



Detection of marine toxins using cell-based assays and Characterization of toxin profiles in ciguatera-related natural samples: microalgae and fish.

***(Detecció de toxines marines mediante ensayos celulares y
Caracterización del perfil tóxico en muestras naturales
asociadas a la ciguatera: microalgas y pescado)***

Amandine Caillaud

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UNIVERSIDAD DE BARCELONA
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Memoria presentada por

Amandine Caillaud

Para optar al grado de

Doctor en Biología, mención doctor europeo

Directores:

Dr. Jorge Diogène Fadini

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Certifican

Que la memoria titulada “**Detection of marine toxins using cell-based assays and characterization of toxin profiles in ciguatera -related natural samples: microalgae and fish**” (Detección de toxinas marinas mediante ensayos celulares y caracterización del perfil toxínico en muestras naturales asociadas a la ciguatera: microalgas y pescado) presentada por Amandine Caillaud para optar al Grado de Doctora, mención doctor europeo, ha sido realizada bajo su dirección en el centro IRTA Sant Carles de la Ràpita.

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TABLE OF CONTENTS

Figures and Tables of contents.....	iv
List of abbreviations.....	vi
I. Introduction.....	1
1.General overview	2
2.Generalities on marine toxins produced by dinoflagellates: CTXs, MTX and OA.....	5
2.1. Diversity of marine dinoflagellates producers of toxins. The genus <i>Gambierdiscus</i> and <i>Prorocentrum</i>	5
2.2. Diversity of targets of marine toxins: CTXs, MTXs and OA	10
3.Cell-based assay (CBA) as a potent strategy for marine toxins detection.....	14
3.1. Methods for marine toxins detection: CTXs, MTXs and OA	14
3.1.1 Current methods for CTXs determination.....	15
3.1.2. Current methods for MTXs determination	15
3.1.3. Methods for OA determination	16
3.2. Generalities on the use of cell-based assay (CBA) for marine toxin detection.....	17
3.2.1. Sensitivity of CBA to marine toxins.....	18
3.2.2. Specificity of CBA for the different groups of marine toxins	20
3.2.3. Interferences of biological matrices with CBA	21
3.3. The neuroblastoma Neuro-2a cell-based assay (Neuro-2a CBA).....	22
II. Context and Objectives.....	24
III. Results and Discussion.....	30
3.1 CHAPTER I. Development of methodological approaches for the application of <i>in vitro</i> cell-based assay to marine toxins detection and characterization in natural samples.....	31

3.1.1 Results and Discussion.....	32
3.1.2 Publications	37
Article 1. Cell-based assay coupled with chromatographic fractioning: a strategy for marine toxins detection in natural samples. <i>Toxicology in vitro</i> (2009), 53, 1591-1596	38
Article 2. Detection and quantification of maitotoxin-like compounds using a neuroblastoma (Neuro-2a) cell based assay. Application to the screening of maitotoxin-like compounds in <i>Gambierdiscus</i> spp. <i>Toxicon</i> (2010), 56, 36-44.....	40
3.2 CHAPTER II. Application of cell-based assay to toxin detection in natural samples: microalgal samples. The genus <i>Gambierdiscus</i> and <i>Prorocentrum</i>.....	42
3.2.1 Results and Discussion.....	43
3.2.2 Publications	51
Articulo 3. Comparative study of the CTX- and MTX-like toxicity of various <i>Gambierdiscus</i> spp. from distinct geographical origin using a Neuroblastoma (Neuro-2a) cell-based assay. Article in preparation for publication in <i>Toxicon</i>	52
Articulo 4. Monitoring of dissolved ciguatoxin and maitotoxin using solid-phase adsorption toxin tracking devices: Application to <i>Gambierdiscus pacificus</i> in culture. Submitted for publication in <i>Harmful Algae</i>	79
Articulo 5. Evidence of okadaic acid production in a cultured strain of the marine dinoflagellate <i>Prorocentrum rhathymum</i> from Malaysia. <i>Toxicon</i> (2010), 55, 633-637.	82
3.3 CHAPTER III. Application of cell-based assay to toxin detection in natural samples: fish samples. Contribution to ciguatera risk assessment.	84
3.3.1 Results and Discussion.....	85
3.3.2 Publications	91
Articulo 6. Update on the methodologies available for ciguatoxin determination. A perspective for facing up the onset of ciguatera in Europe. <i>Marine Drugs</i> (2010), 8, 1838-1907.	92
Articulo 7. Towards the standardization of the neuroblastoma (neuro-2a) cell-based assay for ciguatoxin-like toxicity detection in fish. Application to fish caught in the Canary Islands. Article submitted in <i>Food Additives and Contaminants</i>	95
3.4 General Discussion and Perspectives.....	97
IV. Conclusions.....	106
V. Resumen en castellano	109
VI. Bibliography	121

Annexes.....	143
Annex 1 : First approach towards the implementation of passive sampling adsorption devices for the identification of lipophilic toxins in the coastal embayments of the Ebro Delta. <i>Sixth International Conference on Molluscan Shellfish Safety</i> (2009). 336–342.....	144
Annex 2 : Toxin production and cell abundance of <i>Prorocentrum</i> spp. in Sabah coastal waters, Malaysia. <i>UMT Annual Seminar 2010, submitted</i>	145
Annex 3 : Informe de los directores de la tesis: Factor de Impacto y Contribución del doctorante en cada artículo.....	146

FIGURES and TABLES OF CONTENTS

FIGURES

Figure 1: *Gambierdiscus toxicus* (left ventral view of the hypotheca), Scanning Electron Microscopy, scale bar = 30 μm [1].

Figure 2: Diversity of *Gambierdiscus* species based on SsU rDNA gene phylogeny [1].

Figure 3: *Prorocentrum lima*, Scanning Electron Microscopy, scale bar = 10 μm [2]

Figure 4: Transmembrane organization of sodium channel subunits [3].

● CTXs binding sites

Figure 5: Neuroblastoma (Neuro-2a) cells exposed for 24 hours to CTX1B in the presence or absence (■) of ouabain (0.1 mM) and veratridine (0.01 mM) [4].

Figure 6: Neuroblastoma Neuro-2a cells. Inverted optical microscopy (Nikon Eclipse TE 2000-5), phase contrast.

Figure 7: SPATT-discs filled with DIAON[®] HP20 styrene divinylbenzene resin (Mitsubishi Chemical Corporation).

Figure 8: Phylogenetic tree inferred from the SsU sequences of *Prorocentrum* species. S= valve symmetry A= valve asymmetry [5].

Figure 9: The three components of risk analysis, adapted from Fazil [6]

Figure 10: Dose-response curve : threshold approach in toxicology. Adapted from Johns Hopkins Bloomberg School of Public Health, available at <http://ocw.jhsph.edu>.

TABLES

Table 1: Diversity of toxins produced by marine dinoflagellates and associated syndrome in human intoxication.

Table 2: General aspects addressed for the implementation of CBAs in the field of marine toxins: actual knowledge, requirements and perspectives.

