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 toxinico en muestras naturales asociadas a la ciguatera: microalgas y pescado)

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III. RESULTS AND DISCUSSION

3.1.

CHAPTER I

METHODOLOGICAL APPROACHES FOR THE DETECTION AND CHARACTERIZATION OF MARINE TOXINS IN NATURAL SAMPLES

3.1.1. Results and Discussion

The future applicability of cell-based assays for marine toxin detection is conditioned to the development of suitable methods. Two issues have been examined to favor the use of cell culture for marine toxins detection in natural samples in the context of the present thesis: i) The elimination of the interferences of the accompanying matrix in natural samples that may be toxic to cells (**Article 1**) and ii) The specificity of CBA for the detection one group of toxins, the particular case of MTX (**Article 2**).

i) Elimination of the interferences of the accompanying matrix in natural samples:

Our first contribution (**Article 1**) demonstrates the advantages of implementing CBA coupled to chromatographic fractionation in order to overcome matrix interferences for the evaluation of marine toxins in natural samples. The examples of application of CBA/coupled to chromatographic fractionation in the publication include the identification of the toxic potential of *Gambierdiscus* spp. cultures, but also the application to the identification of toxins in shellfish, an issue that is not the object of this thesis, but that will be summarized. Presence of fatty acids in natural samples has been reported to severely affect the detection and quantification of a given compound e.g toxins. This effect has not only been observed in toxicological studies [109, 137] but also in chemical and biochemical studies [138].

The use of specific purification procedures of extracts previous to exposure of cells is necessary to overcome the possible interferences of the biological matrix. In the present study we proposed the use of HPLC- or solid phase extraction (SPE)-based chromatographic fractioning (or fractionation) as a strategy for the elimination of biological interferences of the matrix of natural samples to favor the detection of toxins using CBA. Basically, the different compounds within the biological samples are separated and distribute according to the different principles of chromatography (reversed-phase chromatography in the study case). Fraction collection of eluates is set according to the elution time or the volume of eluate.

Details are presented in both examples of **Article 1**, each fraction being further tested using CBA.

This approach is not new as it has already been used in past studies but it has received special attention in our study as it showed to present many advantages for the implementation of CBA in the field of marine toxins, especially for diagnostic and research purposes. We emphasis: (i) An increase of the concentration of TE equivalent exposed to cells. As an example. Caillaud et al. [53] described for the first time the production of levels of OA as low as 4.7 ng OA per 10^6 cells of the marine dinoflagellate *Prorocentrum rhathymum* using an HPLC-fractioning protocol; (ii) Chromatographic fractioning combined with CBA serves as a bioguided purification procedure of toxins in natural samples. In our study, SPE-fractioning of an extract of the marine dinoflagellate *Gambierdiscus* sp allowed the identification of non toxic and CTX-containing fractions. This bioguided purification procedure may facilitate the recovery of toxins while eliminating biological matrix containing-fractions. Chemical analysis may be further conducted for the confirmation of the identity of toxins in toxic fractions. This approach has been used for the confirmation of the identity of the different CTXs congeners present in CTXs-containing fish samples [117, 128]. The chromatographic fractioning combined with various detection methods i.e toxicological, chemical or biochemical is also susceptible to identify unidentified toxins or congeners of toxins responsible for the toxicity of a given sample.

Chromatographic fractioning combined with CBA is an interesting methodological approach susceptible to improve the applicability of CBA for diagnostic and research purposes as it may discriminate between non toxic and toxic samples while reducing matrix interferences and may help for the isolation and purification of toxins in natural samples.

ii) The specificity of CBA for the detection one group of toxins, the particular case of MTX

