang, J., Su, B., Chen, G., 2011. Gold nanoparticles-decorated amine-terminated poly(amidoamine) dendrimer for sensitive electrochemical immunoassay of brevetoxins in food samples. Biosens. Bioelectron. 26, 2090–2096.
Tian, R.Y., Lin, C., Yu, S.Y., Gong, S., Hu, P., Li, Y.S., Wu, Z.C., Gao, Y., Zhou, Y., Liu, Z.S., Ren, H.L., Lu, S.Y., 2016. Preparation of a specific ssDNA aptamer for brevetoxin-2 using SELEX. J. Anal. Methods Chem. 2016, 9241860.
Tsumuraya, T., Fujii, F., Inoue, M., Tatatami, A., Miyazaki, K., Hirama, M., 2006. Production of monoclonal antibodies for sandwich immunoassay detection of ciguatoxin 51-hydroxyCTX3C. Toxicon 48, 287–294.
Tsumuraya, T., Fujii, I., Hirama, M., 2010. Production of monoclonal antibodies for sandwich immunoassay detection of Pacific ciguatoxins. Toxicon 56, 797–803.
Tsumuraya, T., Fujii, I., Hirama, M., 2014. Preparation of anti-ciguatoxin monoclonal antibodies using synthetic haptens: sandwich ELISA detection of ciguatoxins. J. AOAC Int. 97, 373–379.
Tsumuraya, T., Sato, T., Hirama, M., Fujii, I., 2018. Highly sensitive and practical fluorescent sandwich ELISA for ciguatoxins. Anal. Chem. 90, 7318–7324.
Tubaro, A., Florio, C., Luxich, E., Sosa, S., Della Loggia, R., Yasumoto, T., 1996. A protein phosphatase 2A inhibition assay for a fast and sensitive assessment of okadaic acid contamination in mussels. Toxicon 34, 743–752.
Tsumuraya, T., Takeuchi, K., Yamashita, S., Fujii, I., Hirama, M., 2012. Development of a monoclonal antibody against the left wing of ciguatoxin CTX1B: thiol strategy and detection using a sandwich ELISA. Toxicon 60, 348–357.
Tubaro, A., Del Favero, G., Beltramo, D., Ardizzone, M., Forino, M., De Bortoli, M., Pelin, M., Poli, M., Bignami, G., Ciminiello, P., Sosa, S., 2011. Acute oral toxicity in mice of a new palytoxin analog: 42-Hydroxy palytoxin. Toxicon 57, 755–763.
Turner, A.D., Hatfield, R.G., 2012. Refinement of AOAC official method 2005.06 liquid chromatography-fluorescence detection method to improve performance characteristics for the determination of paralytic shellfish toxins in king and queen scallops. J. AOAC Int. 95, 129–142.
Turner, A.D., Norton, D.M., Hatfield, R.G., Morris, S., Reese, A.R., Algoet, M., Lees, D.N., 2009. Refinement and extension of AOAC method 2005.06 to include additional toxins in mussels: single-laboratory validation. J. AOAC Int. 92, 190–207.
Turner, A.D., Dhanji-Rapkova, M., Algoet, M., Suarez-Isla, B.A., Cordova, M., Caceres, C., Murphy, C.J., Casey, M., Lees, D.N., 2012. Investigations into matrix

components affecting the performance of the official bioassay reference method for quantitation of paralytic shellfish poisoning toxins in oysters. Toxicon 59, 215–230.
Turner, A.D., Dhanji-Rapkova, M., Baker, C., Algoet, M., 2014a. Assessment of semiquantitative liquid chromatography-fluorescence detection method for the determination of paralytic shellfish poisoning toxin levels in bivalve molluscs from Great Britain. J. AOAC Int. 97, 492–497.
Turner, A.D., Stubbs, B., Coates, L., Dhanji-Rapkova, M., Hatfield, R.G., Lewis, A.M., Rowland-Pilgrim, S., O'Neil, A., Stubbs, P., Ross, S., Baker, C., Algoet, M., 2014b. Variability of paralytic shellfish toxin occurrence and profiles in bivalve molluscs from Great Britain from official control monitoring as determined by pre-column oxidation liquid chromatography and implications for applying immunochemical tests. Harmful Algae 31, 87–99.
Turner, A.D., Powell, A., Schofield, A., Lees, D.N., Baker-Austin, C., 2015a. Detection of the pufferfish toxin tetrodotoxin in European bivalves, England, 2013 to 2014. Euro Surveill. 20, 2–8.
Turner, A.D., McNabb, P.S., Harwood, D.T., Selwood, A.I., Boundy, M.J., 2015b. Single-laboratory validation of a multitoxin ultra-performance LC-hydrophilic interaction LC-MS/MS method for quantitation of paralytic shellfish toxins in bivalve shellfish. J. AOAC Int. 98, 609–621.
Turner, A.D., Boundy, M.J., Rapkova, M.D., 2017a. Development and single-laboratory validation of a liquid chromatography tandem mass spectrometry method for quantitation of tetrodotoxin in mussels and oysters. J. AOAC Int. 100, 1–14.
Turner, A.D., Dhanji-Rapkova, M., Coates, L., Bickerstaff, L., Milligan, S., O'Neill, A., Faulkner, D., McEneny, H., Baker-Austin, C., Lees, D.N., Algoet, M., 2017b. Detection of tetrodotoxin shellfish poisoning (TSP) toxins and causative factors in bivalve molluscs from the UK. Mar. Drugs 15, 277.
Twiner, M.J., Doucette, G.J., Rasky, A., Huang, X.P., Roth, B.L., Sanguinetti, M.C., 2012. The marine algal toxin azaspiracid is an open state blocker of hERG potassium channels. Chem. Res. Toxicol. 25, 1975–1984.
UNE-EN 14526, 2017. Foodstuffs. Determination of Saxitoxin-Group Toxins in Shellfish. HPLC Method Using Pre-Column Derivatization with Peroxide or Periodate Oxidation. Spanish Association for Standardization, pp. 1–68.
Urdaneta, A., 1580. Relación de los sucesos de la Armada del comendador Loaisa a las islas de la Especiería o Molucas en 1525 y sucesos acaecidos en ellas hasta 1536. Real Biblioteca II/1465.
US FDA, 2020. Fish and Fishery Products Hazards and Controls Guidance, fourth ed. United States Food and Drug Administration. March 2020.
Vale-Gonzalez, C., Pazos, M.J., Alfonso, A., Vieytes, M.R., Botana, L.M., 2007. Study of the neuronal effects of ouabain and palytoxin and their binding to Na,K-ATPases using an optical biosensor. Toxicon 50, 541–552.
Van Dolah, F.M., Finley, E.L., Haynes, B.L., Doucette, G.J., Moeller, P.D., Ramsdell, J.S., 1994. Development of rapid and sensitive high throughput pharmacologic assays for marine phycotoxins. Nat. Toxins 2, 189–196.
Vieytes, M.R., Fontal, O.I., Leira, F., Baptista de Sousa, J.M., Botana, L.M., 1997. A fluorescent microplate assay for diarrheic shellfish toxins. Anal. Biochem. 248, 258–264.
Vilariño, N., Fonfría, E.S., Molgó, J., Aráoz, R., Botana, L.M., 2009. Detection of gymnodimine-A and 13-desmethyl C spirolide phycotoxins by fluorescence polarization. Anal. Chem. 81, 2708–2714.
Vlamis, A., Katikou, P., Rodríguez, I., Rey, V., Alfonso, A., Papazachariou, A., Zacharaki, T., Botana, A.M., Botana, L.M., 2015. First detection of tetrodotoxin in Greek shellfish by UPLC-MS/MS potentially linked to the presence of the dinoflagellate *Prorocentrum minimum*. Toxins 7, 1779–1807.

Wandscheer, C.B., Vilariníño, N., Espiniña, B., Louzao, M.C., Botana, L.M., 2010. Human muscarinic acetylcholine receptors are a target of the marine toxin 13-desmethyl C spirolide. Chem. Res. Toxicol. 23, 1753–1761.
Wiese, M., D'Agostino, P.M., Mihali, T.K., Moffitt, M.C., Neilan, B.A., 2010. Neurotoxic alkaloids: saxitoxin and its analogs. Mar. Drugs 8, 2185–2211.
Yasumoto, T., Igarashi, T., Legrand, A.M., Cruchet, P., Chinaim, M., Fujita, T., Naoki, H., 2000. Structural elucidation of ciguatoxin congeners by fast-atom bombardment tándem mass spectroscopy. J. Am. Chem. Soc. 122, 4988–4989.
Zamolo, V.A., Valenti, G., Venturelli, E., Chaloin, O., Marcaccio, M., Boscolo, S., Castagnola, V., Sosa, S., Berti, F., Fontanive, G., Poli, M., Tubaro, A., Bianco, A., Paolucci, F., Prato, M., 2012. Highly sensitive electrochemiluminescent nanobiosensor for the detection of palytoxin. ACS Nano 6, 7989–7997.
Zheng, X., Hu, B., Gao, S.X., Liu, D.J., Sun, M.J., Jiao, B.H., Wang, L.H., 2015. A saxitoxin-binding aptamer with higher affinity and inhibitory activity optimized by rational site-directed mutagenesis and truncation. Toxicon 101, 41–47.
Zhou, J., Qiu, X., Su, K., Xu, G., Wang, P., 2016. Disposable poly(o-aminophenol)-carbon nanotubes modified screen print electrode-based enzyme sensor for electrochemical detection of marine toxin okadaic acid. Sensors Actuators B Chem. 235, 170–178.

UNIVERSITAT ROVIRA I VIRGILI
DEVELOPMENT OF BIOANALYTICAL DEVICES FOR THE DETECTION OF CIGUATOXINS AND THE CIGUATOXIN PRODUCING GENERA GAMBIERDISCUS AND FUKUYOA
Greta Gaiani



Annex 5

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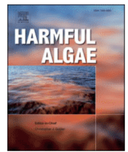

Nucleic acid lateral flow dipstick assay for the duplex detection of *Gambierdiscus australes* and *Gambierdiscus excentricus*

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A B S T R A C T

The proliferation of harmful microalgae endangers aquatic ecosystems and can have serious economic implications on a global level. Harmful microalgae and their associated toxins also pose a threat to human health since they can cause seafood-borne diseases such as ciguatera. Implementation of DNA-based molecular methods together with appropriate detection strategies in monitoring programs can support the efforts for effective prevention of potential outbreaks. A PCR-lateral flow assay (PCR-LFA) in dipstick format was developed in this work for the detection of two *Gambierdiscus* species, *G. australes* and *G. excentricus*, which are known to produce highly potent neurotoxins known as ciguatoxins and have been associated with ciguatera outbreaks. Duplex PCR amplification of genomic DNA from strains of these species utilizing species-specific ssDNA tailed primers and a common primer containing the binding sequence of scCro DNA binding protein resulted in the generation of hybrid ssDNA-dsDNA amplicons. These were captured on the dipsticks via hybridization with complementary probes and detected with a scCro/carbon nanoparticle (scCro/CNPs) conjugate. The two different test zones on the dipsticks allowed the discrimination of the two species and the assay exhibited high sensitivity, 6.3 pg/μL of genomic DNA from both *G. australes* and *G. excentricus*. The specificity of the approach was also demonstrated using genomic DNA from non-target *Gambierdiscus* species and other microalgae genera which did not produce any signals. The possibility to use cells directly for amplification instead of purified genomic DNA suggested the compatibility of the approach with field sample testing. Future work is required to further explore the potential use of the strategy for on-site analysis and its applicability to other toxic species.

1. Introduction

Ciguatera fish poisoning (CFP) is a highly common seafood-borne disease (Lewis, 2001) caused by the potent marine microalgal neurotoxins named ciguatoxins (CTXs) (Yasumoto, 2005). Ciguatera has become a global health concern due to the severe symptoms elicited after intoxication, including cardiovascular, gastrointestinal and neurological alterations which might last a few days but can also persist for longer periods of time (Friedman et al., 2017). Several species of the marine dinoflagellate genus named *Gambierdiscus* have been confirmed to produce CTXs (Caillaud et al., 2011; Litaker et al., 2017; Reverté et al., 2018). Ingestion of CTX-producing microalgae by herbivorous fish and subsequent bioaccumulation of CTXs facilitates their introduction into the food chain, which ultimately may reach humans. This genus of microalgae is epibenthic and endemic of tropical and subtropical waters such as the Pacific and Indian Ocean or the Caribbean Sea (Hamilton et al., 2002; Lewis 2001; Litaker et al., 2017). However, their presence has expanded to non-endemic areas. Recently, Tester et al. (2020) reviewed the global distribution of the genus during the last decade or so (2009 – 2018), underlying zones in which the amount and diversity of *Gambierdiscus* species is higher, such as the French Polynesia, Caribbean coasts and Canary Islands (Spain). In fact, several *Gambierdiscus* species have been reported during the last years in the Canary Islands (Fraga et al., 2011; Rodriguez et al., 2017), the Madeira archipelago (Portugal) (Kaufmann and Bohm-Beck, 2013) and the Mediterranean Sea (Aligizaki and Nikolaidis 2008; Tudó et al., 2018). *Gambierdiscus australes and G. excentricus* strains exhibiting high CTX-like toxicities have been recently found in the Canary Islands and are considered the dominant species associated with ciguatera outbreaks in this area (Rossignoli et al., 2020).

Monitoring the presence of these microalgae and the toxins they produce in the marine environment is essential for implementing

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appropriate risk prevention strategies. A battery of methods has been developed for the direct detection of CTXs in fish and have been extensively reviewed elsewhere (Pasinzski et al., 2020; Reverté et al., 2014). These include animal and cell-based toxicity assays, biochemical assays employing receptors and antibodies, as well as liquid chromatography coupled with mass spectrometry. However, the complex chemical structures and scarce availability of pure CTXs, the low concentration at which they are encountered, and the complexity of fish matrices make the development of highly sensitive and specific assays for CTXs detection very challenging (Pasinzski et al., 2020; Reverté et al., 2014). Biosensors on the other hand, extensively used for the detection of various foodborne pathogens (Lazcka et al., 2007; Velusamy et al., 2010), can offer the required specificity and sensitivity when combined with highly specific biorecognition molecules. The recent generation of CTX specific monoclonal antibodies using synthetic CTX fragments (Tsumuraya et al., 2014) has indeed allowed the development of sensitive sandwich-type immunoassays and biosensors for CTX detection in fish (Leonardo et al., 2020) and microalgal samples (Gaiani et al., 2020; Tudó et al., 2020).