LIST OF ABBREVIATIONS

ATCC: American Type Culture Collection

AZA: azaspiracids

Ca²⁺: calcium

CBA: cell-based assay

CFP: ciguatera fish poisoning

CTX: ciguatoxin

DA: domoic acid

DSP: diarrheic shellfish poisoning

DTX: dinophysistoxin

ELISA: Enzyme linked immunosorbent assay

GTX: gonyautoxin

GYM: gymnodimine

HAB: harmful algal bloom

HPLC: high-performance liquid chromatography

IC₅₀: concentration producing 50% of cell viability

iCa²⁺: intracellular calcium

K⁺: potassium

LC-MS: liquid chromatography coupled with mass spectrometry detection

LC-MS/MS: liquid chromatography coupled with tandem mass spectrometry detection

MBA: mouse bioassay

MRM: multiple reaction monitoring

MTT: 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium

MTX: maitotoxin

Na⁺: sodium

NCX: Na⁺-Ca²⁺ exchanger

Neuro-2a: neuroblastoma

NG108-15: neuroblastoma x glioma hybrid

NSCC: Non-selective Ca²⁺ channel

NSP: neurotoxic shellfish poisoning
O: ouabain
OA: okadaic acid
PbTX: brevetoxin
PKS: Polyketide synthase
PLTX: palytoxin
PP: protein phosphatase
PP2a: protein phosphatase type 2a
PPIA: protein phosphatase inhibition assay
PSP: paralytic shellfish poisoning
PTX: pectenotoxin
RBA: receptor binding assay
rDNA: ribosomal DNA
RE: resin exposure
RMCE: receptor mediated Ca²⁺ entry
S: Segment
SPATT: solid-phase adsorption toxin tracking
SPE: solid-phase extraction
SPL: spirolide
SsU: small sub-unit
STX: saxitoxin
TE: tissue equivalent
V: veratridine
VGSC: voltage-gated sodium channel
VSCC: voltage-sensitive Ca²⁺ channel
YTX: yessotoxin

I. INTRODUCTION

1. General Overview

«...and the waters that were in the river were turned to blood. And all the fish that was in the river died; and the river stank, and the Egyptians could not drink of the water of the river...»

Bible, Exodus : Chapter 7, versus 20-21

Microalgae form the basis of the aquatic food chain and are responsible for the bulk of the primary production. When the combination of physical, chemical and biological conditions are suitable, they show significant population increase known as blooms [7]. These events are also known as “red tides” in which water is stained a red, brown or yellowish color because of the temporary abundance of a particular pigmented species of microalgae. These proliferations in marine or brackish waters may cause massive fish kills, contaminate seafood with toxins and alter ecosystems [7]. These events are referred to “Harmful Algal Blooms (HABs)”. HAB species involve a wide range of organisms: dinoflagellates, flagellates, cyanobacteria, diatoms and others. It is worth noting that among these different groups, by far the group of dinoflagellates constitutes the most abundant group involved in HABs. The group of dinoflagellates includes autotrophic, but also mixotrophic or heterotrophic species. In that sense, the use of the term “algae” in HABs may be an abuse, since some heterotrophic dinoflagellates such as *Dinophysis* spp. may cause HABs.

HAB may cause damage in several ways [7]: i) HAB species which may proliferate resulting in high biomass which can cause hypoxia or anoxia and may cause mortality of marine fauna after reaching high densities, ii) HAB species that may proliferate and their high densities may cause physical damage, for example to fish gills or also to man-made structures such as water filtering or water cooling systems, iii) HAB species that may proliferate and cause foams that have an incidence on the quality of bathing waters and tourism, and iv) HAB species which produce toxins that contaminate seafood products or that may kill fish and other marine organisms. Toxins are secondary metabolites (which do not participate in vital functions of microalgae) that may exhibit potent biological activities. The ecological role played by those metabolites is debatable and may probably serve as allelochemical (advantages for the emitter) defenses, conferring advantages over microorganisms or discourage predation by higher trophic levels organism [8].

While HABs are natural phenomena, it is well-known that the frequency, distribution and severity of HABs may be increasing worldwide for several reasons [9, 10, 11]: (i) the increasing impact of human activities by nutrient enrichment in coastal waters [12, 13], the transfer of HAB species via ballast waters or associated to the translocation of shellfish stocks from one area to another [14], increased utilization of coastal waters for aquaculture [9], (ii) long-term climatic changes [15] which are likely to be related to human activities and (iii) an improved and more intense scientific research leading to the detection of new species, toxins and HAB events [9].

The impact of HABs on human health is of major concern in addition to the damages caused to specific economies (aquaculture, fisheries and tourism) and is more worrisome in a context where HABs may be increasing. Food borne intoxications may occur after the consumption of shellfish and fish that have accumulated toxins by filtration of HAB species containing water or after accumulation of toxins through the food web [7]. For example,

ciguatera fish poisoning (CFP), the subject of study of the present work, is a human food borne intoxication occurring in tropical and sub-tropical areas after consumption of contaminated fish that contains ciguatoxins (CTXs) which are potent neurotoxins produced by dinoflagellates and that are transferred through the food webs [16, 17]. Of additional concern are the deleterious effects of some toxins on the human respiratory apparatus linked to the onshore transport of toxins in aerosols under specific wind and wave conditions [18].

In Europe as in many other countries, the presence of toxins in seafood products is regulated in order to assess the consumer safety and protect the fishery activity. Preventive measures rather than remediation actions are taken through dynamic monitoring programs that determine the presence of marine toxins in seafood products and the presence of toxin producing HAB species in fishery harvesting areas [19]. Additionally, thorough legislation for the most important marine toxins regulates the maximum permitted levels of toxins in food [20] and determines official methods for toxin quantification [21]. In complement to monitoring programs, research provides support in the identification of hazards that may threaten consumers and aquaculture. Some important actions for research on HABs are (i) the development of methods for aquatic toxin detection in natural samples (e.g. fish, microalgae shellfish), (ii) the identification and characterization of toxins in water, microalgae and food, and (iii) field work for the identification of the HAB species and toxins present in ecosystems. Research allows responding to limitations in the legislation and goes beyond the legal frame in order to predict conflictive or emerging issues, consequently contributing to the revision and establishment of regulations on marine toxins.

In vitro cell cultures have been used as toxicological models for the study of marine toxins [22] and have been proposed as a possible alternative or complementary approach for replacing or reducing the use of living animals in seafood safety monitoring programs [23].

The scope of the present work is the development and application of *in vitro* cell-based assays (CBAs) for the detection and characterization of marine toxins in natural samples (microalgae and fish). The range of toxins studied includes toxins produced by marine benthic dinoflagellates found in CFP endemic areas, i.e the genus *Gambierdiscus* and *Prorocentrum*. The genus *Gambierdiscus* is the responsible for the production of ciguatera related toxins. However other toxin-producing dinoflagellates coexist with *Gambierdiscus* in CFP endemic areas and have sometimes been associated with ciguatera such as the genera *Prorocentrum*, *Coolia*, *Ostreopsis*, or *Amphidinium*.