Maitotoxins (MTXs) may concomitantly be produced with CTXs by the marine dinoflagellate of the genus *Gambierdiscus* [139]. It is therefore important to establish specific CBAs in order to discriminate between these two toxins. **Article 2** of the present study describes the settlement of a CBA specifically conceived for the detection and quantification of MTX in extracts of *Gambierdiscus* based on the inhibitory effect of SK&F 96365 on the MTX-induced toxic effects as previously described elsewhere [133]. Optimal conditions for the assay were set according to the response obtained with a standard solution of MTX. The assay was developed using the Neuroblastoma (Neuro-2a) cell line as it showed great sensitivity to MTX (IC_{50}=3.38±0.4 µM and 2.72±0.2 µM MTX after respectively 2.5 and 24 hour exposure). SK&F 96365 was toxic to Neuro-2a cells after 3 hour exposure and for concentrations above 30 µM SK&F 96365. According to the sensitivity of the Neuro-2a cells to MTX standard solution and SK&F 96365, optimal conditions of the CBA specific for the detection of MTX were set as following: 30 minutes exposure of Neuro-2a cells to 30µM SK&F 96365 previous additional 2.5 hour exposure to MTX standard solution or extract of *Gambierdiscus* to test. The assay showed a clear inhibition of the MTX-induced toxic effects in presence of SK&F 96365 treatment, with 100% inhibition of the MTX-induced toxic effects for concentrations of MTX up to 11.4 nM MTX. In extracts of *Gambierdiscus*, presence of MTX was qualitatively evidenced by a significant decrease of toxic effects in the presence of SK&F 96365 treatment. The content of MTX in *Gambierdiscus* extracts was further quantitatively estimated according to the MTX calibration curve.

The Neuro-2a CBA for MTX is sensitive to and specific for MTX. Furthermore, the assay is rapid with a reduce time of exposure of 3 hours. Although the use of cell viability measurement for the assessment of toxic effects may be faster than previously reported

endpoint based on the observation of morphological alteration induced by MTX [88], and hence may be more advantageous as a screening tool of MTX.

The Neuro-2a CBA for MTX was successful for the qualitative and quantitative determination of MTX in both crude extracts of *Gambierdiscus* examined in the study. As a consequence, it may contribute to taxonomical studies as it can discriminate between MTX- and non-MTX producing species of *Gambierdiscus* or to physiological studies regarding toxin production within the genus. The assay is also likely to identify the interferences of MTX during purification procedures of CTXs. Examples of application of the assay will be further presented in the second chapter of the present thesis.

Chromatographic fractioning of natural crude extracts and the use of CBA specific for one group of marine toxins (e.g MTX, CTXs) are two methodological approaches likely to favor the detection and characterization of toxins in natural samples. Chapter II and III of the present thesis provides results of the application of both approaches to microalgal samples (i.e genus *Gambierdiscus* and *Prorocentrum*) (Chapter II) and to fish samples (Chapter III).

3.1.2. Publications

Article 1

Cell-based assay coupled with chromatographic fractioning: a strategy for marine toxins detection in natural samples.

Published in *Toxicology in Vitro* 23 (2009), 1591-1596

RESUMEN DE LA PUBLICACIÓN

La aplicación de los ensayos celulares a la detección de toxinas en muestras naturales (moluscos, pescados, microalgas) es un desafío debido a la elevada variedad de compuestos presentes en dichas muestras. Experimentalmente, un control negativo de mejillón no tóxico directamente expuesto a una concentración de 2.5 mg mL^{-1} a las células Neuro-2a produce 20% de mortalidad. Con el objetivo de eliminar las interferencias de la matriz con la respuesta celular, varios estudios describen la necesidad de purificar los extractos. En este artículo, se describe una estrategia experimental para detectar toxinas marinas en muestras naturales. Esta estrategia consiste en la separación de los diferentes compuestos de la muestra con un fraccionamiento por cromatografía acoplado a un sistema de detección de los compuestos tóxicos con ensayos sobre cultivos celulares. Dos ejemplos de aplicación de esta estrategia a muestras de mejillón y de microalgas son descritos.

El uso combinado del fraccionamiento por cromatografía y de los ensayos celulares permite distribuir los diferentes compuestos de la matriz a lo largo de las fracciones. Para las muestras de molusco, esta estrategia permite minimizar las interferencias de la matriz y por consecuencia permite incrementar la cuantidad de extracto analizado, lo que facilita la detección de toxinas en dichas muestras. Para las muestras de microalgas, esta estrategia no sólo es válida para el fin ya descrito sino también como ayuda a la separación de toxinas que pueden ser producidas conjuntamente por una misma especie y a la vez permite identificar la presencia de nuevas toxinas o toxinas que contribuyen a la descripción de las especies.

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 *Gambierdiscus* spp.