Detection of harmful microalgae like the CTX-producing *Gambierdiscus* species based on molecular methods has also been introduced over the last years to implement the monitoring and containment efforts of harmful algae blooms (Toldrà et al., 2020). These methods are considered as easier, faster and more specific alternatives to the traditional light microscopy. They rely on the amplification of genomic DNA from the target species using specific primers in combination with colorimetric, fluorescent or electrochemical detection techniques. Even though the gold standard for amplification is PCR, isothermal amplification has also been reported (Toldrà et al., 2020). Nevertheless, few reports can be found in the literature exploiting molecular methods for the identification of *Gambierdiscus* species (Vandersea et al., 2012; Nishimura et al., 2016; Lyu et al., 2017; Lozano-Duque et al., 2018; Pitz et al., 2021; Gaiani et al., 2021).

In this work, a nucleic acid lateral flow assay (LFA) in a dipstick format was developed for the duplex detection of two toxin-producing *Gambierdiscus* species, *G. australes* and *G. excentricus*. This is the first report of an LFA employed for the identification of *Gambierdiscus* species. It relies on PCR amplification of genomic DNA using specifically modified primers followed by detection with dipsticks. Two reverse primers were designed, each one modified with a distinct single-stranded DNA (ssDNA) tail to allow discrimination of the two species. The amplicons were captured on two separate test lines of the dipsticks via hybridization with capture probes complementary to these ssDNA tails. On the other hand, the common forward primer contained the binding sequence of scCro DNA binding protein to facilitate detection with scCro/carbon nanoparticles (scCro/CNPs) conjugate. Enzyme Linked Oligonucleotide Assay (ELONA) was used to demonstrate correct amplification of both targets before transferring the assay to its final LFA format. The performance of the approach in terms of sensitivity and specificity was evaluated. Genomic DNA from several microalgae genera, different combinations of genomic DNA from five *Gambierdiscus* and one *Fukuyoa* species, and DNA extracted and amplified directly from cells of the two target species, were used to test the potential applicability of the strategy on field samples.

2. Materials and methods

2.1. Materials

Phosphate buffered saline (PBS; 10 mM phosphate, 137 mM NaCl, 2.7 mM KCl, pH 7.4), DreamTaq DNA polymerase, neutravidin and neutravidin-coated microplate strip plates and Tween-20 were obtained from Fisher Scientific (Spain). The BioFX TMB Super Sensitive One Component HRP Microwell Substrate was from Surmodics (USA), the FF170HP nitrocellulose membrane from Cytiva (Spain), the C083 cellulose fiber absorbent pad and Empigen BB from Merck (Spain). The preparation of scCro was based on a previous report (Aktas et al., 2015) with some modifications as described in the Supplementary Data (Fig. S1). HRP-scCro and scCro/CNPs conjugates were prepared as previously described (Aktas et al., 2015; 2019). Primers and DNA probes were purchased from Biomers.net (Germany) and their sequences are shown in Table 1.

2.2. Genomic DNA from microalgae

The microalgae strains used in this work are shown in Table 2. Maintenance of the cultures and genomic DNA extraction from each strain were performed as in previous works (Gaiani et al., 2021). Briefly, cultures were maintained under a photon flux of 100 µmol photons m^{-2} s^{-1} with a 12:12-h light/dark regime and at 24 ± 1 °C. For DNA extraction, cell pellets were re-suspended in lysis buffer (1 M NaCl, 70 mM Tris, 30 mM EDTA, pH 8.6), 1:8 vol of 10% (w/v) DTAB and 1 vol of chloroform. Subsequently, cellular disruption was achieved adding zirconium beads (0.5 mm diameter) to the mixture and using a Bead Beater-8 (BioSpec, USA). Then, DNA was extracted from the aqueous phase using standard phenol/chloroform method. Precipitation of the DNA was achieved by the addition of 2 vol of absolute ethanol and 0.1 vol of 3 M sodium acetate (pH 8.0). Finally, DNA was rinsed with 70% (v/v) ethanol and then dissolved in 1:4 vol of molecular DNAse/RNAse-free water. The concentration and purity of the extracted DNA were evaluated using a NanoDrop 2000 spectrophotometer (Thermo Fisher Scientific, Spain) and it was stored at −20 °C until analysis. Each strain was analyzed individually or in mixtures.

2.3. PCR amplification of genomic DNA

The primers used for amplification of genomic DNA from *G. australes* and *G. excentricus* were designed within the D1-D3 region of the 28 S large subunit (LSU) ribosomal DNA (Gaiani et al., 2021) and are shown in Table 1. The strategy for duplex PCR amplification of the target *Gambierdiscus* species with the modified primers is illustrated in Fig. 1A. Each reverse primer was extended at its 5′ end with a distinct ssDNA tail to allow the discrimination of the two species. The common forward primer for the two species was extended at its 5′ end with the DNA binding site for the scCro DNA binding protein. For simplex PCR, 200 nM of each primer were used whereas duplex PCR was performed using 200 nM of *G. australes* reverse primer, 300 nM of *G. excentricus* and 500 nM of the common forward primer for both *G. australes* and *G. excentricus*. Purified genomic DNA from each species was added to the PCR reactions at final concentrations of 400 pg/µL down to 400 fg/µL. PCR was performed using an initial denaturation step for 3 min at 95 °C, 30 cycles of denaturation for 10 s at 95 °C, annealing for 10 s at 58 °C and extension of 10 s at 72 °C, and a final extension step for 5 min at 72 °C. Strains *G. australes* IRTA-SMM-13-11 and *G. excentricus* IRTA-SMM-17-407 were used in all experiments unless otherwise stated. The compatibility of the primers with other strains of these species was also tested (Fig. S2). The PCR reactions were analyzed by agarose gel (2.6% w/v agarose in Tris/borate/EDTA buffer) electrophoresis.

2.4. Enzyme linked oligonucleotide assay (ELONA) for colorimetric detection of PCR amplicons

Correct incorporation of the ssDNA tails and scCro DNA binding site to the *G. australes* and *G. excentricus* PCR amplicons was verified by a colorimetric ELONA. Several parameters of the assay were optimized as shown in the Supplementary Data (Fig. S3). For the ELONA, biotinylated capture probes (Table 1) specific for each species (50 µL of 50 nM in PBS with 0.05% (v/v) Tween-20 (PBST)) were immobilized on separate wells of neutravidin-coated microplates for 15 min at room temperature. After washing (3 × 300 µL of PBST), 1 µL of PCR reaction was mixed with 49 µL of PBS and added to the wells for a 30-min incubation step. After washing, the HRP-scCro conjugate was added (50 µL of 15 nM in PBS)

Table 1
Oligonucleotides used in this work. The scCro DNA binding site is in bold and the DNA tails are in italics.

Oligonucleotide	Sequence (5′ – 3′)
G. australes & *G. excentricus* Forward primer	**TATCACTTGCGGTGATA**TGCTGCATGYGGAGATTCTTTYYTKG
G. australes Reverse primer	*GTTTTCCCAGTCACGAC*-C3-ATGCATAACTCTTCATTGCCAGTAG
G. excentricus Reverse primer	*TCTACAGGCTCGTATATGTA*-C3-AGCTTGGGTCACAGTGCAACAGAG
G. australes Capture probe	GTCGTGACTGGGAAAACTTTTTTTTTTTTTTT-TEG-biotin
G. excentricus Capture probe	TACATATACGAGCCTGTAGATTTTTTTT TTTTTTT-TEG-biotin
NALFA control line probe	biotin-AGTCCGTGGTAGGGCAGGTTGGGGTGACTTTTTTTT**TATCACCGCAAGTGATA**TTTT**TATCACTTGCGGTGATA**

Table 2
Microalgae strains used in this work.

Species	Strain	Origin	GenBank accession
Gambierdiscus australes	IRTA-SMM-13-09	Hierro Island, Spain	KY564322
	IRTA-SMM-13-11	Selvagem Grande Island, Portugal	KY564324
	IRTA-SMM-16-286	Lanzarote Island, Spain	MT119197
Gambierdiscus balechii	VGO920	Manado, Indonesia	KX268469
Gambierdiscus belizeanus	IRTA-SMM-17-421	Hierro Island, Spain	MT379471
Gambierdiscus caribaeus	IRTA-SMM-17-03	Hierro Island, Spain	MT119203
Gambierdiscus excentricus	IRTA-SMM-17-407	Gomera Island, Spain	MT119200
	IRTA-SMM-17-428	Gomera Island, Spain	MT119201
	VGO791	Tenerife, Spain	JF303066; JF303075
Fukuyoa paulensis	VGO1185	Ubatuba, Brazil	KM886379
	IRTA-SMM-17-211	Menorca Island, Spain	MT119205
Coolia monotis	IRTA-SMM-16-285	Formentera Island, Spain	MW328563
Ostreopsis cf. *ovata*	IRTA-SMM-16-133	Catalonia, Spain	MH790463
Prorocentrum lima	IRTA-SMM-17-47	Lanzarote Island, Spain	MW328564

and the plate was incubated for another 30 min. After a final washing step, 50 μL of TMB ELISA substrate were added and signal generation was stopped after 10 min with the addition of equal volume of 1 M H_2SO_4. The absorbance was finally recorded at 450 nm. Target genomic DNA calibration curves were constructed using 1/50 diluted PCR reactions performed with serially two-fold diluted genomic DNA from each species (final concentrations of 8 pg/μL down to 8 fg/μL). The absorbance data was fitted to four-parameter logistic model using the GraphPad Prism software and the limits of detection (LOD) were interpolated from the curves as the blank signals (ntc) plus three times their standard deviation (ntc + $3xSD_{ntc}$). Four replicates were prepared for each concentration examined.

2.5. Preparation of the LFA dipsticks

Nitrocellulose FF170HP membrane (height 3 cm) was used to prepare the dipsticks. The control line was constructed at 1.8 cm from the bottom of the membrane and separated from the two test lines by 0.4 cm and 0.8 cm. To facilitate ssDNA immobilization, each biotinylated probe was mixed with neutravidin in PBS at final concentrations of 35 μM and 8.3 μM, respectively. The mixtures were incubated for 15 min at ambient temperature and then dispensed on the membrane using a Lateral Flow Reagent Dispenser (Gentaur, Belgium). *G. australes* and *G. excentricus* capture probes were used for the construction of the two test lines, whereas a hairpin probe forming the scCro dsDNA binding site was used for the control line (see Table 1 for sequences). The membranes were dried at room temperature for at least 2 h and then blocked for 30 min with 2% (w/v) skim milk and 0.1% (v/v) Empigen BB in 10 mM carbonate-bicarbonate buffer pH 9.4. The strips were assembled on backing cards with a 2 cm absorbent pad overlapping the nitrocellulose membrane by 2 mm to ensure correct wicking. Finally, the dipsticks were cut at a width of 4 mm using an Autokun Cutter (Hangzhou Autokun Technology, India), packaged in plastic pouches and stored at 4 °C until use.

2.6. Detection of the PCR amplicons with the LFA dipsticks

The PCR amplicons were detected on the dipsticks using scCro/CNPs conjugate for carbon black signal (Fig. 1B and 1C). The scCro/CNPs conjugate was prepared as previously described (Aktas et al., 2019). The dipsticks were dipped vertically in the wells of a microtiter plate containing 10 μL of PCR reaction, 1 μL of scCro/CNPs conjugate suspension (0.2% w/v) and 39 μL of PBS with 1% (w/v) skim milk and 0.1% (v/v) Tween-20. Black color signals were observed within 15 min and the dipsticks were dried and finally imaged by flatbed scanning. Each experiment was performed in triplicate for the construction of the calibration curves or at least in duplicate for other experiments. The volume of the PCR reaction used for analysis with the dipsticks was optimized beforehand as described in the Supplementary Data (Fig. S4).

2.7. Direct PCR amplification of G. australes and G. excentricus cells and detection with LFA dipsticks

The possibility of detecting the two *Gambierdiscus* species using directly cells instead of purified genomic DNA was also evaluated. Cell suspensions containing 40 cells/μL from each strain (*G. australes* IRTA-SMM-16-286 and *G. excentricus* VGO791) were prepared in milli-Q water and heated for 5 min at 95 °C to promote lysis and release of genomic DNA. Crude cell extracts (2 μL) from each species were then added directly, individually or in a mixture, to PCR reactions containing primers for both strains to a final volume of 20 μL. Purified genomic DNA (100 pg/μL) was used for PCR in parallel as a control. PCR amplification was performed as detailed in Section 2.3 but with an extended initial denaturation step of 10 min at 95° C instead of 5 min. Amplicons were finally detected with the LFA dipsticks as described in Section 2.6.

3. Results

3.1. Specificity of the primers and PCR amplicon detection by ELONA

The specificity of the primers was first evaluated with simplex PCR. DNA from each species was added to a PCR reaction containing only its corresponding primers, and after amplification, the reactions were analyzed by agarose gel electrophoresis. As shown in Fig. 2A, only the specific genomic DNA was amplified with its corresponding primers

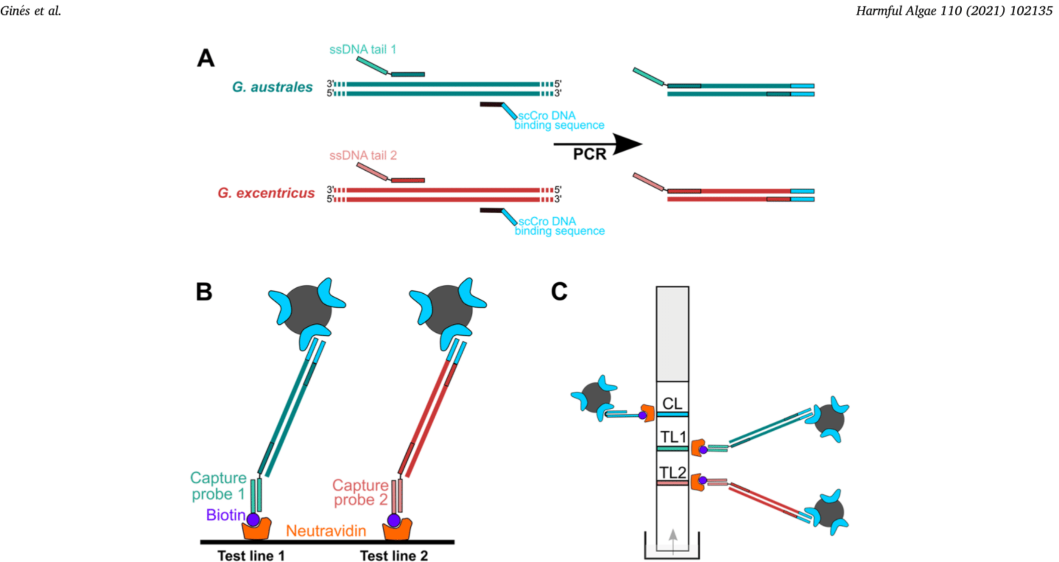

Fig. 1. Strategy for the detection of the target *Gambierdiscus* species with the PCR-LFA dipstick. (A) PCR amplification of *G. australes* and *G. excentricus* genomic DNA using modified primers. (B) PCR amplicon detection by LFA. Capture of the ssDNA-dsDNA hybrid amplicons on the dipsticks by complementary probes and detection with scCro/CNPs conjugate. (C) Design of the dipsticks. CL: control line; TL1: test line 1 for *G. australes*; TL2: test line 2 for *G. excentricus*.