2. Generalities on marine toxins produced by dinoflagellates: CTXs, MTX and OA

2.1. Diversity of toxin producing marine dinoflagellates. The genus *Gambierdiscus* and *Prorocentrum*.

Dinoflagellates are unicellular eukaryotic organisms termed “flagellates” because of the use of pair flagella that allow them swimming with rotation [24]. They can be whether planktonic (present in the water column) or benthic (associated with the bottom using macroalgae, rocks or detritus as a support), or parasites [25]. As previously state before, the majority of dinoflagellates are photosynthetic (autotrophic) and some species are heterotrophic or mixotrophic (e.g *Dinophysis*). Dinoflagellates constitute the protist group with the largest number of harmful species and responsible for the production of the widest array of toxins.

However of the several thousand species of dinoflagellates known, only few species appear to be toxigenic [24]. **Table 1** lists marine toxins produced by marine dinoflagellates with the respective intoxication type in human food borne intoxication.

Table 1: List of toxins according to intoxication type produced by marine dinoflagellates and associated syndrome in human intoxication.

Type of Intoxication	Related toxin	Toxin producer (genera)
Paralytic Shellfish Poisoning (PSP)	Saxitoxin (STX) Gonyautoxin (GTX)	<i>Alexandrium</i> , <i>Gymnodinium</i> <i>Pyrodinium</i>
Diarrheic Shellfish Poisoning (DSP)	Okadaic acid (OA) Dinophysistoxin (DTX) Pectenotoxin (PTX)	<i>Dinophysis</i> , <i>Prorocentrum</i>
Neurotoxic Shellfish Poisoning (NSP)	Brevetoxin (PbTX)	<i>Gymnodinium</i>
Ciguatera Fish Poisoning (CFP)	Ciguatoxin (CTX)	<i>Gambierdiscus</i>
Others	Spirolide (SPL)	<i>Alexandrium</i>
	Yessotoxin (YTX)	<i>Protoceratium</i>
	Prorocentrolide Eorbotoxin	<i>Prorocentrum</i>
	Gymnodimine (GYM)	<i>Gymnodinium</i> <i>Karenia</i>
	Azspiracid (AZA)	<i>Azadinium</i>
	Maitotoxin (MTX) Gambierol Acid gambieric	<i>Gambierdiscus</i>
	Palytoxin (PLTX) Ovatoxin	<i>Ostreopsis</i>
	Cooliatoxin	<i>Coolia</i>
	Amphidinolide	<i>Amphidinium</i>
	Karlotoxin	<i>Karlodinium</i>

a. The genus *Gambierdiscus*

Species of the genus *Gambierdiscus* are marine epibenthic dinoflagellates that have been found attached on microalgae in coral reef ecosystems [26], on artificial surfaces [27] or sand [28]. Their geographical repartition was initially found to be restricted to subtropical and tropical areas but some specimens were isolated in 2004 in temperate waters in the Eastern Atlantic Ocean (Canary Island) [29] and quite recently (2008) in the Mediterranean Sea (Crete Island) [30].

The genus *Gambierdiscus* is a producer of CTXs [31] and is considered as the responsible for the occurrence of CFP [26]. No organisms belonging to other genus have ever been identified as CTX producers. The same genus may also concomitantly produce other toxins such as maitotoxin (MTX), gambierol and gambieric acid (see **Table 1**), however these compounds are unlikely to participate within the symptom of ciguatera. We invite readers of the present dissertation to consult the **Article 6** of the **Chapter III** in which the broad aspects associated with the concept of ciguatera have been reviewed (transfer of CTXs through the food web, the epidemiology and symptomatology of ciguatera, etc.).

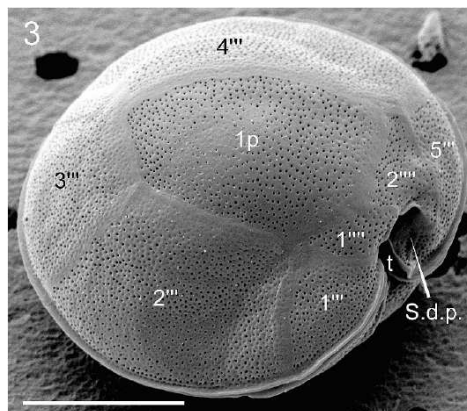
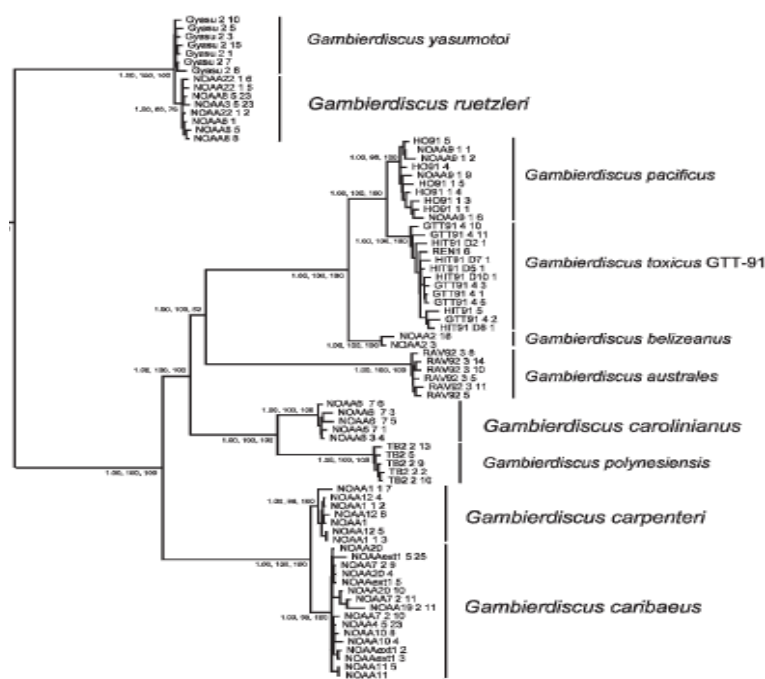


Figure 1 : *Gambierdiscus toxicus* (left ventral view of the hypotheca), Scanning Electron Microscopy, scale bar = 30 μm [1].

Gambierdiscus toxicus [32] (**Figure 1**) was the first species described in 1979 within the genus and data regarding toxin production were all originally obtained from studies conducted with *G. toxicus* [31, 33]. Later on, five additional species were described [28, 34, 35] for which production of CTXs has been described in three of them: *G. polynesiensis*, *G. pacificus* and *G. australes* [35]. However their direct implication within CFP has never been reported. In 2008, doubts raised regarding the species-level description of *G. toxicus* conducted to the hypothesis that the original description of *G. toxicus* [32] might include multiple species [1, 36, 37]. In 2009, Litaker et al. [1] have conducted an extensive revision of the taxonomy of the genus *Gambierdiscus* based on morphological and molecular analysis, which led to the description of four new species (**Figure 2**). Toxin production by these four new species remains to be determined.