Published in *Toxicon* 56 (2010), 36-44

RESUMEN DE LA PUBLICACIÓN

Un ensayo con células de Neuroblastoma (Neuro-2a) ha sido desarrollado para detectar con especificidad los compuestos de tipo maitotoxina (MTX) en extractos de microalgas del genero *Gambierdiscus.* La MTX incrementa el nivel de calcio intracelular y el compuesto SK&F 96365 inhibe la entrada de calcio intracelular por bloqueo de los canales calcio dependiente de voltaje y por medio de receptores. SK&F 96365 ha sido previamente descrito como un inhibidor de los efectos tóxicos producidos por la MTX (Soergel et al, 1992). El ensayo celular especifico para MTX consiste en tratar las células con SK&F 96365 durante 30 minutos previos a la exposición de las células durante 2.5 horas a la solución de MTX o a los extractos objeto de análisis. La concentración en MTX que inhibe 50% de la viabilidad celular (IC_{50}) fue estimada a 3.38 ± 0.04 nM MTX después de 2.5 horas de exposición y 11.31 ±3.38 nM para las células previamente tratadas con SK&F 96365; indicando una significativa inhibición de los efectos tóxicos inducidos por la MTX en presencia de SK&F 96365. Aplicando este mismo ensayo a dos cepas de *Gambierdiscus* spp., se identificó la producción de compuestos de tipo MTX por las dos cepas estudiadas y cuantitativamente estimados a 1.382 y 0.125 mg MTX 10^6 células.

Este ensayo celular especifico para MTX constituye una herramienta para identificar las cepas de *Gambierdiscus* spp. productoras de compuestos de tipo MTX, y permite identificar las interferencias de la MTX que pueden dificultar la detección y purificación de CTXs a partir de extractos de microalgas.

3.2.

CHAPTER II

APPLICATION OF CELL-BASED ASSAY TO TOXIN DETECTION IN NATURAL SAMPLES:

MICROALGAL SAMPLES.

THE GENUS *GAMBIERDISCUS* AND *PROROCENTRUM*

3.2.1. Results and Discussion

Cell-based assays (CBA) have been applied to the study of toxin production by the marine dinoflagellates of the genus 1) *Gambierdiscus* and 2) *Prorocentrum*. Both methodological approaches previously presented which favor the use of CBA for toxin detection in natural samples have been applied: (i) the specificity of CBA for the detection and quantification of CTXs and MTXs in extracts of *Gambierdiscus* spp. and (ii) the chromatographic fractioning of microalgal crude extract as a bioguided purification procedure of toxic compounds produced concomitantly in microalgal extracts. Results presented in the second Chapter of the present thesis are clear examples of the broad aspects CBA contribute to, especially for taxonomical and physiological studies on microalgae, as well as for the identification of the hazard that may treaten aquaculture in a given area.

1) The genus *Gambierdiscus*

The Neuro-2a CBAs for CTX and MTX were suitable for the determination of CTX- and MTX-like toxicity in crude extracts of *Gambierdiscus* spp. and as a consequence they were suitable for the description of the toxicity of species of the genus *Gambierdiscus* (**Article 3**), especially for novel species. Results confirmed the CTX and MTX production by *G. pacificus* from Malaysia as previously reported in the literature [35, 140] and identified *Gambierdiscus* sp3 from Indonesia as a CTX and MTX producer. *Gambierdiscus* sp1 proposed novel species *G. excentricus* was identified for the first time as a CTX and MTX producer. Although quantification given by the Neuro-2a CBA identified *Gambierdiscus* sp1 as a high CTX and MTX producer respect to *G. pacificus* and *Gambierdiscus* sp3. *Gambierdiscus* sp2 proposed novel species from Crete was identified as a non toxic species and is the first species already analyzed for its toxicity [34, 35], for which no MTX is detected. The identification of different pattern of CTX and MTX production between the different species of *Gambierdiscus* spp, especially for *Gambierdiscus* sp1 and sp2 in this study, might be used as additional chemotaxonomical character to differentiate these two

distinct novel species. Furthermore the identification of the CTX production by the different *Gambierdiscus* spp. analyzed in the present study suggested Malaysia, Indonesia and the Canary Islands susceptible to suffer CFP. Toxicity analysis of the different *Gambierdiscus* spp. have been realized using cell pellet obtained under culture conditions and analysis of natural populations of *Gambierdiscus* spp. would definitively help to understand the risk of CFP in the distinct areas examined in the study. In the Canary Islands, examination of natural population of *Gambierdiscus* spp. would definitively help to understand if *G. excentricus* is responsible for the recently reported CFP events in that area [134, 135].