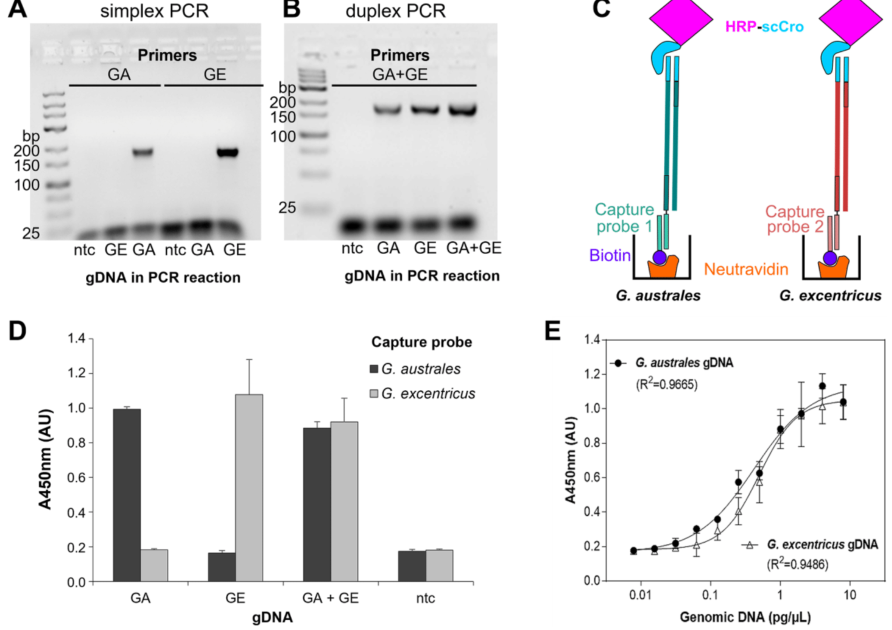

Fig. 2. PCR amplification of *G. australes* (GA) and *G. excentricus* (GE) genomic DNA (gDNA) and detection by ELONA. (A) Simplex PCR using primers for each species and (B) duplex PCR using primers for both strains. (C) Format of the ELONA for the detection of the two *Gambierdiscus* species. (D) Specificity of the duplex PCR-ELONA for the detection of the amplicons. (E) Calibration curves for the detection of genomic DNA from *G. australes* and *G. excentricus* by ELONA. ntc: no template control.

whereas no amplification was observed for the other one. Duplex PCR was then performed using a mixture of the two reverse primers and the common forward primer. Again, successful amplification for each strain was observed when only one of the targets was present (Fig. 2B). Since the expected size of the two amplicons is very similar (approximately 150 bp), it is not possible to differentiate the two amplicons in the duplex PCR reaction solely by gel electrophoresis. Therefore, ELONA was performed using capture probes specific to each target, which were complementary to the ssDNA tails at one end of the generated PCR amplicons for each species. For colorimetric detection, the HRP-scCro conjugate was used to bind the dsDNA binding site of scCro formed at the other end of the amplicons after incorporation of the specific sequence in the common forward primer of the two species (Fig. 2C). Correct amplification of each target genomic DNA in the duplex PCR reaction was verified when added individually or simultaneously whereas no signal was observed when the non-specific capture probe was used (Fig. 2D). The sensitivity of the ELONA was finally assessed using optimized conditions for PCR amplification and ELONA detection as described in the Supplementary Data (Fig. S3). Genomic DNA from each species was used for PCR amplification in a master mix containing primers for both species and representative agarose gels are shown in Fig. S5. The calibration curves are shown in Fig. 2E and the LODs were calculated to be 22.8 fg/µL and 52.3 fg/µL of genomic DNA for *G. australes* and *G. excentricus*, respectively. These correspond to 1.1 pg of genomic DNA for *G. australes* and 2.6 pg for *G. excentricus*, considering the 50 µL sample volume used for analysis.

3.2. Detection of G. australes and G. excentricus PCR amplicons by dipstick LFA

The approach used for the detection of the PCR amplicons by LFA and the design of the dipsticks, based on two test lines, one for each of the *Gambierdiscus* species, are illustrated in Fig. 1B and 1C, respectively. Duplex PCR reactions were performed using 100 pg/µL of genomic DNA from each species, separately or in a mixture, and the amplicons were detected with the dipsticks as described in Section 2.6. As it can be seen in Fig. 3, successful detection was achieved when genomic DNA from each strain was added separately or simultaneously, while no signal was observed in the absence of both targets.

3.3. Sensitivity of the dipsticks for G. australes and G. excentricus

To assess the sensitivity of the LFA dipsticks, PCR reactions were performed using a series of genomic DNA concentrations from each species (1.6 – 400 pg/µL) and a mixture of the primers for the two species. After amplification, the PCR reactions, previously optimized as

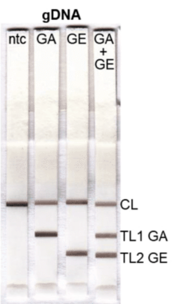

Fig. 3. LFA dipsticks for the detection of genomic DNA (gDNA) from *G. australes* (GA) and *G. excentricus* (GA) after duplex PCR amplification of genomic DNA (100 pg/µL) from each species. CL: control line; TL1 GA: test line 1 for *G. australes*; TL2 GE: test line 2 for *G. excentricus*.

shown in Fig. S4, were analyzed by agarose gel electrophoresis (Fig. S5). Finally, each PCR reaction was mixed with the scCro/CNPs conjugate and analyzed with the dipsticks. The visual LODs were determined as the minimum amounts of genomic DNA used for PCR amplification which resulted in visible test lines on the strips. As it can be seen in Fig. 4, the visual LODs were 6.3 pg/µL of genomic DNA for both *G. australes* and *G. excentricus*, corresponding to 63 pg of genomic DNA for each species considering that 10 µL of PCR reactions were analyzed with the dipsticks.

3.4. Specificity of the PCR-LFA

The dipsticks were tested with PCR reactions performed with genomic DNA from different non-target microalgae species to evaluate the specificity of the developed approach. Each PCR reaction contained the three primers for *G. australes* and *G. excentricus* and 100 pg/µL of genomic DNA from each species. Only the PCR reactions containing genomic DNA from *G. australes* and *G. excentricus* resulted in amplification as seen after agarose gel electrophoresis (Fig. S6) and positive test lines signals on the dipsticks (Fig. 5). The genomic DNA from the other microalgae did not cross-react with the primers, the capture probes or the reporter scCro/CNPs conjugate used for detection, thus demonstrating the specificity of the approach.

3.5. Detection of genomic DNA from G. australes and G. excentricus in the presence of non-target Gambierdiscus or Fukuyoa species

The effect of various non-target *Gambierdiscus* and a *Fukuyoa* species on the detection of genomic DNA from *G. australes* and *G. excentricus* was evaluated next. Mixtures containing equal concentrations of genomic DNA from each of the *Gambierdiscus* and *Fukuyoa* species (Fig. 6) were thus prepared and used for PCR amplification with the *G. australes* and *G. excentricus* primers. Analysis of the PCR reactions by agarose gel electrophoresis revealed successful amplification only in mixtures containing genomic DNA either from *G. australes* or *G. excentricus* or both (Fig. S7). Likewise, amplicons were detected as black lines on the dipsticks when either one or both target *Gambierdiscus* species were present (Fig. 6). The presence of the non-target *Gambierdiscus* or the *Fukuyoa* species included in this study did not interfere with the detection of the two target species since the intensity of the test lines was similar in all cases regardless of the composition of the DNA mixtures.

3.6. Detection of G. australes and G. excentricus cells

The possibility to detect the two target *Gambierdiscus* species with the PCR-LFA approach developed in this work using directly cells was finally evaluated. To this end, cell suspensions from each species were heated briefly and the crude cell lysates were used directly for PCR amplification instead of purified genomic DNA. PCR amplicons were detected by agarose gel electrophoresis for both species when used individually or in a mixture (Fig. S8). Similar amplification efficiency of the duplex reaction was observed when using cells or 100 pg/µL of purified genomic DNA from each species. The amplicons were finally detected with the LFA dipsticks (Fig. 7). However, the intensity of the test lines of the duplex reactions using crude cell extracts was slightly lower compared to when purified genomic DNA from each strain was used for amplification.

4. Discussion

The main objective of this work was to detect two toxin-producing *Gambierdiscus* species, *G. australes* and *G. excentricus*, associated with ciguatera outbreaks using PCR combined with lateral flow dipsticks. The spread of ciguatoxin-producing microalgae of the *Gambierdiscus* and *Fukuyoa* genera in non-endemic areas has increased the demand for fast and reliable detection methods to monitor these microalgae and prevent

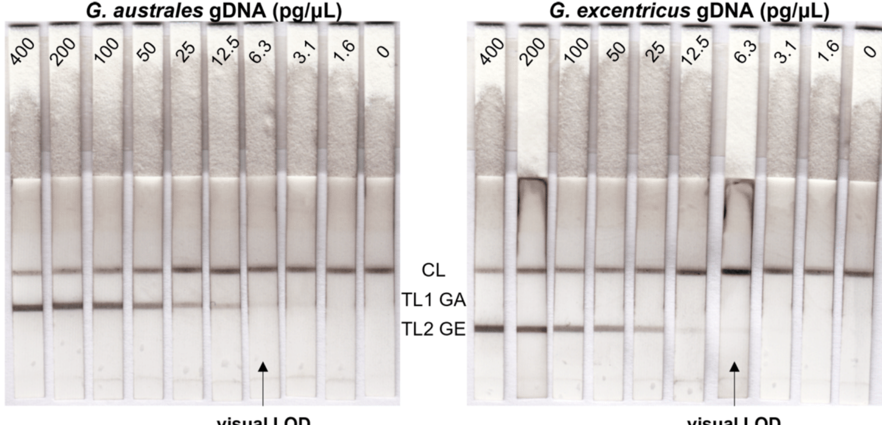

Fig. 4. Sensitivity of the PCR-LFA for each *Gambierdiscus* species. Serially two-fold diluted genomic DNA (gDNA) from each strain (400 to 1.6 pg/μL) was used in each PCR reaction containing primers for both species and added to the dipsticks for visual detection. CL: control line; TL1 GA: test line 1 for *G. australes*; TL2 GE: test line 2 for *G. excentricus*.

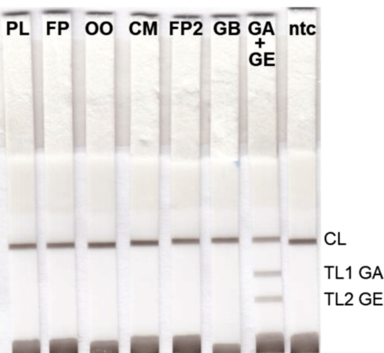

Fig. 5. Specificity of the PCR-LFA in the presence of various microalgae genera. PCR reactions containing the *G. australes* and *G. excentricus* primers were performed in the presence of 100 pg/μL of genomic DNA from each strain. PL: *P. lima* (IRTA-SMM-17-47); FP: *F. paulensis* (IRTA-SMM-17-211); OO: *O.* c.f. *ovata* (IRTA-SMM-16-133); CM: *C. monotis* (IRTA-SMM-16-285); FP2: *F. paulensis* (VGO1185); GB: *G. balechii* (VGO920); GA: *G. australes* (IRTA-SMM-13-11); GE: *G. excentricus* (IRTA-SMM-17-407); ntc: no template control; CL: control line; TL1 GA: test line 1 for *G. australes*; TL2 GE: test line 2 for *G. excentricus*.

future outbreaks. Hence, a lot of effort has been invested over the last years into moving from laboratory-based techniques, which are inherently slow and equipment-dependent, to decentralized, user-friendly platforms such as biosensors which are suitable for on-site detection (McPartlin et al., 2017). The sensitivity and specificity provided by nucleic acid amplification tests has allowed the implementation of molecular methods as promising tools for the detection of microalgae (Ebenezer et al., 2012; Medlin and Orozco, 2017). There is a plethora of reports on the detection of microalgae nucleic acids using both PCR and isothermal amplification (Toldrà et al., 2020).

Studies on the detection of ciguatoxin-producing microalgae based on molecular methods are scarce though. The first one reported the development of a qPCR assay for the simultaneous detection of several *Gambierdiscus* species with an LOD of 10 cells (Vandersea et al., 2012). In another qPCR-based study, identification and enumeration of four *Gambierdiscus* species was demonstrated with LODs of 10 gene copies (Nishimura et al., 2016). Alternatively, restriction fragment length polymorphism (RFLP) was used to identify *Gambierdiscus* and *Fukuyoa* species in field samples (Lyu et al., 2017; Lozano-Duque et al., 2018). In addition to these methods, species-specific fluorescence in situ hybridization (FISH) probes were also designed for the multiplex detection of several *Gambierdiscus* species in another report (Pitz et al., 2021). Finally, isothermal recombinase polymerase amplification in combination with a sandwich hybridization assay was recently developed for the detection of single cells from *Gambierdiscus* and *Fukuyoa* strains (Gaiani et al., 2021). Most of these studies targeted the detection of *G. australes* strains (Nishimura et al., 2012; Lyu et al., 2017, Lozano-Duque et al., 2018; Pitz et al., 2021, Gaiani et al., 2021), among other species, however only two of them focused also on *G. excentricus* (Lyu et al., 2017; Gaiani et al., 2021).