Figure 2 : Diversity of *Gambierdiscus* species based on SsU rDNA gene phylogeny [1].



The most recent species identified within the genus and proposed as a novel species (proposed name: *G. excentricus*) is the first representative of the genus isolated from temperated water (Canary Islands, Spain) [38]. Data on toxicity will be presented in the second Chapter of the thesis (**Article 3**). Another specimen has been isolated from Crete (Greece), and is likely to belong to another undescribed species (Aligizaki, K., personal communication), although morphological and taxonomical identification are currently ongoing. Data on toxicity of the specimen from Crete are presented in the **Article 3 (Chapter 2)**.

b. The genus *Prorocentrum*

The genus *Prorocentrum* has a worldwide geographical repartition with approximately 70 species already described [39]. Two distinct life form are known, planktonic and benthic [24]. Benthic species have been found to live on numerous support : macroalgal or floating detritus, sand, sediment, coral rubble, algal turf, oyster racks [40]. The genus *Prorocentrum* belongs to the community of benthic dinoflagellates usually found in association with *Gambierdiscus* (the major source of ciguatera toxins) in ciguatera endemic areas and still, has sometimes been associated with the ciguatera [41].

Benthic *Prorocentrum* species have been reported to produce okadaic acid (OA) (and its derivatives) and dinophysistoxins (DTXs), and are considered putative links to the Diarrheic Shellfish Poisoning (DSP) (**Table 1**). Many times *P. lima* (**Figure 3**) has been detected in DSP areas [42, 43] and its direct implication during DSP event has been reported in the Atlantic coast of Nova Scotia [44]. Production of OA and its derivatives was reported for 8 species of *Prorocentrum* i.e. *P. lima* [45], *P. arenarium* [46], *P. concavum* [47], *P. hoffmanianum* [48], *P. belizeanum* [49], *P. faustiae* [50], *P. maculosum* [51] and *P. levis*

[52]. Recently, *P. rhathymum* has been added for the first time to the list of the DSP-toxin producers [53] and this result will be presented in the **Article 5 (Chapter 2)**.

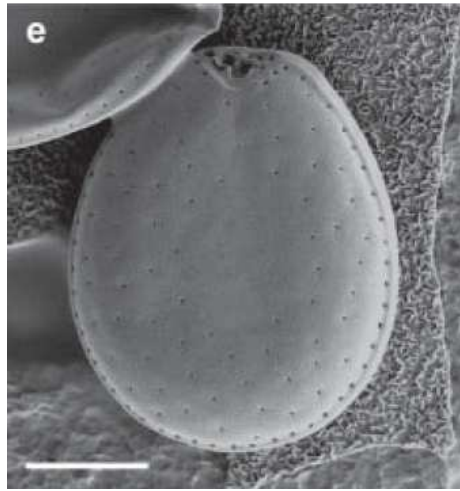


Figure 3 : *Prorocentrum lima*, Scanning Electron Microscopy, scale bar = 10 μm [2]

In addition to the production of DSP toxins, the genus *Prorocentrum* may produce other toxins such as prorocentrolides [54] or borbotoxins [55], however involvement of these toxins in food borne intoxication has never been reported (**Table 1**).

2.2. Diversity of targets of marine toxins CTXs, MTXs and OA

Marine toxins produced by dinoflagellates are structurally complexes and diversifies, including a variety of fused (ladder-like) or linear polyethers compounds (example: CTXs, MTXs, OA, DTXs, PLTX, YTX), alkaloids (STX) or cyclic imines (spiroolides, gymnodimine or prorocentrolides). The synthesis of this variety of biological compounds is likely to derive from a unique biosynthesis pathway *via* polyketide synthases (PKS) [56, 57, 58], although

the high structural diversity of toxins may be a result of the diversity of substrates and modification processes used during the PKS pathway [58].

The structural diversity of toxins directly implicates that toxins will interact with different targets, leading to the wide variety of symptoms elicited during seafood intoxications (see **Table 1**). At a cellular level, toxins from dinoflagellates may interact with ion channels implicated with the flux of sodium (Na^+) (CTX, PbTX, STX, PLTX) [59, 60, 61], calcium (Ca^{2+}) (MTX, YTX) [62, 63], potassium (K^+) (gambierol) [64], enzymes such as protein phosphatases (PP) (OA and DTX1) [65], or nicotinic acetylcholine receptors (spirolides, gymnodimine, prorocentrolides) [66, 67]. Since CTX, MTX and OA will be subject to further analysis in the course of the present dissertation, a more in deep description of the interaction of these toxins with their respective target is detailed below.

a. Interaction of CTXs with voltage-gated sodium channels (VGSC)

Voltage gated sodium channel (VGSC) are responsible for action potential initiation and propagation in excitable and non excitable cells and consist of a highly processed α subunit, which is approximately 260 kDa, associated with auxiliary β subunits [3]. The pore-forming α subunit is responsible for functional expression and is organized in four repeated homologous domains (I-IV), each containing six putative transmembrane spanning α -helical segments (S1-S6) [3].

Ciguatoxins specifically bind the S5 (domain IV)-S6 (domaine I) segments (= receptor site 5), inducing a shift in the voltage dependence of activation that results in Na^+ influx through the channel at membrane potentials where the sodium channel is normally closed [68].

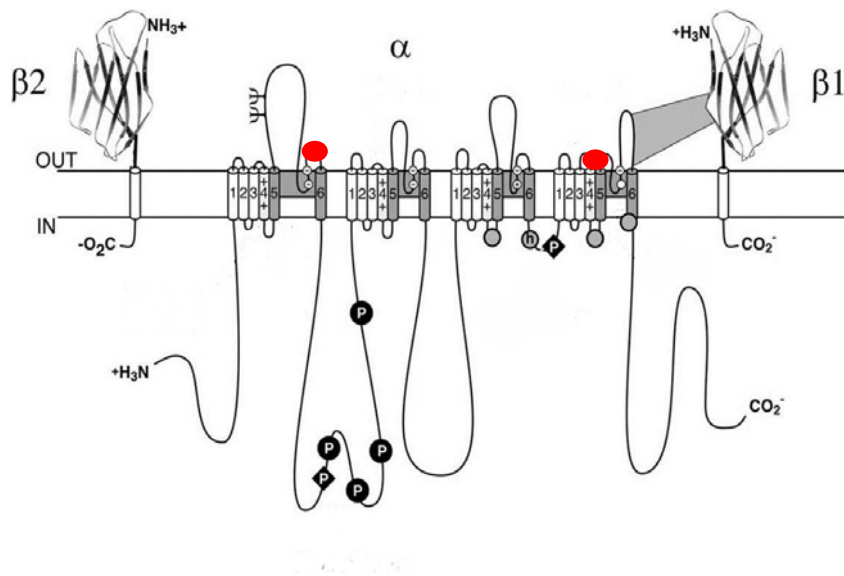


Figure 4: Transmembrane organization of sodium channel subunits [3].