Since CFP may occur 3 months after occurrence of toxic (CTX-producing) *Gambierdiscus* blooms [141], the monitoring of the presence and toxicity of *Gambierdiscus* blooms may be used as a preventive action to reduce CFP [142, 143]. Additionally, the use of solid phase toxin tracking (SPATT) devices for the detection of dissolved CTXs in seawater has been proposed in our study as a strategy for simulating the presence of toxic *Gambierdiscus* blooms (**Article4**). For that purpose, the suitability of the HP20 Diaon® resin for tracking of dissolved CTX and MTX was verified under *in vitro* condition (i) using standard solutions of CTX1B and MTX and (ii) then applied to a culture of *G. pacificus*.

Numerous studies have reported the use of biochemical and chemical studies for the detection of toxins tracked by the resin [144, 145, 146, 147]. Our study is the first report of the use of *in vitro* toxicological assay for the detection of tracked toxins by the resin. The Neuro-2a CBA endures moderately high content of resin equivalent (RE) with a limit a RE exposure set at 100 mg mL^{-1} in our study in order to avoid negative interferences of the matrix of resin (**Article 4**). Presence of polymeric material lixiviated from resin HP20 after desorption of toxins from the resin, as previously reported [148] and confirmed by LC-MS in our study, may be responsible for the toxic effects elicited by the matrix of resin. Under the

limit of RE exposure established, the Neuro-2a CBAs specific for CTXs and MTXs were suitable for the detection and quantification of CTX1B and MTX tracked by the resin with respective recoveries of 85.5 (±13.2) and 66.2 (±11.9) %, 72h exposure of the resin to dissolved CTX1B and MTX being set as the optimal exposure time in our study (**Article 4**). When applied to a culture of *G. pacificus* and under the limit of RE exposure, the use of SPATT combined with Neuro-2a CBAs specific for CTX and MTX allowed a quantitative estimation of the CTX- and MTX-like compounds dissolved in the culture medium of *G. pacificus*.

LC-MS analysis was performed on resin extracts of *G. pacificus* culture in order to tentatively identify the CTXs congeners responsible for the CTX-like toxicity in resin extracts (**Article 4**). Due to the lack of proper analytical standards for all CTXs congeners, identification of CTXs congeners was conducted using Multiple Reaction Monitoring (MRM) analysis performed on the basis of structural similarities of the precursor and products ions provided by LC-MS/MS analysis and described in the literature. In order to confirm the CTX-like activity of the different CTXs congeners identified by MRM analysis, chromatographic fractioning of resin extract (at T44-T47) was combined with the Neuro-2a CBA for CTXs and allowed identifying CTX-like toxic fractions which corresponded to the retention time in which CTX congeners and isomeric forms were identified by MRM analysis. The MRM approach allowed identifying and quantifying various CTXs congeners in resin extracts from days 16 to 47 of *G. pacificus* culture, congeners 51-hydroxyCTX3C and 2,3-dihydroxyCTX3C being identified as the most abundant.

Quantifications given by both the Neuro-2a CBA and LC-MS analysis were estimated according to their respective CTX-1 calibration curves. Both methods agreed in the detection of a higher content of dissolved CTX1B equivalents at the stationary (T34-T37) and senescent (T44-T47) phases of *G. pacificus* culture (**Article 4**). However quantifications given by LC-

MS were higher than that estimated by the Neuro-2a CBA. As an example, LC-MS quantification at the decade phase (38 ng CTX1B L^{-1}) was approximately one hundred time higher than the content estimated using the Neuro-2a CBA (400 pg CTX1B L^{-1}). One of the explanations proposed is that estimations of the Neuro-2a CBA are given in equivalent of CTX-1 which is one of most potent CTX among the different CTXs congeners; however the different CTXs congeners may display distinct toxic potency even lower than CTX1B. Results of the Neuro-2a CBAs for CTX and MTX, and LC-MS analysis for CTXs have confirmed the suitability of the resin HP20 for the recovery of dissolved CTX and MTX dissolved in the culture medium of *G. pacificus*. This study encourages the use of SPATT *in situ* in order to evaluate the suitability of SPATT for the monitoring of the incidence and prevalence of toxic *Gambierdiscus* blooms, especially in ciguatera endemic areas.