The combination of molecular methods for target DNA amplification with a sensitive detection strategy that is also simple, cost-effective and compatible with field testing would be ideal for monitoring the presence of toxic algae species in the marine environment. LFAs can potentially serve this purpose. The low cost and easy operation of these simple devices have encouraged their use for the detection of a wide variety of target analytes such as proteins, nucleic acids, toxins, drugs and others even in complex samples, and are especially suitable for on-site testing (Bahadir and Sezgintürk, 2016). For nucleic acid targets, detection of DNA amplicons generated by PCR or isothermal amplification is generally achieved using different labels (Bahadir and Sezgintürk, 2016; Zheng et al., 2021). Gold nanoparticles have been the mainstay in nucleic acid LFAs (Aveyard et al., 2007; Jauset-Rubio et al., 2016), mainly for qualitative or semiquantitative detection. Other labels have also been reported, such as CNPs (Noguera et al., 2011; Aktas et al., 2019; El-Tholoth et al., 2019), fluorescent nanoparticles (Takalkar et al., 2017), colored latex beads (Mao et al., 2013), enzymes (Aktas et al., 2019) or enzymes combined with nanoparticles (He et al., 2011; Aktas et al., 2019). Label materials such as quantum dots, upconversion nanoparticles, NIR dye-doped beads, SERS tags and semiconductor polymer dots were further developed to improve sensitivity and achieve quantitative analysis when combined with specific reader devices (Gui et al., 2014; Liu et al., 2021). Magnetic nanoparticles are also extremely

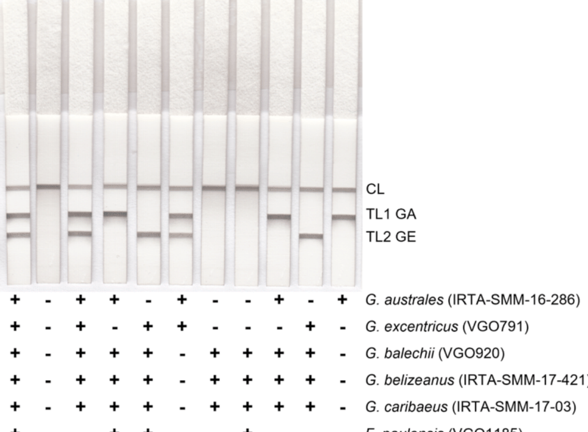

Fig. 6. Specificity of the PCR-LFA in the presence of non-target *Gambierdiscus* and *Fukuyoa* species. Each combination used for PCR amplification contained 400 pg/μL genomic DNA from each strain. CL: control line; TL1 GA: test line 1 for *G. australes*; TL2 GE: test line 2 for *G. excentricus*.

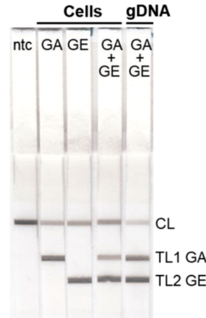

Fig. 7. Detection of *G. australes* (GA) and *G. excentricus* (GE) cells. Each PCR reaction contained 4 cells/μL while 100 pg/μL of purified gDNA (gDNA) from each species was used in parallel as a control. ntc: no template control; CL: control line; TL1 GA: test line 1 for *G. australes*; TL2 GE: test line 2 for *G. excentricus*.

useful as labels because of their dual magnetic and optical properties (Liu et al., 2021; Yan et al., 2019). On the other hand, CNPs are particularly attractive because of their low cost, high stability, easy modification and high signal-to-noise ratio (Posthuma-Trumpie et al., 2012; Zheng et al., 2021). CNPs were previously shown to provide enhanced sensitivity in LFAs when compared to gold nanoparticles, silver-enhanced gold nanoparticles and blue latex beads (Linares et al., 2012). Generic lateral flow immunoassay strips based on CNPs are in fact commercially available by Abingdon Health (UK) for the detection of double-labeled (biotin/fluorophore) DNA amplicons. CNPs were chosen as the colorimetric label for this work as well. Even though CNPs are not typically used for quantitative analysis, the main advantage they provide compared to the more sophisticated labels mentioned above is the simplicity of use and facile interpretation of the visual results without the need of a specific reader when a yes/no response is appropriate for the target analyte.

The primers used for PCR amplification of DNA from the two target toxic microalgal *Gambierdiscus* species were based on a previous work (Gaiani et al., 2021). They were designed within the large subunit of ribosomal DNA (rDNA) which provides the necessary sequence variability to allow specific amplification of the target strain and high copy number for increased sensitivity. The same region of rDNA was also targeted in previous studies with regards to the detection of several *Gambierdiscus* species including *G. australes* and *G. excentricus* (Lyu et al., 2017; Lozano-Duque et al., 2018; Pitz et al., 2021). To facilitate capture of the amplicons on the dipsticks, each reverse primer was modified at its 5′ end with a ssDNA sequence (tail) separated from the rest of the sequence with a carbon-based spacer. For detection, the common forward primer for both species was extended with the specific DNA sequence recognized by scCro DNA binding protein. In this way, the generated hybrid ssDNA-dsDNA amplicons were tagged at one end with distinct ssDNA tails to allow discrimination of the two species on the two individual test lines comprised of complementary ssDNA probes. The other end of the amplicons contained the dsDNA binding site for scCro DNA binding protein to facilitate detection with the scCro/CNPs conjugate. The performance of HRP-scCro, scCro/CNPs and HRP-scCro/CNPs conjugates regarding the detection of *Escherichia coli* bacterial DNA by PCR-LFA was compared in a previous report (Aktas et al., 2019). Even though all three approaches exhibited very similar sensitivity, the use of scCro/CNPs provided the fastest (less than 20 min) and more reliable detection with no false positives, and this conjugate was chosen for this work as well. The use of tailed primers for amplification combined with LFAs for detection via hybridization with complementary probes has also been reported previously (Jauset-Rubio et al., 2016, 2018). Isothermal recombinase polymerase amplification (RPA) was used in these reports for amplification of target DNA from the bacterial biowarfare agents *Yersinia pestis* and *Francisella tularensis*, whereas gold nanoparticles served as the colorimetric reporter achieving high sensitivity (< 1 pg of genomic DNA). However, purified amplicons were used in these studies for detection, resulting in extended time-to-result and requirement of additional material or infrastructure. In the current work, the assay was simplified by solely diluting the PCR reactions prior to analysis. Since scCro specifically binds only to dsDNA, no interference from unreacted primers was expected thus eliminating the need for amplicon purification.

For the initial validation of the primer design and to ensure correct incorporation of the tags at the two ends of the generated amplicons (ssDNA tail and dsDNA binding site for scCro), an ELONA was developed. Since the expected length of the two amplicons was similar, it was not possible to discriminate them by agarose gel electrophoresis. Therefore, the same capture probes later used for the preparation of the LFA dipsticks were employed for the ELONA which allowed the validation of the specificity of the primers and of the overall strategy for amplification and detection. Similar enzyme-based DNA hybridization assays have been developed before for other toxic algae like *Karlodinium* (Toldrà et al., 2018) and *Ostreopsis* (Toldrà et al., 2019). In these studies, ssDNA tails were used for capturing the amplicons with complementary ssDNA probes whereas a ssDNA-HRP conjugate was utilized for detection. The LODs reported for example for *Ostreopsis* (50 – 70 pg of gDNA) were more than 10-fold higher compared to the ones achieved in this work for the two *Gambierdiscus* species (1.1 – 2.6 pg). However, it is important to note that these LODs are expressed in genomic DNA amount. In *Gambierdiscus* species, the rDNA copy number per cell has been reported to be as high as 4560–21,500 (Vandersea et al., 2012) or even up to 3197,000 (Nishimura et al., 2016), probably due to the large cell size and high amount of genomic DNA. This means that the genomic DNA amount detected in this work for *Gambierdiscus* species may have a higher number of copies that of *Ostreopsis* species. Additionally, the rDNA copy number can vary between genus, species, strains, geographic origins, and even cell growth phases and thus sample harvesting times (Gaiani et al., 2021). Also, in this work, detection of the amplicons was achieved using an HRP-scCro conjugate. Because of the dimeric nature of scCro and the imperfect dyad symmetry of its specific DNA binding sequence, stoichiometries of scCro/DNA binding sites can range from 1:1 to 2:1, potentially allowing more than one HRP-scCro conjugate to associate with each amplicon and resulting in increased sensitivity. This target-independent HRP-scCro conjugate can be very useful for colorimetric assays and it could be potentially used in any assay provided that the dsDNA binding site for scCro is incorporated in the target amplicon.

As mentioned earlier, there are very few reports in the literature on the detection of harmful algae using DNA amplification combined with visual LFAs and they are summarized in Table S1. In these reports, amplification of the target microalgae species was performed with PCR, loop-mediated isothermal amplification (LAMP), RPA and rolling circle amplification (RCA), exploiting one or two labeled primers. Commercially available generic dipsticks were used for detection of the generated amplicons via hybridization or by sandwich formation with capture/reporter probes in almost all the reports. In the case of hybridization, only one labeled (biotinylated) primer was required for amplification, and the biotinylated amplicon was hybridized with a ssDNA probe labeled with a fluorophore, followed by capture on the test line using a biotin-binding biomolecule and detection with an AuNPs/anti-fluorophore IgG conjugate. On the other hand, for sandwich detection, two labeled primers were used and the biotin-fluorophore double-labeled amplicon was captured on the test line and detected again with an AuNPs/anti-fluorophore IgG conjugate. The generic LFA dipsticks used were obtained from two different companies (Ustar Biotech Ltd, China and Milenia Biotec GmbH, Germany) and were used directly for the detection of the amplicons. The reported LODs were in the range of 0.34 pg/μL – 10 ng/μL, with few exceptions reporting $\leq$ 1 fg/μL when variations of RCA were employed for the amplification step. The sensitivity achieved in this work for the two *Gambierdiscus* species with the PCR-LFA dipsticks (6.3 pg/μL) was in line with the previous reports on the other microalgal species where an isothermal amplification method was used. Two reports were found in the literature showing the combination of PCR with LFAs for the detection of toxic microalgae. In these reports, ssDNA tagged primers were used for PCR amplification and the detection of the ssDNA double-tagged DNA amplicons was performed with LFA dipsticks via hybridization with complementary DNA probes (Nagai et al., 2016; Chen et al., 2020). In one of these works, several *Alexandrium* species were detected using commercially available universal dipsticks from Kaneka Co. (Japan) and detection limits of $<$ 0.1 – 10 pg were achieved (Nagai et al., 2016). In the second work, homemade dipsticks were prepared for the detection of *Karlodinium veneficum* exhibiting a sensitivity of 91.3 pg/μL (Chen et al., 2020) in comparison to the 63 pg (6.3 pg/μL) of genomic DNA from the two *Gambierdiscus* species demonstrated in this work also exploiting homemade LFA dipsticks.

The PCR-LFA strategy developed in this work was not only sensitive but also very specific. DNA from other microalgae genera potentially present in the same habitat as the two target *Gambierdiscus* species like *Prorocentrum lima, Ostreopsis* cf. *ovata* and *Coolia monotis* as well as non-target *Gambierdiscus* (*G. balechii, G. belizeanus, G. caribaeus*) or *Fukuyoa* (*F. paulensis*) species did not produce positive signals on the dipsticks. This is not only due to the high specificity of the primers but also the specific DNA binding properties of the scCro reporter protein. The analysis of mixtures containing equal concentrations of genomic DNA from non-target *Gambierdiscus* species further demonstrated the specificity of the approach since positive signals on the dipsticks were obtained only when the target species were present. These samples were analyzed in an effort to mimic field samples potentially containing more than one microalgae species. These findings further highlight the importance of the strategy shown herein which is highly specific and does not produce any false positive signals. Research efforts should focus on the validation of the PCR-LFA system for the analysis of field samples and compared to light microscopy. Nevertheless, the results obtained in this work demonstrate that the strategy should be implementable in situ monitoring and research activities, since portable PCR devices can be brought to the field, whereas LFA is easy and fast to perform, and results are easy to interpret by simple visual inspection.

5. Conclusions

In this work, a PCR-LFA dipstick was developed for the simultaneous detection of the toxin-producing *G. australes* and *G. excentricus* species. Duplex PCR amplification of genomic DNA from these species with specifically modified primers allowed facile visual detection with the dipsticks employing a scCro/CNPs conjugate generating black colored line signals. The strategy was highly sensitive whereas the presence of non-target *Gambierdiscus* species or other microalgae genera potentially co-habiting in the same waters did not interfere with the assay. The approach combines the specificity and sensitivity provided by PCR amplification with the simplicity, low cost and on-site testing compatibility of LFAs. Preliminary results demonstrating DNA amplification directly from cells instead of purified genomic DNA and subsequent detection with the LFA dipsticks suggests that the approach could be applied to field sample testing, further highlighting its potential to be implemented in monitoring programs.

Declaration of Competing Interest

The authors declare that there is no conflict of interest.

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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.2021.102135.