● CTXs binding sites

The CTX induced activation of VGSC is responsible for numerous Na^+ dependent effects [69] such as membrane depolarization with spontaneous and repetitive action potentials in excitable cells, an increase in intracellular Ca^{2+} (iCa^{2+}) through alteration of the Na^+ gradient driving the Na^+ - Ca^{2+} exchanger [70], a repetitive synchronous and asynchronous neurotransmitter release which conduct to transient increases and decreases in the quantal content of synaptic responses [71] that causes spontaneous and titanic muscle contractions, an impairment of synaptic vesicle recycling and ultimately the swelling of axons, nerve terminals and perisynaptic Schwann cells [3, 68, 69]

b. Interaction of MTX with calcium entry pathway

Maitotoxin induces massive Ca^{2+} influx [72, 73] leading to cell death cascade after a succession of events at the cellular level (phosphoinositide breakdown, arachidonic acid release, neurotransmitter release and calpain activation) [74]. Although the precise

mechanism in which MTX induces Ca^{2+} entry remains unclear. Thus MTX is considered a unique pharmacological tool for research on Ca^{2+} dependent mechanism [74].

The initial MTX trigger was thought to be activation of a non-selective Ca^{2+} channel (NSCC) permeable to Na^+ and K^+ [75] but with low permeability to Ca^{2+} . MTX may also increase intracellular Ca^{2+} ($i\text{Ca}^{2+}$) via other Ca^{2+} entry pathway such as the L-type voltage-sensitive Ca^{2+} channels (VSCCs) [76] or receptor operated Ca^{2+} channels [77] or via other uncharacterized pathway. A recent study described the contribution of the Na^+ - Ca^{2+} exchanger (NCX) (that corrects significant increase in $i\text{Ca}^{2+}$) with the MTX-evoked Ca^{2+} influx [78]. This report supported the hypothesis that NCX is a primary target of MTX, and that the toxin causes an early and rapid reversal of the NXC from Ca^{2+} efflux (forward mode) to Ca^{2+} influx (reverse mode) following binding to NXC, converting NCX into a Ca^{2+} entry pathway [78].

c. Interaction of OA and DTXs with type 1 and type 2A serine/threonine protein phosphatases (PP1 and PP2A)

Phosphorilation is a post translational modification of proteins important for the regulation of many cellular functions such as cell growth and death, differentiation, signal transduction and metabolism. Serine/Threonine PP remove phosphate groups from serine and threonine amino acids. Different PP subtypes have been identified (PP1, PP2A, PP4, PP5 or PP6) which differ according to their catalytic domain and biochemical properties [79].

OA and DTX (type 1 and 2) are specific inhibitors of PP1 and PP2A, acting through a specific binding and inhibition of the catalytic subunits of PP1 and PP2A [80]. The strong inhibition of PP1 (implicated in the regulation of numerous cell functions, cell regulation, smooth muscle contraction) and PP2A (implicated in the control of cell events, metabolism, apoptosis, DNA replication) contributes to numerous toxic effects which have suggested OA and DTX to be either potent tumor promoters or apoptotic agents arresting cell cycle [81].

3. Cell-based assay (CBA) as a potent strategy for marine toxins detection

3.1. Methods for marine toxins detection: CTXs, MTXs and OA

Management systems are in place in numerous countries to reduce the risk of seafood to consumers through dynamic monitoring programs based on regular coastal sampling with testing of seafood for toxins and testing for the presence of toxic phytoplankton in seafood harvested areas [19]. In Europe, regulations have promulgated governing maximum levels of some toxins in seafood products [20] and establishing the official methods of analysis that have to be applied [21]. Presently and according to the group of toxins to be consider, official methods in Europe include the mouse bioassay (MBA) for OA, YTX, PTXs, AZA, the MBA for PSP group, the high-performance liquid chromatography (HPLC) with fluorimetric detection and pre-column oxidation for PSP-group of toxins and HPLC with UV detection for domoic acid (DA), an amnesic toxin produced by diatoms of the genus *Pseudo-nitzschia* or enzyme-linked immunosorbent assay (ELISA) (for DA) [82, 83]. The development of alternative methods to the MBA is strongly supported by many countries in order to decrease the number of animals used during the assay [23], whenever their implementation provides an equivalent level of public health protection [21]. In addition, numerous functional methods based on the effect of toxins (CBA, tissue assays, *in vivo* assays, biochemical assays, ELISAs, and more) [22] or their physico-chemical properties (HPLC, LC-MS/MS) have been used in research for a several purposes: first identification of toxins in microalgae and food, understanding the mechanism of action of toxins, elucidating the toxin structure, ...Here we focus on a more detailed description of the methods developed for the toxins CTXs, MTXs and OA involved in this work.

3.1.1 Current methods for CTXs determination

Current European legislation regarding the presence of CTXs in fish states that “fishery products containing biotoxins such as ciguatoxins...must not be placed on the market” [84]. The regulation neither set maximum permitted levels of CTXs in fish sample nor official testing method to assess the presence of CTXs in fish. Numerous methods have been developed for the determination of CTXs, based on *in vivo* toxicological assays with animals, *in vitro* toxicological CBA, immunoassays, a pharmacological receptor-binding assay (RBA) and instrumental analytical approaches [4]. These methodological approaches for CTXs determination (in fish and *Gambierdiscus* spp. samples) are fully described in the **review article** of the **Chapter III (Article 6)** and their suitability as a screening tool for CTXs for diagnostic confirmation and monitoring programs purposes discussed.

3.1.2. Current methods for MTXs determination

No regulations exist regarding the presence of MTX in fish samples. This is understandable as: i) MTX are hydrosoluble polycyclic polyethers [85] which may not bioaccumulate in fish tissue and still are unlikely to participate within the symptoms of ciguatera and ii) MTX may be found in the viscera of herbivorous fishes [85] but its low potency by oral route suggests that MTX may not produce human illness [86]. However the interest for the development of methods for MTX determination comes from research requirement, first for the understanding of the structure and function of MTX which is an extraordinary complex molecule that presented extreme intra-peritoneal potency, and further on for the evaluation of the production of MTX by *Gambierdiscus* spp. for taxonomical and physiological purposes, for the detection of possible MTX-like interferences that may difficult CTX detection and purification from *Gambierdiscus* spp. extracts [87]. Methods for MTX determination include:

- Qualitative *in vitro* CBA based on the distribution of the F-actin microfilaments stained with fluorescent probes [88].
- Qualitative and quantitative *in vitro* CBA based on the inhibitory effects of SK&F 96365 on the MTX-induced toxic effects [87]. This approach will be further presented in the **first Chapter** of the present study (**Article 2**).
- An immunoassay using maitotoxin-specific antibodies [89].
- A fluorimetric assay for membrane potential measurement based on the detection of ion influx-induced toxins [90].
- Analytical detection of MTX by structural identification using liquid chromatography with time of flight (TOF) mass spectrometry detection [91].