Additionally to the applicability of SPATT as a warning method for the presence of toxic *Gambierdiscus* spp. blooms, the use of SPATT was described in our study as an interesting tool for increasing the knowledge on CTX and MTX production by *Gambierdiscus* spp. The Neuro-2a CBAs allowed quantifying the intracellular and dissolved CTX-1 and MTX content per cell of *G. pacificus* according to the different growth phase of the *G. pacificus* culture. We emphasis a negative correlation between intracellular toxin biosynthesis and high division rate as previously reported in the literature [149]. Although a higher dissolved CTX content per cell was evidenced at the exponential and senescent growth phase. Hypothesis of a release of CTXs after cell membrane disruption at the senescent growth phase and a possible excretion or release of CTXs at the exponential growth phase were proposed. Although the high abundance of hydroxyl derivates of CTXs found in resin extracts suggested that *G. pacificus* may oxidize CTXs in order to favor their release in the culture medium. These preliminary results encourage the use of SPATT combined with the Neuro-2a CBAs for investigating toxin production during physiological studies with *Gambierdiscus* spp.

1) The genus *Prorocentrum*

Production of OA and derivatives has been previously described for various species of the genus *Prorocentrum* which are considered as a putative link to the Diarrheic Shellfish Poisoning (DSP). A study on the diversity and toxicity (**Annex 2**) of various species of *Prorocentrum* spp isolated from Malaysia was conducted in order to evaluate the risk of DSP in that area. A fibroblast cell-based assay was used for discriminating between toxic and non-toxic species based on a decrease of cell viability when exposed to crude extracts of three strains of *P. lima*, one strain of *P.* cf *faustiae* and one strain of *P. rhathymum*. All strains studied were toxic to fibroblasts cells. Although as no CBA specific for the detection of OA is currently available, LC-MS analysis was required in order to confirm the presence of OA and derivatives by the different strains. Only *P. lima* strains were confirmed to produce OA and various derivatives.

A more in deep study on *P. rhathymum* (**Article 5**) was conducted in order to try to resolve the identity of toxins responsible for the toxic effects previously reported (**Annex 2**). For that purpose, HPLC- based chromatographic fractioning of *P. rhathymum* crude extract was combined with various detection methods, i.e the Neuro-2a CBA, the biochemical PPIA for the detection of OA and derivatives (see Introduction, section 3.1.3), and LC-MS analysis for lipophilic toxins. The approach allowed increasing the concentration of extract of *P. rhathymum* cells (at a concentration of 2.0 x 10^6 cells equivalents mL^{-1}) to which Neuro-2a cells were exposed. Chromatographic fractioning also allowed increasing the amount of *P. rhathymum* extract up to 3.7 x 10^6 cells equivalents used for the PPIA. The combination of both the Neuro-2a CBA and PPIA allowed the identification of one fraction toxic to Neuro-2a cells with inhibiting activity on PP2a, thus suggesting the presence of OA or derivatives in fraction number 29. Quantifications of OA equivalents given by both methods were estimated

according to their respective OA calibration curve and were 4.7 (Neuro-2a CBA) and 8.3 ng (PPIA) OA equivalents per 1 x 10^6 cells. The presence of OA and OA isomer in fraction 29 was confirmed by LC-MS analysis and was estimated at 2.9 ng OA equivalents in 1 x 10^6 cells. These results show the suitability of the chromatographic fractioning for the detection of low levels of toxins in microalgal samples and are the first report of the production of OA by P. *rhathymum*. This finding suggests *P. rhathymum* as a putative DSP risk species and contrast the hypothesis of previous phylogenetical studies [5] that suggested OA production by *Prorocentrum* species to be limited to the symmetric *Prorocentrum* species of Clade 2 (**Figure 8**) which did not include *P. rhathymum*.

Additionally to fraction 29, other fractions were found toxic to Neuro-2a cells with no effects or activation of PP2a (fraction 6 and fraction 7), and hence suggested that *P. rhathymum* may produce other toxins. The use of chromatographic fractioning combined with toxicological and biochemical tools in microalgal extracts is likely to identify the production of new toxins or bioactive compounds.