References

Aktas, G.B., Skouridou, V., Masip, L., 2015. Novel signal amplification approach for HRP-based colorimetric genosensors using DNA binding protein tags. Biosens. Bioelectron. 74, 1005–1010.
Aktas, G.B., Wichers, J.H., Skouridou, V., van Amerongen, A., Masip, L., 2019. Nucleic acid lateral flow assays using a conjugate of a DNA binding protein and carbon nanoparticles. Microchim. Acta 186, 426.
Aligizaki, K., Nikolaidis, G., 2008. Morphological identification of two tropical dinoflagellates of the genera *Gambierdiscus* and *Sinophysis* in the Mediterranean Sea. J. Biol. Res.-Thessalon. 9, 75–82.
Aveyard, J., Mehrabi, M., Cossins, A., Braven, H., Wilson, R., 2007. One step visual detection of PCR products with gold nanoparticles and a nucleic acid lateral flow (NALF) device. Chem. Commun. 4251–4253.
Bahadir, E.B., Sezgintürk, M.K., 2016. Lateral flow assays: principles, designs and labels. Trac. Trends Anal. Chem. 82, 286–306.
Caillaud, A., de la Iglesia, P., Barber, E., Eixarch, H., Mohammad-Noor, N., Yasumoto, T., Diogéne, J., 2011. Monitoring of dissolved ciguatoxin and maitotoxin using solid-phase adsorption toxin tracking devices: application to *Gambierdiscus pacificus* in culture. Harmful Algae 10, 433–446.
Chen, Q., Zhang, C., Liu, F., Ma, H., Wang, Y., Chen, G., 2020. Easy detection of *Karlodinium veneficum* using PCR-based dot chromatography strip. Harmful Algae 99, 101908.
Ebenezer, V., Medlin, L.K., Ki, J.S., 2012. Molecular detection, quantification, and diversity evaluation of microalgae. Mar. Biotechnol. 14, 129–142.
El-Tholoth, M., Branavan, M., Naveenathayalan, A., Balachandran, W., 2019. Recombinase polymerase amplification-nucleic acid lateral flow immunoassays for Newcastle disease virus and infectious bronchitis virus detection. Mol. Biol. Rep. 46, 6391–6397.
Fraga, S., Rodríguez, F., Caillaud, A., Diogène, J., Raho, N., Zapata, M., 2011. *Gambierdiscus excentricus* sp. nov. (Dinophyceae), a benthic toxic dinoflagellate from the Canary Islands (NE Atlantic Ocean). Harmful Algae 11, 10–22.
Friedman, M., Fernandez, M., Backer, L., Dickey, R., Bernstein, J., Schrank, K., Kibler, S., Stephan, W., Gribble, M.O., Bienfang, P., Bowen, R.E., Degrasse, S., Quintana, H.A. F., Loeffler, C.R., Weisman, R., Blythe, D., Berdalet, E., Ayyar, R., Clarkson-Townsend, D., Swajian, K., Benner, R., Brewer, T., Flemming, L.E., 2017. An updated Review of Ciguatera Fish Poisoning: clinical, Epidemiological, Environmental, and Public Health Management. Mar. Drugs 15, 72.
Gaiani, G., Leonardo, S., Tudó, A., Toldrà, A., Rey, M., Andree, K.B., Tsumuraya, T., Hirama, M., Diogéne, J., O'Sullivan, C.K., Campàs, M., 2020. Rapid detection of ciguatoxins in *Gambierdiscus* and *Fukuyoa* with immunosensing tools. Ecotoxicol. Environ. Saf. 204, 111004.
Gaiani, G., Toldrà, A., Andree, K.B., Rey, M., Diogéne, J., Alcaraz, C., O'Sullivan, C.K., Campàs, M., 2021. Detection of *Gambierdiscus* and *Fukuyoa* single cells using recombinase polymerase amplification combined with a sandwich hybridization assay. J. Appl. Phycol. 33, 2273–2282.
Gui, C., Wang, K., Li, C., Dai, X., Cui, D., 2014. A CCD-based reader combined with CdS quantum dot-labeled lateral flow strips for ultrasensitive quantitative detection of CagA. Nanoscale Res. Lett. 9, 57.
Hamilton, B., Hurbungs, M., Vernoux, J.P., Jones, A., Lewis, R.J., 2002. Isolation and characterisation of Indian Ocean ciguatoxin. Toxicon 40, 685–693.
He, Y., Zhang, S., Zhang, X., Baloda, M., Gurung, A.S., Xu, H., Zhang, X., Liu, G., 2011. Ultrasensitive nucleic acid biosensor based on enzyme-gold nanoparticle dual label and lateral flow strip biosensor. Biosens. Bioelectron. 26, 2018–2024.
Jauset-Rubio, M., Svobodová, M., Mairal, T., McNeil, C., Keegan, N., Saeed, A., Abbas, M. N., El-Shahawi, M.S., Bashammakh, A.S., Alyoubi, A.O., O'Sullivan, C.K., 2016. Ultrasensitive, rapid and inexpensive detection of DNA using paper based lateral flow assay. Sci. Rep. 6, 37732.
Jauset-Rubio, M., Tomaso, H., El-Shahawi, M.S., Bashammakh, A.S., Al-Youbi, A.O., O'Sullivan, C.K., 2018. Duplex lateral flow assay for the simultaneous detection of Yersinia pestis and Francisella tularensis. Anal. Chem. 90, 12745–12751.
Kaufmann, M., Böhm-Beck, M., 2013. *Gambierdiscus* and related benthic dinoflagellates from Madeira archipelago (NE Atlantic). Harmful Algae News 47, 18–19.
Lazcka, O., Del Campo, F.J., Muñoz, F.X., 2007. Pathogen detection: a perspective of traditional methods and biosensors. Biosens. Bioelectron. 22, 1205–1217.
Leonardo, S., Gaiani, G., Tsumuraya, T., Hirama, M., Turquet, J., Sagristà, N., Rambla-Alegre, M., Caixach, J., Diogéne, J., O'Sullivan, C.K., Alcaraz, C., Campàs, M., 2020. Addressing the analytical challenges for the detection of ciguatoxins using an electrochemical biosensor. Anal. Chem. 92, 4858–4865.
Lewis, R.J., 2001. The changing face of ciguatera. Toxicon 39, 97–106.
Linares, E.M., Kubota, L.T., Michaelis, J., Thalhammer, S., 2012. Enhancement of the detection limit for lateral flow immunoassays: evaluation and comparison of bioconjugates. J. Immunol. Methods 375, 264–270.
Litaker, R.W., Holland, W.C., Hardison, D.R., Pisapia, F., Hess, P., Kibler, S.R., Tester, P. A., 2017. Ciguatoxicity of *Gambierdiscus* and *Fukuyoa* species from the Caribbean and Gulf of Mexico. PLoS ONE 12, e0185776.
Liu, X., Wu, W., Cui, D., Chen, X., Li, W., 2021. Functional micro-/nanomaterials for multiplexed biodetection. Adv. Mater. 33, 2004734.
Lozano-Duque, Y., Richlen, M.L., Smith, T.B., Anderson, D.M., Erdner, D.L., 2018. Development and validation of PCR-RFLP assay for identification of *Gambierdiscus* species in the Greater Caribbean region. J. Appl. Phycol. 30, 3529–3540.
Lyu, Y., Richlen, M.L., Sehein, T.R., Chinain, M., Adachi, M., Nishimura, T., Xu, Y., Parsons, M.L., Smith, T.B., Zheng, T., Anderson, D.M., 2017. LSU rDNA based RFLP assays for the routine identification of *Gambierdiscus* species. Harmful Algae 66, 20–28.
Mao, X., Wang, W., Du, T.E., 2013. Dry-reagent nucleic acid biosensor based on blue dye doped latex beads and lateral flow strip. Talanta 114, 248–253.
McPartlin, D.A., Loftus, J.H., Crawley, A.S., Silke, J., Murphy, C.S., O'Kennedy, R.J., 2017. Biosensors for the monitoring of harmful algal blooms. Curr. Opin. Biotechnol. 45, 164–169.
Medlin, L.K., Orozco, J., 2017. Molecular techniques for the detection of organisms in aquatic environments, with emphasis on harmful algal bloom species. Sensors 17, 1184.
Nagai, S., Miyamoto, S., Ino, K., Tajimi, S., Nishi, H., Tomono, J., 2016. Easy detection of multiple Alexandrium species using DNA chromatography chip. Harmful Algae 51, 97–106.
Nishimura, T., Hariganeya, N., Tawong, W., Sakanari, H., Yamaguchi, H., Adachi, M., 2016. Quantitative PCR assay for detection and enumeration of ciguatera-causing dinoflagellate *Gambierdiscus* spp. (Gonyaulacales) in coastal areasa of Japan. Harmful Algae 52, 11–22.
Noguera, P., Posthuma-Trumpie, G.A., van Tuil, M., van der Wal, F.J., de Boer, A., Moers, A.P.H.A., van Amerongen, A., 2011. Carbon nanoparticles in lateral flow methods to detect genes encoding virulence factors of Shiga toxin-producing Escherichia coli. Anal. Bioanal. Chem. 399, 831–838.
Pasinszki, T., Lako, J., Dennis, T., 2020. Advances in detecting ciguatoxins in fish. Toxins (Basel) 12, 494.
Pitz, K.J., Richlen, M.L., Fachon, E., Smith, T.B., Parsons, M.L., Anderson, D.M., 2021. Development of fluorescence in situ hybridization (FISH) probes to detect and enumerate *Gambierdiscus* species. Harmful Algae 101, 101914.
Posthuma-Trumpie, G.A., Wichers, J.H., Koets, M., Berendsen, L.B.J.M., van Amerongen, A., 2012. Amorphous carbon nanoparticles: a versatile label for rapid diagnostic (immuno)assays. Anal. Bioanal. Chem. 402, 593–600.
Reverté, L., Soliño, L., Carnicer, O., Diogéne, J., Campàs, M., 2014. Alternative methods for the detection of emerging marine toxins: biosensors, biochemical assays and cell-based assays. Mar. Drugs 12, 5719–5763.
Reverté, L., Toldrà, A., Andree, K.B., Fraga, S., de Falco, G., Campàs, M., Diogéne, J., 2018. Assessment of cytotoxicity in ten strains of *Gambierdiscus australes* from Macaronesian Islands by neuro-2a cell-based assays. J. Appl. Phycol. 30, 2447–2461.
Rodríguez, F., Fraga, S., Ramilo, I., Rial, P., Figueroa, R.I., Riobó, P., Bravo, I., 2017. Canary Islands (NE Atlantic) as a biodiversity 'hotspot' of *Gambierdiscus*: implications for future trends of ciguatera in the area. Harmful Algae 67, 131–143.
Rossignoli, A.E., Tudó, A., Bravo, I., Díaz, P.A., Diogène, J., Riobó, P., 2020. Toxicity characterisation of *Gambierdiscus* species from the Canary Islands. Toxins (Basel) 12, 134.
Takalkar, S., Baryeh, K., Liu, G., 2017. Fluorescent carbon nanoparticle-based lateral flow biosensor for ultrasensitive detection of DNA. Biosens. Bioelectron. 98, 147–154.
Global distribution of the genera Gambierdiscus and Fukuyoa Tester, P., Wickliffe, L., Jossart, J., Rhodes, L., Enevoldsen, H., Adachi, M., Nishimura, T., Rodriguez, F., Chinain, M., Litaker, W., 2020. Global distribution of the genera Gambierdiscus and Fukuyoa. In: Hess, P. (Ed.), Harmful Algae 2018 – from ecosystems to socioecosystems. Proceedings of the 18th Intl. Conf. on Harmful Algae. International Society for the Study of Harmful Algae, Nantes, pp. 138–143.
Toldrà, A., Jauset-Rubio, M., Andree, K.B., Fernández-Tejedor, M., Diogéne, J., Katakis, I., O'Sullivan, C.K., Campàs, M., 2018. Detection and quantification of the toxic marine microalgae *Karlodinium veneficum* and *Karlodinium armiger* using recombinase polymerase amplification and enzyme-linked oligonucleotide assay. Anal. Chim. Acta 1039, 140–148.
Toldrà, A., Alcaraz, C., Andree, K.B., Fernández-Tejedor, M., Diogéne, J., Katakis, I., O'Sullivan, C.K., Campàs, M., 2019. Colorimetric DNA-based assay for the specific detection and quantification of *Ostreopsis* cf. *ovata* and *Ostreopsis* cf. *siamensis* in the marine environment. Harmful Algae 84, 27–35.
Toldrà, A., O'Sullivan, C.K., Diogène, J., Campàs, M., 2020. Detecting harmful algal blooms with nucleic acid amplification-based biotechnological tools. Sci. Total Environ. 749, 141605.
Tsumuraya, T., Fujii, I., Hirama, M., 2014. Preparation of anti-ciguatoxin monoclonal antibodies using synthetic haptens: sandwich ELISA detection of ciguatoxins. J. AOAC Intl. 97, 373.
Tudó, A., Toldrà, A., Andree, K.B., Rey, M., Fernández-Tejedor, M., Campàs, M., Diogène, J., 2018. First report of Gambierdiscus in the Western Mediterranean Sea (Balearic Islands). Harmful Algae News 59, 22–23.
Tudó, A., Gaiani, G., Ray, Varela, M, Tsumuraya, T, Andree, K.B, Fernández-Tejedor, M, Campàs M., Diogéne, J., 2020. Further advances of *Gambierdiscus* species in the Canary Islands, with the first report of *Gambierdiscus belizeanus*. Toxins (Basel) 12, 692.
Vandersea, M.W., Kibler, S.R., Holland, W.C., Tester, P.A., Schultz, T.F., Faust, M.A., Holmes, M.J., Chinain, M., Litaker, R.W., 2012. Development of semi-quantitative PCR assays for the detection and enumeration of *Gambierdiscus* species (Gonyaulacales, Dinophyceae). J. Phycol. 48, 902–915.

Velusamy, V., Arshak, K., Korostynska, O., Oliwa, K., Adley, C., 2010. An overview of foodborne pathogen detection: in the perspective of biosensors. Biotechnol. Adv. 28, 232–254.

Yan, W., Wang, K., Xu, H., Huo, X., Jin, Q., Cui, D., 2019. Machine learning approach to enhance the performance of MNP-labeled lateral flow immunoassay. Nano-Micro Lett 11, 7.

Yasumoto, T., 2005. Chemistry, etiology, and food chain dynamics of marine toxins. Proc. Jpn. Acad. 81, 43–51.

Zheng, C., Wang, K., Zheng, W., Cheng, Y., Li, T., Cao, B., Jin, Q., Cui, D., 2021. Rapid development in lateral flow immunoassay for nucleic acid detection. Analyst 146, 1514–1528.

Annex 6

UNIVERSITAT ROVIRA I VIRGILI
DEVELOPMENT OF BIOANALYTICAL DEVICES FOR THE DETECTION OF CIGUATOXINS AND THE CIGUATOXIN PRODUCING
GENERA GAMBIERDISCUS AND FUKUYOA
Greta Gaiani

New information about the toxicological profile of *Prorocentrum panamense* (Prorocentrales, Dinophyceae) and its global distribution

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Abstract

Dinoflagellates of the genera *Prorocentrum* and *Dinophysis* are known producers of toxic compounds belonging to the okadaic acid (OA) group. The ingestion of shellfish contaminated with these toxins cause a human disease named diarrheic shellfish poisoning (DSP). In this study, the first record of *Prorocentrum panamense*, a potential toxin-producer species, was reported in the Canary Islands together with its toxicological characterization. *Prorocentrum panamense* cells were collected during April 2017 from natural pools located in the Northeastern part of Gran Canaria. This new record represents an expansion of *P. panamense* distribution area, previously restricted to the Pacific Ocean, Indian Ocean, Arabian Gulf and the Caribbean, and its introduction mechanisms is discussed. Laboratory cultures of *P. panamense* were settled and toxin production was assessed in both cell pellets and culture media at four different growth phases (latency, exponential, early stationary and late stationary) implementing LC-MS/MS and neuro-2a cell-based assay (CBA). LC-MS/MS allowed the identification of OA in the fraction corresponding to the late stationary phase, and tests performed on neuro-2a cells showed, for most of the fractions, OA-like activity observable by both cell morphology changes and cell mortality. This information is fundamental for a better understanding of the genus *Prorocentrum* global distribution, its ecology and risks associated to toxic producing species.