3.1.3. Methods for OA determination

In Europe, levels of 160 µg OA (and analogues) equivalents per kg of shellfish flesh are established as maximum permitted levels as values above are considered to constitute a non acceptable DSP risk for human [20]. The MBA remains actually the official method for OA and related toxins detection in seafood products, although LC-MS analysis methods for lipophilic toxins is currently accepted as a complementary approach to the MBA [92]. However, other functional assays have been developed for OA and related toxins detection:

- *In vitro* CBA in which toxic effects elicited by OA and related toxins may be quantitatively measured with a simple cell viability assay [93] or qualitatively observed after checking morphological alterations in cultured cells [88, 94].
- Protein phosphatases (PP) assays in which the activity of PP (inhibited by OA and DTXs) can be determined by measuring the release of inorganic phosphate-labeled ³²P (PP radioassay) [95]; or colorimetric [96], bioluminescent [97] and fluorescent [98] assays measuring respectively the inhibition of the activity of PP on different substrates : p-

nitrophenyl phosphate, luciferin phosphate, 4-methylumbelliferyl phosphate and fluorescein phosphate.

- Immunoassays with OA-specific antibodies [99].
- Instrumental analytical methods using liquid chromatography with fluorescent detection [100] and with mass spectrometry detection [101].

3.2. Generalities on the use of cell-based assay (CBA) for marine toxin detection

Culture cells are truly representative of the tissue from they derive [102] and have been widely used for drug discovery, studies of the mechanism of action of drugs and toxic compounds, and for toxicity assessment. Cell culture models include the use of i) primary cells which are directly explanted from organism and capable of some divisions and survival only for some time; these constitute the best experimental model for *in vivo* situations, ii) secondary cells originally explanted from organism that may divide and grow for 50-100 generation but with physical characteristic that may have change, and iii) immortalized or transformed cells which continue to grow and divide indefinitely *in vitro* as long as correct culture conditions are maintained and for which growth properties have been altered by infection by transforming tumor viruses or chromosomal changes. Contrarily to the use of primary and secondary cells, the use of immortalized cells is likely to reduce the cost for cell isolation, establishment of cultures and improve the repeatability of experimental conditions [103], leading to a cellular model more suitable for routine toxicity screening.

In the field of seafood contaminants and especially toxins, primary cell cultures [104, 105] and immortalized cell cultures [53, 87, 103, 106, 107, 108, 109, 110, 111, 112] have been widely used for the detection, toxicity assessment and study of the mechanism of action of numerous toxins. The development of cytotoxicity assays for toxin detection represents a clear methodological advancement since the use of culture cells is likely to replace the use of

living animals in regulatory purposes [106]. Numerous endpoints have been developed to assess cytotoxic effects *e.g.*: i) qualitatively after checking for cellular morphological alterations [88, 94] or ii) quantitatively after checking for cell viability based on the measure of alterations in the metabolism of cells [113, 114, 115, 116]. Still, the [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium] MTT test [116] is actually the most widely used endpoint for cytotoxicity screening in natural samples (fish, shellfish and microalgae) [53, 87, 93, 103, 106, 107, 108, 109, 117], requiring minimal processing and allowing for accurate and reproducible quantification of toxic effects [93]. MTT is reduced by the mitochondrias of metabolically active cells into a blue formazan product that can be easily quantified using a standard spectrophotometer [116].

In order to assess the applicability of CBA as a method for marine toxins detection in natural samples, various factors have to be address: i) the sensitivity of cell cultures to marine toxins, ii) the specificity of CBA for the different groups of toxins, and iii) the resistance of CBA to biological matrices.

3.2.1. Sensitivity of CBA to marine toxins

The sensitivity of CBA to marine toxins is considered as the capacity of marine toxins in producing toxic effects that can be quantitatively measured or qualitatively observed in a determined period of exposure. In numerous studies, sensitivity of CBA to toxins is assessed using the concentration of toxin producing a reduction of 50% of cell viability, the Inhibition Concentration fifty (IC_{50}). This toxicological parameter allows the comparison of the toxic potency of various toxins and their respective derivatives, and hence contributes to a better knowledge of the risk associated to the presence of a determined toxin in natural samples. Although sensitivity of CBA may vary according to different parameters:

- The cellular model used for cytotoxicity assay: Cell lines may differ according to different characteristics features such as the expression of specific receptors or physical characteristics, or according to their metabolism. Since marine toxins target diverse receptors, the choice of a specific cell line for the detection of a particular group of toxins is likely to improve sensitivity of CBA to these toxins. As an example, neuronal cell lines such as the neuroblastoma (Neuro-2a) cells [103, 106, 107, 117, 118, 119, 120] or neuroblastoma x glioma hybrid cells (NG108-15) [70, 108, 109] have been used for the detection of neurotoxins for which toxic effects depend on the presence of VGSCs.

- Experimental conditions: Time of incubation of cells before exposure to toxins and time of exposure to toxins are likely to influence the sensitivity of the response of cells to toxins [103, 108]. As an example, Cañete and Diogène [103] described that toxic effects elicited after exposure of Neuro-2a cells to DTX-1 were improved for an incubation period of cells equivalent to 1 hour respect to 24 hour incubation . One hour incubation in addition to 48 hour exposure of YTX and AZA to NG108-15 cells improved the sensitivity of cell response compared to 24 hour incubation and 24 exposure [108]. It is thus important to set maximal experimental conditions for the detection of one group of toxins in order to improve the applicability of CBA to natural samples.

- The use of specific agonist/antagonist of the mechanism of action of toxins: Binding of toxins to their receptors, and especially neurotoxins on the VGSC, may induce or block the influx of Na^+ [121]. However the diversity of ion channel systems in different cell types is likely to compensate for these disorders such that the action of neurotoxins alone is not sufficient to cause cell death. To address this issue, the addition of other agonist/antagonist of Na^+ influx increments or hinders Na^+ influx-induced by neurotoxins [122]. As an example, the addition of ouabain (O) and veratridine (V) which respectively blocks Na^+ influx through an inhibition of the ATP dependent Na^+/K^+ pump [123] and increases Na^+ permeability

through a blockage of the voltage-gated Na⁺ channel in the open position [121], increases the sensitivity of CBA to neurotoxins (STX, CTXs, PbTX) and results in cell death. The increase sensitivity of CBA to neurotoxins in presence of such agonist/antagonist has been improve for the development of CBA able to specifically detect the presence of neurotoxins [115, 124, 125], further described below.

3.2.2. Specificity of CBA for the different groups of marine toxins

Cytotoxicity assays are toxicological tools that allow the quantification of toxic effects elicited by toxins but are unlikely to discriminate between different groups of toxins. Thus the need for confirmation of the identity of toxicity-induced toxins usually required complementary approaches based on instrumental analytical methods [53, 109, 120]. However the development of CBA specific for a particular group of toxins acting in the same way is a challenge and would represent a clear advantage for the application of CBA to toxin detection in natural samples. Still, various CBA specific for neurotoxins (CTXs, PbTXs, STX), PLTX and MTX have been developed [87, 107, 115, 124, 125, 126]. These CBA are all dependent of the use of specific agonist/antagonist of the mechanism of action of toxins.