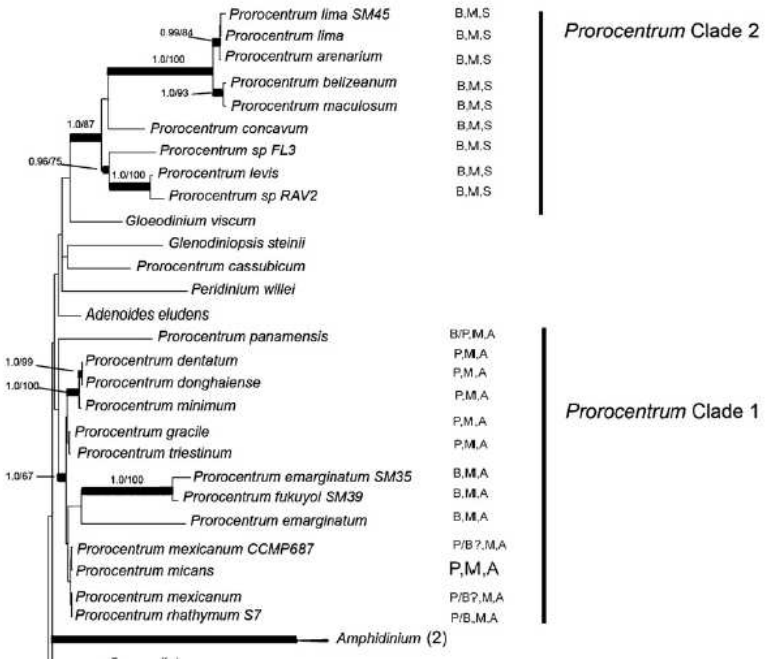

Figure 8: Phylogenetic tree inferred from the SsU sequences of *Prorocentrum* species. S= valve symmetry A= valve asymmetry [5].

3.2.2. Publications

Article 3

Comparative study of the CTX- and MTX-like toxicity of various *Gambierdiscus* spp. from distinct geographical origin using a Neuroblastoma (Neuro-2a) cell-based assay

For submission in *Toxicon*

RESUMEN DE LA PUBLICACIÓN

Varias cepas de las dinoflageladas marina *Gambierdiscus* sp1 procedente de las Islas Canarias (España) (cepas VGO790, VGO791, VGO792) propuesta como una nueva especies *G.excentricus*, *Gambierdiscus* sp2 procedente de Creta (Grecia) (cepa KC81), *G. pacificus* procedente de Malasia (cepas G10DC, GDSA01, GPSi) and *Gambierdiscus* sp3 procedente de Indonesia (cepas VGO917, VGO920) han sido examinadas por su contenido en compuestos de tipo ciguatoxina (CTX) y tipo maitotoxina (MTX). Dos ensayos con células de Neuroblastoma (Neuro-2a) específicamente concebidos para la detección de toxicidad de tipo CTX y de tipo MTX han sido aplicados a los extractos crudos de las diferentes cepas de *Gambierdiscus* spp. Todas las cepas de *Gambierdiscus* sp1, *G. pacificus* y *Gambierdiscus* sp3 han sido identificadas como productoras de cantidades significativas de compuestos de tipo CTXs y estimadas entre 0.06 (±0.01) y 1.10 (±0.19) pg equivalentes de CTX tipo 1 (CTX-1) por célula. La producción de compuestos tipo CTX fue mayor para las cepas de *Gambierdiscus* sp1 respecto a las cepas procedentes de Malasia e Indonesia. La producción de compuestos de tipo MTX fue significativa para *Gambierdiscus* sp1, *G. pacificus* y *Gambierdiscus* sp3, y estimada entre 0.02 (±0.01) y 1.38 (±0.31) ng equivalentes de MTX por célula. *Gambierdiscus* sp1 ha sido identificada como una potente productora de compuestos de tipo MTX respecto a las demás cepas de Malasia e Indonesia. La producción de compuestos de tipo CTX por *Gambierdiscus* sp3 no es significativa tratándose de la primera cepa para la cual no se ha detectado producción de MTX. El análisis de la toxicidad de las diferentes cepas de *Gambierdiscus* spp. Contribuye a la evaluación del riesgo de ciguatera en las zonas de estudio e incrementa el conocimiento del potencial toxico de las diferentes especies de *Gambierdiscus* spp.

Comparative study of the CTX- and MTX-like toxicity of *Gambierdiscus* spp. cultures from distinct geographical origin using a Neuroblastoma (Neuro-2a) cell-based assay.