1. Introduction

Diarrheic shellfish poisoning (DSP) is a foodborne disease reported in several areas worldwide (Gestal et al. 2008). It is associated with the consumption of bivalves contaminated with toxins of the okadaic acid (OA) group, mainly produced by dinoflagellates of the genera *Prorocentrum* and *Dinophysis* (Yasumoto et al. 1985). Before implementing efficient monitoring programs, DSP outbreaks affected a large number of people, causing the closing of shellfish harvesting areas even for several months (Economou et al. 2007), resulting in dramatic losses in both the aquaculture and fisheries sectors. The toxins responsible for DSP include OA, dinophysistoxin-1 (DTX-1) and

dinophysistoxin-2 (DTX-2). Their mechanism of action is based on phosphatase inhibition, which can interfere with several mammalian physiological processes, such as the cell cycle regulation and the metabolism of intracellular protein, potentially causing the inflammation of the intestinal tract (*i.e.*, abdominal pain, vomiting) and diarrhea (Yasumoto et al. 1985). Furthermore, OA and DTX-1 have tumor promoting activity (Fujiki et al. 1991). Currently, the genus *Prorocentrum* is composed of 80 species, divided among planktonic and epibenthic species (Hoppenrath et al. 2013). Furthermore, the taxonomic status of some species is in flux, because the *Prorocentrum* genus presents a large variety in terms of cell shape, length and width, number and shape of lateral plates and marginal pores, thus making difficult species identification with microscope techniques (Aligizaki et al. 2009). Recent progress in molecular techniques has improved species identification and has contributed to the clarification of taxonomy of this genus. Currently, within the genus, 6 planktonic and 9 epibenthic species form high-biomass blooms and are considered potentially harmful (Glibert et al. 2012). Among the six planktonic species, only *P. minimum* has been described as potentially toxic (Glibert et al. 2012; Grzebyk et al. 1998). Instead, all the benthic species have been described as toxic, with *Prorocentrum lima* (Ehrenberg) F.Stein 1878 being the most toxic (Moreira-González et al. 2019). However, since the genus *Prorocentrum* counts a huge number of species, it is probable that there may be more unidentified harmful species in addition to the currently described (Glibert et al. 2012). Some species of the genus *Prorocentrum* have a global distribution, such as *Prorocentrum emarginatum* Y. Fukuyo 1981, *Prorocentrum mexicanum* Osorio-Tafall 1942*, Prorocentrum hoffmannianum* M. A. Faust 1990 and *P. lima* (Glibert et al. 2012). Nevertheless, in the last years, other species that were previously considered as endemic to certain areas have been reported in other regions, exhibiting an extremely disjunct global distribution. This is the case of the epibenthic *Prorocentrum panamense* D. Grzebyk, Y. Sako & B. Berland 1998, which was identified and described from the waters of Contadora Island on the Pacific coast of Panama in 1998, and since then, it has been recorded in La Réunion (Indian Ocean), Martinique (Caribbean Sea), Revillagigedo Islands (Mexican Pacific Ocean), and Hainan in the northern South China Sea (see review in Chomérat et al. (2019)). In the past decade, several microalgae species associated to toxin production have been newly recorded in the Canaries, and the majority of them are dinoflagellates (Fraga et al. 2011; Rodríguez et al. 2018). In fact, the particular position of the Canary Islands makes the archipelago a key point for marine transport routes (Tichavska and Tovar 2015), and its peculiar environmental conditions (Glibert et al. 2012) can facilitate the settlement of microalgae species introduced via marine transport (Hallegraeff 1998). In this work, the first record of *P. panamense* in the Canary

Islands is reported, together with the toxicological characterization by LC-MS/MS and CBA. This information is crucial to better understand the global distribution, ecology and risks associated to species of the genus *Prorocentrum*.

2. Materials and methods

2.1. Field sampling and microalgal cultures

Macroalgae samples were collected in April 2017 from three natural rock pools in Las Salinas de Agaete (28° 6' 24.120" N, 15° 42' 40.140" W), Northwestern Gran Canaria (Figure 1). Samples, of *ca*. 150-200 g fresh weight of macroalgae (fwm, hereafter) were collected, placed in polystyrene bottles containing 1 L of seawater, vigorously shaken for 1 min to release epiphytic microalgae, and filtered (200-µm mesh) to remove gross material. From each sample, 100 mL were fixed in 3% of Lugol's iodine solution for species identification and cell counting, and of the remaining 900 mL, 100 mL were used for cells isolation purpose. Samples were kept at room temperature and close to a natural source of light for a maximum of 3 days. Then, they were shipped to IRTA and stored in the incubator (24 °C) upon laboratory procedures. In the laboratory, cells were isolated with a glass pipette under an inverted microscope (Leica, DMIL) by the capillary method (Stein et al. 1973), and cultivated in untreated Nunc 24 well plate (Themo Fisher Scientific) containing filtered and autoclaved local seawater (salinity adjusted at 36 psu) supplemented with modified ES medium (Provasoli 1968). Cells were grown at 24 °C under a 12:12 light/dark cycle with a photon irradiance of 110-µmol photons m^{-2} s^{-1}. Once cultures reached approximately 20–35 cell mL^{-1}, they were transferred to 28 mL round bottom glass tubes. After acclimation to laboratory conditions, four 225 cm^2 cell culture flasks (500 mL volume, vented cap) were prepared with 150 cell mL^{-1} each, from the same mother culture. Aliquots of cell cultures were stained with Calco-fluor White M2R (Sigma Aldrich, Spain) (Fritz and Triemer 1985)and identified to species level under a light microscope (LEICA DMLB). Morphological features were determined according to Hoppenrath et al. (2013). Microphotographs were taken with an Olympus DP-70 camera at different magnification levels.

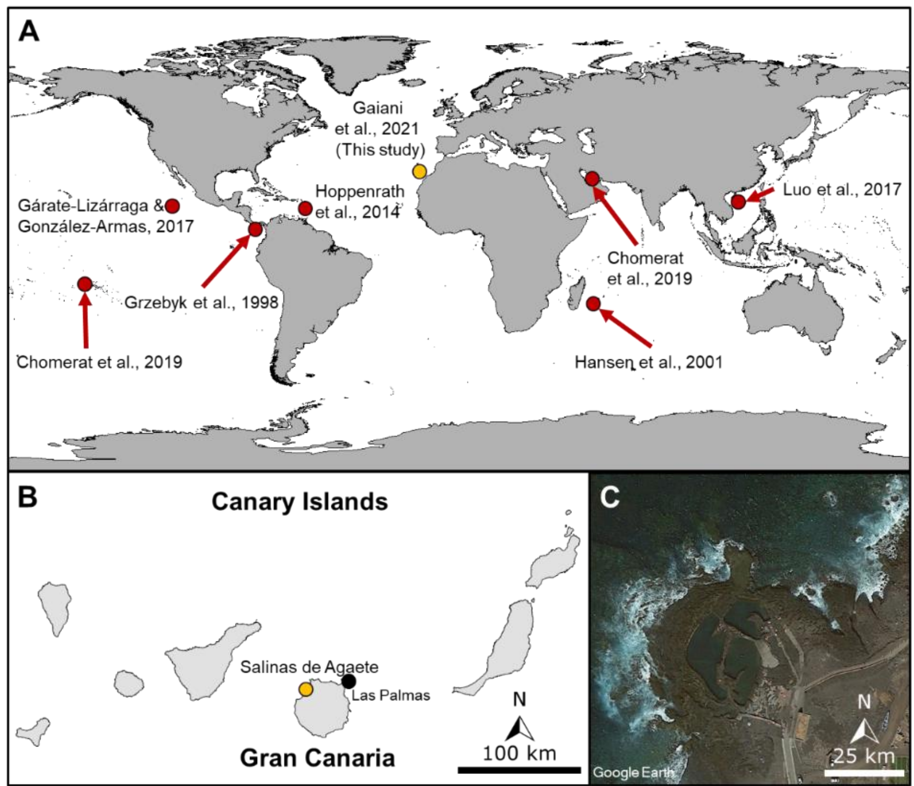

Figure 8. (A) Global distribution of *P. panamense.* Red circles indicate previous records. Yellow circle indicates first record presented in this paper. (B) Sampling site in Gran Canaria where *P. panamense* was found (Salinas de Agaete). (C) © 2020 Google Earth vision of Salinas de Agaete.

2.2 Molecular Analysis

Molecular identification of the species was performed on genomic DNA isolated and purified from 50 mL aliquots of cultures in the stationary phase, following the phenol/chloroform procedure as described in Toldrà et al. (2018). The D1-D3 region of the 28S LSU rRNA gene was amplified by polymerase chain reaction (PCR) using the primers D1R (5'-ACCCGCTGAATTTAAGCATA-3') and D2C (5'-CCTTGGTCCGTGTTTCAAGA-3') (Chomérat et al. 2010). PCR was performed in a 25 µL reaction containing 2.5 µL of 1 × PCR buffer, 1 µL of 2 mM $MgCl_2$, 1.5 µL of 600 µM dNTPs, 0.25 µL of each primer at a final concentration of 0.2 µM, 0.20 µL of 1 U Taq DNA polymerase (Invitrogen™, Thermo Fisher™, Massachusetts, U.S.A), 1.25 µL of 5% dimethyl sulfoxide (DMSO), 2 µL of template DNA at a concentration of 1 ng μL^{-1}, and sterile water to a final volume of 25 µL. The PCR amplification was performed in a Nexus Gradient Thermal Cycler (Eppendorf Ibérica, Madrid, Spain) for an initial denaturation step of 94 °C for 5 min, followed by 40 cycles of 95 °C for 30 s, 54 °C for 30 s, and 72 °C for 1 min, and then a final elongation step of 72

°C for 5 min. Four µL of each PCR product were separated by electrophoresis (1% TAE, 60 V), stained with ethidium bromide and checked under UV-illumination. PCR products were purified using a Thermo Scientific GeneJET PCR purification Kit (Thermo Fisher™, Massachusetts, U.S.A) following manufacturer's instruction. The resulting purified product was sequenced in both directions at Sistemas Genómicos (LLC, Valencia, Spain). Obtained sequence was manually checked and edited using BioEdit v7.0.5.2 (Hall 1999). To assess the evolutionary relationship between the obtained sequence and *Prorocentrum* species sampled globally, we retrieved a set of 36 sequences belonging to 18 different species from GenBank (see Figure S1). *Adenoides eludens* was used as outgroup. Sequences alignments were performed using MUSCLE algorithm implemented in MEGA X (v10.0.5), and the phylogenetic relationships were estimated by Maximum Likelihood (ML) using RaxML v.8 (Stamatakis 2014) and Bayesian Inference (BI) using Mr. Bayes v.3.2.2 (Huelsenbeck and Ronquist 2001). Initial tree(s) for the heuristic search were obtained automatically by applying Neighbor-Join and BioNJ algorithms to a matrix of pairwise distances estimated using the Maximum Composite Likelihood (MCL) approach, and then selecting the topology with superior log likelihood value. There was a total of 334 positions in the final dataset. A discrete Gamma distribution was used to model evolutionary rate differences among sites (5 categories (+ *G*, parameter = 0.5083)).

Phylogenetic relationships were inferred by using the Maximum Likelihood method and Tamura-Nei model. The rate variation model allowed for some sites to be evolutionarily invariable ([+*I*], 0.00% sites). All positions containing gaps and missing data were eliminated.

2.3. Toxin extraction

Each of the four *Prorocentrum* replicates was harvested at four different phases of the culture growth namely: latency phase (2 days, 225 cell mL^{-1}), exponential phase (14 days, 3650 cell mL^{-1}), beginning of the stationary phase (23 days, 5570 cell mL^{-1}), and end of the stationary phase (34 days, 5390 cell mL^{-1}). Pellets were obtained by splitting the entire bottle volume in 50 mL falcon tubes and centrifuging them at 3700 g for 25 min (Alegra X-15R, Beckman Coulter). The obtained supernatants from each 50 mL tube were pooled together according to the harvesting phase (the resulting fractions are referred as "culture media" from here on). Both pellet and culture media from each harvesting phase were analyzed for toxin presence. Cell pellets were extracted with 10 mL of pure methanol and sonicated for 30 minutes (three times) at an amplitude of 37%, 3 sec on/2 sec off, using a 3 mm diameter sonicator probe (Watt ultrasonic processor VCX 750, Newton, USA). Cell disruption after each sonication was evaluated under microscopy. Once solvent was evaporated, the residue was re-suspended in 500 µL of methanol, vortexed, filtered with a 0.2 µm PTFE filter, and transferred to autosampler vials. The

culture media corresponding to the four harvesting stages, instead, underwent a solid phase extraction (SPE). Briefly, the entire volume of each harvested stage (500 mL) was filtered throw an Empore C18 disk (Sigma-Aldrich, Spain) to retain the toxins, which were afterwards eluted with 10 mL of pure methanol. After solvent was evaporated, residue was re-suspended in 500 µL of methanol, vortexed, filtered with 0.2 µm PTFE filter, and transferred to autosampler vials. All samples were stored at -20 ºC until toxin analysis.

2.4. LC-MS/MS analysis

Certified reference materials (CRMs) of okadaic acid (OA), dinophysistoxin-1 (DTX-1), dinophysistoxin-2 (DTX-2), yessotoxin (YTX), homoyessotoxin (hYTX), pectenotoxin-2 (PTX-2), azaspiracid-1 (AZA-1), azaspiracid-2 (AZA-2), azaspiracid-3 (AZA-3), 13-desmethylspirolide C (SPX-1), gymnodimine A (GYM-A) and pinnatoxin G (PnTX-G) were obtained from the National Research Council of Canada (NRC, Halifax, NS, Canada). LC-MS/MS analysis of marine lipophilic toxins was performed following the method described in García-Altares et al. (2013). Briefly, an Agilent 1200 LC (Agilent Technologies, USA) was coupled to a 3200 QTRAP mass spectrometer (Applied Biosystems, USA) through a TurboSpray® ion source operating at atmospheric pressure. Toxins were separated on a XBridge BEH C8 2.5 µm 2.1 × 50 mm column (Waters). A binary gradient was programmed with water (mobile phase A) and acetonitrile/water (mobile phase B), both containing 6.7 mM of ammonium hydroxide All runs were carried out at 30 ºC using a flow rate of 500 µL min^{-1}. The injection volume was 10 µL and the auto-sampler was set at 4 ºC. All lipophilic toxins were analyzed in both negative (-ESI) and positive polarity (+ESI), selecting two product ions per toxin to allow the quantification (the most intense transition) and confirmation; identification was supported by toxin retention time and the multiple reaction monitoring (MRM) ion ratios. An external standard calibration curve was prepared with a six-level curve, from 4 to 40 ng toxin mL^{-1} for OA. The minimum performance criteria were checked out throughout the study such as retention time deviation ± 0.2 min, peak area deviation (RSD ≤ 3.0%), linearity ($R^2 \geq 0.98$), sensitivity (individual toxin LOD should be equal or lower than 1:20th of regulatory level), precision intra-batch ≤ 20% and inter-batch ≤ 25%. All samples were analyzed in duplicate.