The neuronal (Neuro-2a, NG108-15) CBA specific for neurotoxins (CTXs, PbTX, STX) is one of the best established and commonly used CBA for the detection of neurotoxins in natural sample. As an example, the neuro-2a CBA for CTXs takes advantage of the agonist effect of O/V on the CTX-like induced toxic effects to produce a highly sensitive and specific assay for CTX [124, 125, 126] (**Figure 5**). See the full description of the assay in the Review article (**Article 6**) of the third Chapter of the thesis. The assay has been widely used for discrimination between toxic (CTX containing) and non-toxic fish for clinical recognition of CFP or epidemiological purposes [127, 128] and for the identification of the production of CTXs by *Gambierdiscus* spp. [106, 119, 120].

3.2.3. Interferences of biological matrices with CBA

As one of the purposes of developing CBA is the applicability of the methods to the evaluation of toxins in microalgae and fish, it is important to consider the possible interferences of different biological matrices in the evaluation of toxins. Natural samples contain numerous compounds that may be toxic to cells. The efficiency of the different methods of toxin detection is highly dependent of the typology of the sample to test, and these may require efficient preparation procedures of samples for the separation and elimination of biological matrices that may interfere with the detection of toxins. The Review article of the third Chapter (**Article 6**) presents detailed description of sample preparation procedures suitable for the determination of CTXs in fish and *Gambierdiscus* spp. samples as well as for the separation of MTX versus CTXs in extracts of *Gambierdiscus* spp.

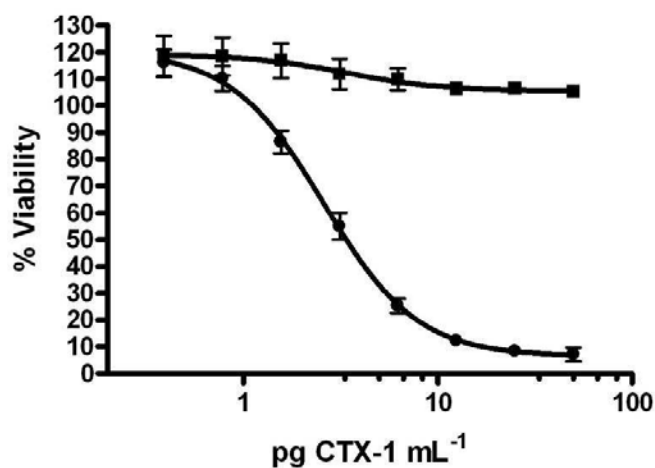


Figure 5 : Neuroblastoma (Neuro-2a) cells exposed for 24 hours to CTX1B in the presence or absence (■) of ouabain (0.1 mM) and veratridine (0.01 mM) [4].

Literature reports the use of purification procedures that are likely to improve the implementation of CBA for marine toxins detection in natural samples. These procedures include the separation and elimination of the different compounds of natural samples according to their nature and polarity, which may be achieved by liquid/liquid partition [128], solid-phase extraction (SPE) clean-up [125], the use of carbon black for the elimination of organic material [129], SPE- or high-performance liquid chromatography (HPLC)- based fractioning [4, 106, 109].

3.3. The neuroblastoma cell-based assay (Neuro-2a CBA)

In the present PhD study, all studies with CBA have been conducted with the use of the Neuro-2a cells. The Neuro-2a cell line (CCL-131) was provided by the American Type Culture Collection (ATCC, Manassas, USA) and was established from a spontaneous brain tumor of strain A albino *Mus musculus* (mouse). Neuro-2a cells are adherent and present neuronal and amoeboid stem cell morphology (**Figure 6**).

Numerous studies reported the use of the neuroblastoma (Neuro-2a) cells for the study of VGSC toxins [87, 103, 106, 107, 115, 117, 119, 120, 124, 125, 126, 128, 130] and non VGSC toxins [53, 103]. A Neuro-2a CBA ready-to-ship kit, consisting of a modification of the Neuro-2a CBA with shippable plates and reagents to be used by unspecialized laboratories, was developed for the determination of VGSC blocking toxins in 1999 [131]. However it proved to be unsuccessful, probably due to a lack of stability during shipment or unreliable laboratory conditions.

Cañete and Diogène [103] recently verified the suitability of the response of Neuro-2a cells to detect a wide variety of marine toxins, i.e STX, PbTX, PLTX, PTX-2, OA and DTX-1. Various reports almost describe the accuracy of the model for CTXs determination and its use as a screening tool for CTXs in fish for the prevention and confirmation of the diagnostic

of CFP [127, 128]. Ledreux et al. [107] proposed an experimental model for the determination of neurotoxins in natural samples, based on the use of the Neuro-2a cell line. Although the Neuro-2a CBA shows great potential as an alternative toxicological method to the MBA, and is proposed as a likely candidate in the quest for a validated reference method of CTX assessment in fish samples [4].

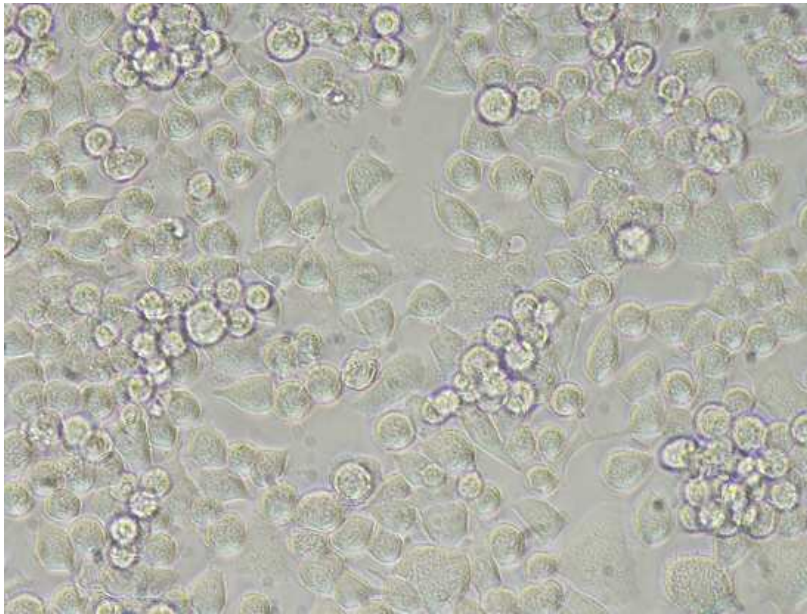


Figure 6: Neuroblastoma Neuro-2a cells. Inverted optical microscopy (Nikon Eclipse TE 2000-5), X200.