2.5 Neuro-2a CBA

CBA are routinely used to study the effect of bioactive compounds. In fact, the alteration of homeostasis caused by these compounds, such as physiological cell disruption and cell mortality, can be easily observed and measured. Indeed, CBA is commonly used for the identification of marine toxins that affect food safety, including OA (see for instance Cañete and Diogène 2008; Diogène et al. 1995; Huynh-Delerme et al. 2003). Experimental conditions followed the procedures described in Cañete and Diogène (2008), with minor modifications. Briefly, for the assay on the evaluation of cells morphology changes, cultivated

neuroblastoma neuro-2a cells were seeded into 96-well plates at an initial density of 40,000 cells well^{-1}. After 24 h, cells were exposed to a 90 mM OA standard solution (positive control), phosphate saline buffer (PBS, negative control), and the *P. panamense* culture extracts (both microalgae pellets and culture media extracts) collected at the four growing stages. Toxin standards and culture extracts were previously evaporated to remove methanol completely, and subsequently re-suspended in a 5% fetal bovine serum (FBS) medium. Then, samples were serially diluted, and initial pellet extracts exposure concentrations were 1.0×10^4 (latency phase), 1.5×10^5 (exponential phase), 2.0×10^5 (early stationary phase), and 1.8×10^5 cell equivalents mL^{-1} (late stationary phase) for the four harvested phases, respectively. Culture media extracts exposure concentration was 2.4 mL culture media equivalents mL $^{-1}$ for the four phases. After 4 h of exposure, the changes in cell morphology were observed under a light microscope (NIKON ECLIPSE TE2000-S), and cells were photographed using phase contrast. All conditions were tested in triplicate. In addition, a semi-quantitative evaluation of *P. panamense* toxicity was performed following the CBA described by Soliño et al. (2015). Cultivated neuro-2a cells were exposed to *P. panamense* pellets and culture media extracts collected at the four harvested phases (4 serial ½ dilutions). Initial pellet exposure concentrations for this test were 1.2×10^4 (latency phase), 1.7×10^5 (exponential phase), 2.4×10^5 (early stationary phase) and 2.1×10^5cell equivalent/mL (late stationary phase) for the four harvested phases, respectively. Initial culture media extracts exposure concentration was 2.7 mL culture media equivalents mL^{-1} for the four phases. Cells were incubated for 24 h, and viability was assessed by the MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assay (Manger et al. 1993). This compound is converted to insoluble formazan crystals by mitochondrial dehydrogenase activity. This activity can be performed only by live cells and results in a violet color that can be measured by a spectrophotometer at 570 nm. All conditions were tested in triplicate.

3. Results and Discussion

3.1. Molecular identification

Isolated cells presented the particular asymmetrical heart shaped form (Hoppenrath et al., 2013), asymmetrical lateral plates and round posterior margin, and measured 46–52 µm in length and 43–46 µm in width (Figure 2), as originally described by Grzebyk et al. (1998). Furthermore, calcofluor white stained cells showed the reticulate-foveate thecal surface with depressions that become shallower towards the plate center (Figure 2). The internal part of this structure presents several pores, only visible in the SEM image (Hoppenrath et al. 2013; Luo et al. 2017), and it is interpreted as a synapomorphy of the *P. panamense* group. Most importantly, cells presented a large sieve like structure close to the thecal margin (Figure 2A), a unique feature of *P. panamense* and *P. pseudopanamense* species. Stained cells also presented the linear periflagellar area (Figure 2B), a typical trait of the *P. panamense* species.

Finally, molecular analysis showed that IRTA-SMM-17-72 (deposited in GenBank with the code MW600273) branches in a subgroup of the other *P. panamense* species (Figure S1). It must be underlined that there are only 4 sequenced strains of *P. panamense,* three of them correspond to the same locality (i.e. Martinique Island; IFR12-210, IFR12-212 and IFR12-218), and one from South China Sea (TIO97). Thus, available data probably not represent the entire genetic variance existing in the D1-D3 region of 28S LSU rDNA. The only species that could arise doubt about the correct species attribution of the strain described in this work is *P. pseudopanamense* Chomérat & Nézan, since studies on the SSU rDNA showed that is genetically close to *P. panamense* (Chomérat et al. 2011). Nevertheless, Chomérat and coworkers Chomérat et al. (2019) stated that *P. panamense* species shape is very peculiar and morphologically easy to recognize and it can be clearly distinguished from *P. pseudopanamense*, which is less asymmetrical and never heartshaped (Hoppenrath et al. 2013; Chomerat et al. 2019).

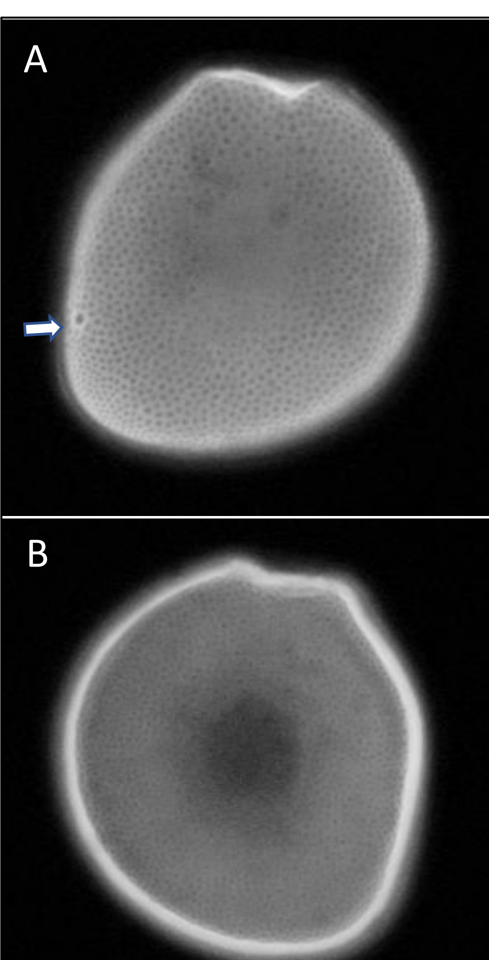

Figure 9. Cell view of *P. panamense* after calcofluor-white staining. It is possible to observe the asymmetrical shape and reticulate-foveate thecal surface. (A) Left thecal view. Arrow indicates the marginal pore. (B) Right thecal view. Linear periflagellar area can be observed. Scale bar is 20 µm.

Knowledge of the ecology of benthic *Prorocentrum* species is very limited, and this is due mostly to the difficulties to discriminate among them in benthic samples (Glibert et al. 2012; Hoppenrath et al. 2013). Species of the dinoflagellate genus *Prorocentrum* mainly occur in marine and brackish waters worldwide. Specifically, the type locality of *P. panamense* is Contadora Island, on the Pacific side of the Gulf of Panama (Grzebyk et al. 1998), but in the last decade its presence has been reported in La Réunion Island, Martinique Island, Revillagigedo Islands (México), China, French Polynesia and in the Arabian Gulf (Hansen et al. 2001; Hoppenrath et al. 2014; Lizárraga and González-Armas 2017; Chomérat et al. 2019). Thus, its presence in the Canary Islands is the first record of *P. panamense* in the central eastern Atlantic Ocean. *Prorocentrum panamense* exhibits an extraordinary disjunct global distribution pattern, with a low level of intraspecific genetic variation in the LSU region (Chomérat et al. 2019). Furthermore, the lack of previous records of this species in the Canary Islands is strongly supported by recent surveys that failed to report the presence of the species in the region (Fraga et al. 2011; Rodriguez et al. 2018). Although this absence may be due to species misidentification, from a morphological perspective this species is very peculiar and easy to recognize and identify (Chomérat et al. 2019). Thus, its presence in the region could have happened naturally, we consider that a human-mediated introduction may be occurred. The method of introduction is unknown, but the transport through ballast water is considered the most

probable vector of introduction. Coastal ship traffic constitutes an effective introduction vector for aquatic organisms (Roy et al. 2012), and ballast water from shipping has been considered responsible for the introduction of several benthic dinoflagellate species in some countries (*e.g.*, Australia, Canada, Japan), sometimes with dramatic economic consequences to aquaculture, fisheries and tourism (Hallegraeff, 1998; Roy et al. 2012). Ballast waters have been identified as a potential source for dinoflagellate species introduction (Hallegraeff and Bolch, 1992), and the Canary Islands play an important role in the global marine transport. Las Palmas Port is a major logistic platform between Europe, Africa and America, with a cargo hub over 19 million tons, being a leading worldwide bunker trader (Tichavska and Tovar, 2015). In addition, ballast water from oil platforms have also suggested as a potential source of marine species introduction to the Canary Islands (Brito et al. 2011).

3.2. Toxin content and profile

The analysis on *P. panamense* extracts showed both the presence of toxic compounds and toxicological activity. In fact, LC-MS/MS analysis showed the presence of OA in the culture media extracted from the late stationary phase (Table S1, Figure S2). No other marine lipophilic toxins were found. Thus, this is the first record of toxin production of *P. panamense*. The evaluation of cell morphology changes (neuro-2a) provided additional information on the toxicity of the extracts (both from microalgae pellets and culture media) obtained at the four different growth phases. Okadaic acid induces cell apoptosis through the disruption of the filamentous actin (F-actin) cytoskeleton, the activation of caspase-3 and the collapse of the mitochondrial membrane potential. This results in a change in cell morphology and substrate detachment (Diogène et al. 1995; Cabado et al. 2004). In this work, morphological changes including cell blebbing and detachment could be observed when control cells (Figure 3A) were compared to the cells exposed to OA (Figure 3B). After 4 h of incubation with the pellets and culture media extracts, neuro-2a cells showed damages in presence of the pellet extract of *P. panamense* from late stationary phase (Figure 3C) and of the culture media extract of the early stationary phase (Figure 3D). Thus, even if OA was detected with LC-MS/MS only in the culture media extract of the late stationary phase, neuro-2a cell anomalies showed the presence of OA-like toxicity in other two extracts, underlying the toxic capacity of the *P. panamense* strain.

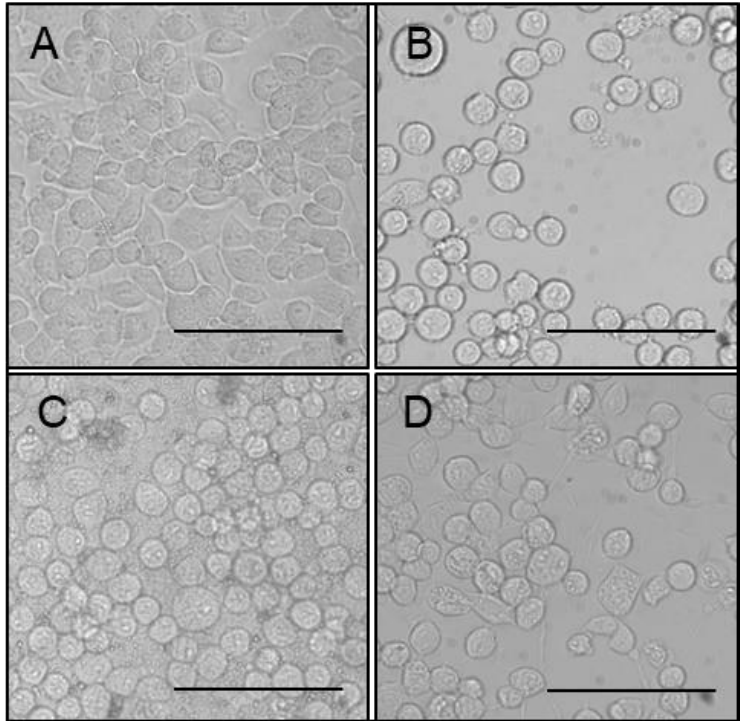

Figure 10. Morphology of neuroblastoma cells after 4h exposure to (A) PBS (control), (B) okadaic acid at 90 nM, (C) pellet extract from late stationary phase and (D) culture media extract from early stationary phase. Scale bar is 100 µm

CBA results (Table S1) showed cell toxicity of all pellet extracts from the different growth phases, with the exception of the latency phase. The cell mortality was as follow: late stationary > early stationary > exponential. Furthermore, all culture media extracts induced cell mortality, with a maximum in the early stationary phase and a minimum in the exponential phase (early stationary > late stationary > latency > exponential). Hence, it seems that the nature and/or concentration of toxic compounds released to the media may vary not only according to the number of cells, and also to the phase of the growth. These results confirmed the toxicity of *P. panamense* IRTA-SMM-17-72. In some treatments a concomitant mortality evaluated by the MTT assay and morphological changes in cells (*e.g.,* early stationary phase) can be observed. Nonetheless, for some treatments, cell mortality has been recorded but no morphological changes were observed. This may be explained by the kinetics of the effects, and the time of observation. It is also possible that changes in morphology end-up in cell detachment of dead cells that would reduce the MTT signal, without observable changes in morphology. Our results revealed the presence of OA and its effects on neuro 2a-cell morphology and viability, in particular in the stationary phases with high concentrations of *P. panamense* cells. Even if there are no studies investigating the cell growth and toxin

production of *P. panamense,* a similar toxin production behavior has been observed in the stationary phase of some *P. lima* cultures (Bravo et al. 2001; Holmes et al. 2001). However, the results derived from the analysis of *P. lima* showed a much higher OA contents (mean of 4.74 pg cell^{-1} in Bravo et al. (2001) and 15 pg cell^{-1} in Holmes et al. (2001)) compared to the ones obtained in this work (Table S1). Even though, the CBA values related to OA-like activity, obtained from the sum of pellet and culture media data, gives OA concentration that are comparable with the ones obtained in the analysis of *P. lima* (Table S1).Only Luo et al. (2017) investigated the toxicity of a *P. panamense* strain from China and described it as non-toxic according to LC-MS/MS. However, in their work, cells were collected during the mid-exponential phase, which also in our experiment show either few (only with CBA) or a total absence of toxic activity or toxins. Thus, the undetected toxicity of the Chinese strain could be related to the growth phase considered. Further studies are needed to characterize the toxic profile of *P. panamense,* involving strains from different regions.

4. Conclusions

Prorocentrum *panamense* strain was detected and identified in samples from Gran Canaria (Canary Islands, Central Eastern Atlantic Ocean). This is the first record of this species in the Macaronesian region and underlines the expansion of the *P. panamense* distribution area. This discovery highlights the importance of monitoring programs and long-term data sets, which facilitate the new detection of species. Additionally, in this work, LC-MS/MS analysis confirmed the presence of OA, and the assay on the evaluation of neuro-2a cell morphology changes together with the viability CBA with colorimetry, identified the presence of OA-like activity in several extracts from both cell pellets and culture media, pointing this species as a possible threat for human health. This is the first toxicity report of a *P. panamense* strain. Hence, there is a need for further studies on the toxicology of several strain belonging to this species to better assess the toxin production. The identification of OA producing species out of their area of distribution can contribute to the DSP risk assessment and help in spotting future outbreaks, so limiting the economic cost associated to DSP events.

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Supplementary material

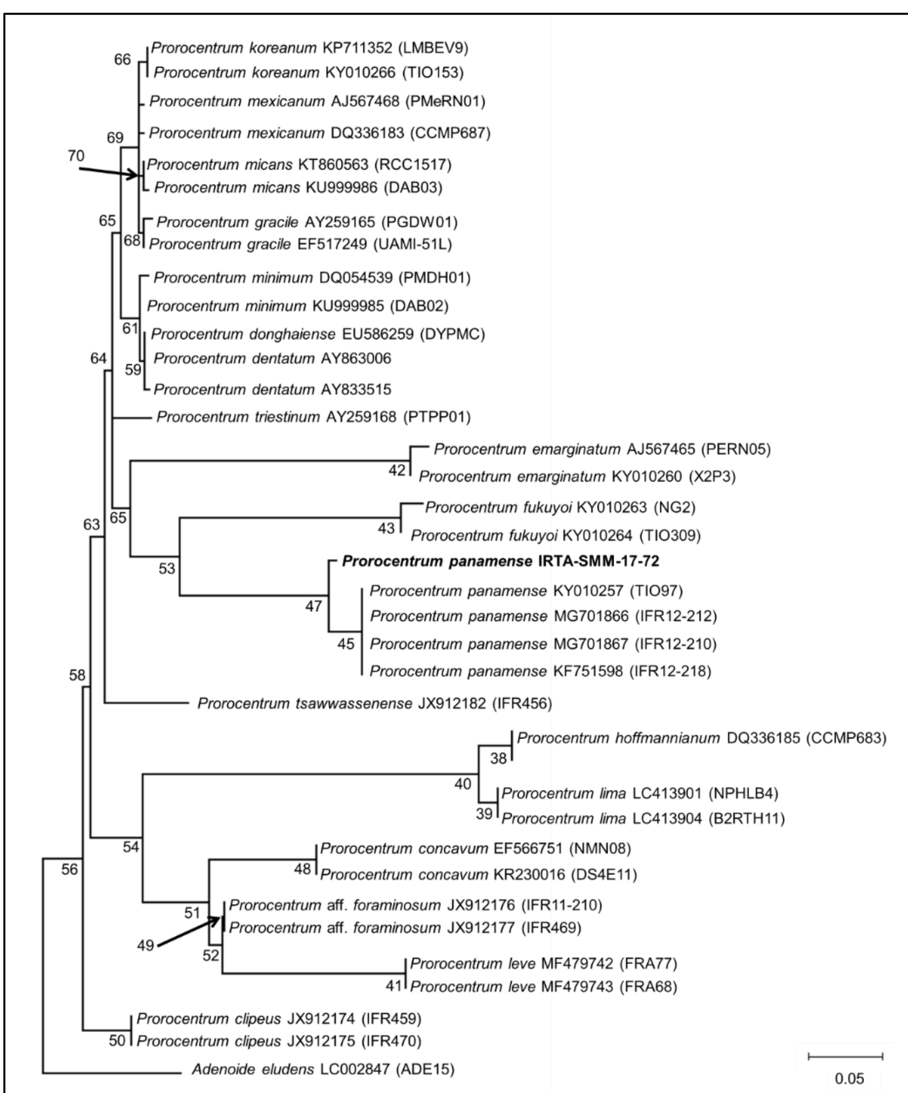

Figure S1. Phylogenetic analysis of LSU 28S rDNA gene of the available Prorocentrum sequences (GenBank) and *P. panamanse* collected in this study (in bold). The tree with the highest log likelihood is shown (-2071.16). Values at nodes are bootstrap values. obtained by the maximum likelihood method. Bootstrap values less than 30 % are not shown.

17-72 MEDIO ESTACIONARIA FINAL - OA MRM1 (Unknown) 803.5 / 255.2
Area: 6.420e2, Height: 1.556e2, RT: 2.80 min

17-72 MEDIO ESTACIONARIA FINAL - OA MRM2 (Unknown) 803.5 / 113.1
Area: 3.506e2, Height: 8.893e1, RT: 2.80 min

Figure S2. Multiple reaction monitoring chromatogram of transitions monitored obtained following the analysis of OA in the late stationary culture media by LC-MS/MS.

References

Aligizaki K, Nikolaidis G, Katikou P, Baxevanis AD, Abatzopoulos TJ (2009). Potentially toxic epiphytic *Prorocentrum* (Dinophyceae) species in Greek coastal waters. Harmful Algae 8:299-311.

Bravo I, Fernández ML, Ramilo I, Martınez A (2001). Toxin composition of the toxic dinoflagellate *Prorocentrum lima* isolated from different locations along the Galician coast (NW Spain). Toxicon 39:1537-1545.

Brito A, Clemente S, Herrera R (2011). On the occurrence of the African hind, *Cephalopholis taeniops*, in the Canary Islands (eastern subtropical Atlantic): introduction of large-sized demersal littoral fishes in ballast water of oil platforms? Biol Invasions 13:2185-2189.

Cabado AG, Leira F, Vieytes MR, Vieites JM, Botana LM (2004). Cytoskeletal disruption is the key factor that triggers apoptosis in okadaic acid-treated neuroblastoma cells. Arch Toxicol 78:74-85.

Cañete E, Diogène J (2008). Comparative study of the use of neuroblastoma cells (Neuro-2a) and neuroblastoma× glioma hybrid cells (NG108-15) for the toxic effect quantification of marine toxins. Toxicon 52:541-550.

Chomérat N, Bilien G, Zentz F (2019). A taxonomical study of benthic Prorocentrum species (Prorocentrales, Dinophyceae) from Anse Dufour (Martinique Island, eastern Caribbean Sea). Mar Biodivers 49:1299-1319.

Chomérat N, Sellos DY, Zentz F, Nézan E (2010). Morphology and molecular phylogeny of *Prorocentrum consutum* sp. nov. (Dinophyceae), a new benthic dinoflagellate from South Brittany (Norwestern France). Journal Phycol 46:183-194.

Chomérat N, Zentz F, Boulben S, Bilien G, van Wormhoudt A, Nézan E (2011). *Prorocentrum glenanicum* sp. nov. and *Prorocentrum pseudopanamense* sp. nov.(Prorocentrales, Dinophyceae), two new benthic dinoflagellate species from South Brittany (northwestern France). Phycologia 50: 202-214.

Diogène G, Fessard V, Dubreuil A, Puiseux-Dao S (1995). Comparative studies of the actin cytoskeleton response to maitotoxin and okadaic acid. Toxicol In Vitro 9:1-10.

Economou V, Papadopoulou C, Brett M, Kansouzidou A, Charalabopoulos K, Filioussis G, Seferiadis K (2007). Diarrheic shellfish poisoning due to toxic mussel consumption: the first recorded outbreak in Greece. Food Addit Contam 24:297-305.

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

Fritz L, Triemer RE (1985). A rapid simple technique utilizing calcofluor white M2R for the visualization of dinoflagellate thecal plates 1. J Phycol 21:662-664.

Fujiki H, Suganuma M, Yoshizawa S, Nishiwaki S, Winyar B, Sugimura T (1991). Mechanisms of action of okadaic acid class tumor promoters on mouse skin. Environ Health Perspect 93:211-214.

García-Altares M, Diogène J, de la Iglesia P (2013). The implementation of liquid chromatography tandem mass spectrometry for the official control of lipophilic toxins in seafood: single-laboratory validation under four chromatographic conditions. J Chromatogr A 1275:48-60.

Gestal C, Roch P, Renault T, Pallavicini A, Paillard C, Novoa B, Oubella R, Venier P, Figueras A (2008). Study of diseases and the immune system of bivalves using molecular biology and genomics. Rev Fish Sci 16:133-156.

Glibert PM, Burkholder JM, Kana TM (2012). Recent insights about relationships between nutrient availability, forms, and stoichiometry, and the distribution, ecophysiology, and food web effects of pelagic and benthic *Prorocentrum* species. Harmful Algae 14:231-259.

Grzebyk D, Sako Y, Berland B (1998). Phylogenetic analysis of nine species of *Prorocentrum* (Dinophyceae) inferred from 18S ribosomal DNA sequences, morphological comparisons, and description of *Prorocentrum panamensis*, sp. nov. J Phycol 34:1055-1068.

Hall TA (1999). BioEdit: a user-friendly biological sequence alignment editor and analysis program for Windows 95/98/NT. Nucleic Acids Symp Ser 41: 95–98.

Hallegraeff GM, Bolch CJ (1992). Transport of diatom and dinoflagellate resting spores in ships' ballast water: implications for plankton biogeography and aquaculture. J. Plankton Res. 14: 1067-84.

Hallegraeff GM (1998). Transport of toxic dinoflagellates via ships ballast water: bioeconomic risk assessment and efficacy of possible ballast water management strategies. Mar. Ecol. Prog. Ser. 168: 297-309.

Hansen G, Turquet J, Quod JP, Ten-Hage L, Lugomela C, Kyewalyanga M, Hirbungs M, Wawiye P, Ogongo B, Tunje S (2001). Potentially harmful microalgae of the western Indian Ocean: a guide based on a preliminary survey.

Holmes MJ, Lee FC, Khoo HW, Teo SLM (2001). Production of 7-deoxy-okadaic acid by a New Caledonian strain of *Prorocentrum lima* (Dinophyceae). J Phycol 37:280-288.

Hoppenrath M, Chomérat N, Horiguchi T, Schweikert M, Nagahama Y, Murray S (2013). Taxonomy and phylogeny of the benthic *Prorocentrum* species (Dinophyceae)-A proposal and review. Harmful Algae 27:1-28.

Hoppenrath M, Murray SA, Chomérat N, Horiguchi T (2014). Marine benthic dinoflagellates-unveiling their worldwide biodiversity. Kleine Senckenberg-Reihe, 276 pp.

Huelsenbeck JP, Ronquist F (2001). MRBAYES: Bayesian inference of phylogenetic trees. Bioinformatics 17:754-755.

Huynh-Delerme C, Fessard V, Kiefer-Biasizzo H, Puiseux-Dao S (2003). Characteristics of okadaic acid-induced cytotoxic effects in CHO K1 cells. Environ Toxicol In J 18:383-394.

Lizárraga IG, González-Armas R (2017). First record of the dinoflagellate *Prorocentrum panamense* (Prorocentrales, Dinophyceae) in the Mexican Pacific from the Archipiélago de Revillagigedo. CICIMAR Oceánides 32:63-66.

Luo Z, Zhang H, Krock B, Lu S, Yang W, Gu H (2017). Morphology, molecular phylogeny and okadaic acid production of epibenthic *Prorocentrum* (Dinophyceae) species from the northern South China Sea. Algal Research 22:14-30.

Manger RL, Leja LS, Lee SY, Hungerford JM, Wekell MM (1993). Tetrazolium-based cell bioassay for neurotoxins active on voltage-sensitive sodium channels: semiautomated assay for saxitoxins, brevetoxins, and ciguatoxins. Anal Biochem 214:190-194.

Moreira-González AR, Fernandes LF, Uchida H, Uesugi A, Suzuki T, Chomérat N, Bilien G, Mafra LL (2019). Variations in morphology, growth, and toxicity among strains of the *Prorocentrum lima* species complex isolated from Cuba and Brazil. J Appl Phycol 31:519-532.

Provasoli L (1968). Media and prospects for the cultivation of marine algae. In: Cultures and collection of algae, proceedings of the US-Japanese conference. Japan Society of Plant Physiology, Hakone, pp 63-75

Rodríguez F, Riobó P, Crespín GD, Daranas AH, de Vera CR, Norte M, Fernández JJ, Fraga S (2018). The toxic benthic dinoflagellate Prorocentrum maculosum Faust is a synonym of Prorocentrum hoffmannianum Faust. Harmful algae 78:1-8

Roy S, Parenteau M, Casas-Monroy O, Rochon A (2012). Coastal ship traffic: a significant introduction vector for potentially harmful dinoflagellates in eastern Canada. Can J Fish Aquat Sci 69:627-644.

Soliño L, Sureda FX, Diogène J (2015). Evaluation of okadaic acid, dinophysistoxin-1 and dinophysistoxin-2 toxicity on Neuro-2a, NG108-15 and MCF-7 cell lines. Toxicol in Vitro 29:59-62.

Stamatakis A (2014). RAxML version 8: a tool for phylogenetic analysis and post-analysis of large phylogenies. Bioinformatics 30:1312-1313.

Stein JR, Hellebust JA, Craigie JS (1973). Handbook of phycological methods: culture methods and growth measurements. Cambridge University Press.

Tichavska M, Tovar B (2015). Environmental cost and eco-efficiency from vessel emissions in Las Palmas Port. Transportation Research Part E: Logistics and Transportation Review 83:126-140.

Toldrà A, Andree KB, Fernández-Tejedor M, Diogène J, Campàs M (2018). Dual quantitative PCR assay for identification and enumeration of *Karlodinium veneficum* and *Karlodinium armiger* combined with a simple and rapid DNA extraction method. J Appl Phycol 30:2435-2445.

Yasumoto T, Murata M, Oshima Y, Sano M, Matsumoto GK, Clardy J (1985). Diarrhetic shellfish toxins. Tetrahedron 41:1019-1025.

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