II. CONTEXT AND OBJECTIVES

The main objective of the present study is to demonstrate the suitability of the Neuro-2a CBA for ciguatera risk assessment through the characterization of toxin profiles in microalgal and fish samples associated to the ciguatera. In order to achieve this objective, some specific issues have been addressed:

- i) The development of methodological approaches to favor the application of *in vitro* CBA as a toxicological tool for marine toxins detection and quantification in natural samples.
- ii) The characterization of toxin profiles in microalgal samples of the genus *Gambierdiscus* and *Prorocentrum* using Neuro-2a CBAs, in order to characterize the hazards associated to the presence of ciguatera-related dinoflagellates in ecosystems. Application to test the suitability of passive samplers as a possible monitoring tool for the presence of toxin-producing populations of *Gambierdiscus*.
- iii) The identification of CTX-containing fish samples caught in the Canary Islands using the Neuro-2a CBA for CTXs in order to assess the risk of ciguatera in the Canary Islands.

i) Development of methodological approaches for the application of *in vitro* cell-based assay for marine toxins detection in natural samples.

As previously described in the introduction of the present dissertation, implementation of CBA for marine toxins detection in natural samples may be hindered by the interferences of biological matrices with cell response and by a lack of specificity of CBA for certain groups of toxins.

- With the aim to eliminate possible matrices interferences within cell response, sample preparation is a key step before exposure of suspected toxic samples to cells. To overcome this limitation, the suitability of the HPLC- and SPE-based chromatographic fractioning for the separation and elimination of toxic compounds unrelated to the presence of

toxins was tested. A proposed experimental strategy favoring the implementation of CBA to toxin detection in natural samples is presented (**Article 1**).

- To improve the specificity of CBA, the use of agonists and antagonists of the different toxins was addressed. The ouabain/veratidine (O/V) dependent Neuro-2a CBA is highly sensitive and specific for CTXs, and was shown to be suitable for the determination of CTXs in fish and *Gambierdiscus* spp. extracts. However *Gambierdiscus* spp. are producers of other toxins that may be found concomitantly with CTXs (See Introduction). Presence of such toxins may produce unspecific toxic effects that would interfere with the detection of CTXs in *Gambierdiscus* spp. extracts. Since MTX are highly toxic compounds commonly produced by *Gambierdiscus* spp., the interest for the availability of a CBA specific for MTX was converted in one of the objective of the present study. SK&F 96365 is an inhibitor of the voltage gated Ca^{2+} channel and of the receptor mediated Ca^{2+} entry (RMCE) [132] which has been previously described as an inhibitor of the MTX-induced toxic effects [133]. This effect will be improved for the settlement of a CBA able to specifically detect the presence of MTX in *Gambierdiscus* spp. samples (**Article 2**).

ii) Characterization of toxin profiles in microalgal samples of the genus *Gambierdiscus* and *Prorocentrum*.

Presence of toxin producing dinoflagellates in ecosystems represents a risk for human health. Knowledge of the toxin potency of the community of dinoflagellates in a given area is likely to better characterize the hazard and eventually diminish the risk for seafood intoxication. In the case of describing novel species, data on toxin production is of great interest for taxonomical purposes. The Neuro-2a cell-based assays were applied for studies on the toxicity and toxin content of various clonal cultures of dinoflagellates *Gambierdiscus* spp. (**Articles 3**) and *P. rhathymum* (**Article 5**). Moreover, in order to assess the risk of

ciguatera in a given area, management systems are implemented and usually consider the monitoring of the presence of CTX-producing populations of *Gambierdiscus* in ecosystems. The Neuro-2a cell-based assays were applied for the study of the suitability of passive samplers as a monitoring tool for dissolved ciguatera-related toxins under laboratory controlled conditions (**Article 4**).

- The Neuro-2a CBA specific for CTXs and for MTX was applied for CTX and MTX toxicity analysis in 9 strains of *Gambierdiscus* spp. The species of study were *G. pacificus* from Malaysia (strains GDSA01, GPSi, G10DC), *G. excentricus* (proposed novel species) from Canary Island (strains Vgo790, Vgo791 and Vgo792), *Gambierdiscus* sp. from Indonesia (strains Vgo917, Vgo920) and from Crete (strain KC81) also proposed novel species. Data on toxicity analysis helps to understand the risk for emerging toxins in Europe since knowledge of the presence of *Gambierdiscus* spp. in Canary Island and Crete is very recent (**Article 3**).

- The use of SPATT (Solid Phase Adsorption Toxin Tracking) was first reported in 2004 as a new monitoring tool for the presence of dissolved lipophilic toxins in seafood harvested areas (**Figure 7**) and is likely to simulate the presence of toxin producing dinoflagellates in ecosystems (**Annex 1**). Since the use of SPATT has never been documented for ciguatera toxins, the suitability of the DIAON[®] HP20 styrene divinylbenzene resin (Mitsubishi Chemical Corporation) for tracking of dissolved ciguatera related toxins was tested *in vitro* using standard solutions of CTX and MTX, and then further applied to a culture of *G. pacificus* (**Article 4**). The Neuro-2a CBA specific for CTXs and MTXs was applied to assess the recovery of CTX and MTX by the resin.



Figure 7: SPATT-discs filled with DIAON[®] HP20 styrene divinylbenzene resin (Mitsubishi Chemical Corporation). Photo: IRTA (2010).

- A study on the diversity of benthic dinoflagellates in Malaysia allowed the isolation and identification of various species of the genus *Prorocentrum*, i.e *P. lima*, *P. cf faustiae*, and *P. rhathymum*. A fibroblast based assay was used to assess the production of DSP toxins by those *Prorocentrum* species (**Annex 2**). Since DSP production by *P. rhathymum* was suspicious, a more in deep study based on a multidisciplinary approach was conducted. Results obtained from the combination of toxicological CBA, biochemical and chemical analysis with HPLC-based fractioning of an extract of *P. rhathymum* is described to assess DSP toxin production by this species (**Article 5**).

iii) Identification of CTX-containing fish samples using the Neuro-2a CBA for CTXs for ciguatera risk assessment in the Canary Islands

Ciguatera Fish Poisoning (CFP) was first confirmed in Canary Island in 2005 after the consumption of a 26-kg amberjack (*Seriola rivoliana*) captured along the coast of Canary Islands [134]. Other CFP outbreaks were further reported in 2008 and 2009 in Canary Islands [135] and Madeira Archipelago [136], which is only 260 miles north from Canary Archipelago. Additionally presence of the CTX-producing dinoflagellate *Gambierdiscus* spp. was reported at the same time in the Canary Islands [29, 38] and Crete [30]. Those events raised the question of a possible onset of ciguatera in areas of the Eastern Atlantic Ocean closed to Europe.

- In light of a possible onset of ciguatera in Europe, ciguatera risk analysis is highly advisable. However European regulation does not specify official reference method for CTXs nor maximum permitted levels of CTXs in fish. Although the current methodologies approaches available for CTXs determination were reviewed and their applicability as a routine monitoring tool for CTXs in fish discussed (**Article 6**). The impact of CFP in Europe on European policies was evaluated and key actions were proposed to prevent the risk of CFP in Europe (**Article 6**).

- The suitability of the Neuro-2a CBA for CTX was tested to verify the sensitivity and accuracy of the assay for the determination of CTXs in fish samples from the Canary Islands (**Article 7**). The assay was applied to various fish samples caught in Canary Islands in order to assess the risk of CFP in that area (**Article 7**).

Results of the present work will be presented following the order of the specific objectives described herein.