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Abstract

Gambierdiscus sp1. from Canary Islands (Spain) proposed *G. excentricus*, *Gambierdiscus* sp2 from Crete (Greece), *G. pacificus* from Malaysia and *Gambierdiscus* sp3 from Indonesia have been examined for their ciguatoxin (CTX) and maitotoxin (MTX) production using the neuroblastoma Neuro-2a cell-based assay specifically conceived for the detection of CTX- and MTX-like toxicity. Production of CTX-like compounds was significant for *Gambierdiscus* spp. from Canary Islands, Malaysia and Indonesia and was ranging between 0.06 (±0.01) and 1.10 (±0.19) pg CTX1B equivalent cell^{-1}; the highest CTX-like content being measured for *Gambierdiscus* sp1 from Canary Islands. Production of MTX-like compounds was significant for *Gambierdiscus* spp. from Canary Islands, Malaysia and Indonesia, and was ranging between 1.38 (±0.31) and 0.02 (±0.01) ng MTX equivalent cell^{-1}. *Gambierdiscus* sp1 from Canary Islands (proposed *G. excentricus*) was identified as a potent MTX producer when compared to the other strains. *Gambierdiscus* sp2 from Crete was identified as a non significant CTX-like producer and was the only strain lacking of the production of MTX-like compounds.

Keywords

Gambierdiscus, ciguatoxin, maitotoxin, neuroblastoma, cell-based assay, cytotoxicity, Mediterranean, Malaysia, Indonesia, Canary Islands

Introduction

Ciguatera fish poisoning (CFP) occurs after consumption of fish contaminated with CTXs (Yasumoto et al., 1977). CFP is found endemic in tropical and subtropical areas, however recent studies suggested a possible extension of the ciguatera to more temperate waters of the North Eastern Atlantic Ocean such as in Canary Islands and Salvagems Islands (Boada et al., 2010; Caillaud et al., 2010a; Gouveia et al., 2009; Otero et al., 2010; Pérez-Arellano et al., 2005). The marine dinoflagellate genus *Gambierdiscus* is a likely producer of precursors of CTXs (Satake et al., 1993b) further responsible for CFP after accumulation and transformation of CTXs along the food web from herbivorous to carnivorous fish that feed on them (Mills, 1956; Randall, 1958). Hence, presence of CTXs producing strains of *Gambierdiscus* spp. is likely to be considered as a bioindicator for the risk of CFP in a given ecosystem (Chinain et al., 2010b; Darius et al., 2007). Additionally to the production of CTXs, the same genus may also produce concomitantly other toxins i.e maitotoxins (MTXs) (Holmes et al., 1990; Yasumoto et al., 1977), gambierol (Satake et al., 1993a) and gambieric acid (Nagai et al., 1993).

Previous to the first report of CFP, by consumption of fish (*Seriola rivoliana*), in the Canary Islands in 2005 (Pérez-Arellano et al., 2005), the first description of the presence of *Gambierdiscus* spp. in the Canary Islands was reported in 2004 by Fraga et al. (2004). Clonal cultures of specimens of *Gambierdiscus* sp1 from the Canary Island were established and further analyzed for morphological and taxonomical characterization. Due to uncertainties in the taxonomy of the genus *Gambierdiscus* (Richlen et al., 2008; Tester et al., 2008), phylogenetical analysis are required to unequivocally determine the species level of *Gambierdiscus* sp according to the recent revision proposed in Litaker et al. (2009). The description based on morphology and genetics of *Gambierdiscus* sp1 as a new species, *G. excentricus,* is presently ongoing (S. Fraga, personal communication).

In 2008, description of the presence of *Gambierdiscus* sp2 in Crete (Greece) was the first evidence of this genus in the Mediterranean sea (Aligizaki et al., 2008). Morphological characterization of the specimens from Crete confirmed it belonged to the genus *Gambierdiscus* (Aligizaki and Nikolaidis, 2008), and currently it is also being described as a new species (K.

Aligizaki, personal communication). Revision of long term samples from Crete proved that *Gambierdiscus* sp was present in Crete at least since 2003 (Aligizaki and Nikolaidis, 2008). However despite of the presence of the genus *Gambierdiscu*s in this area, epidemiological data have not reported any event of CFP in Crete for the time being.

Mohammad-Noor et al. (2007) reported a study on the diversity of benthic dinoflagellates present in Malaysia in order to improve knowledge on the risk of seafood intoxication in that area. Various *Prorocentrum* spp. were recorded in addition to the presence of the benthic dinoflagellate of the genus *Gambierdiscus* which was identified as *G. pacificus* (Mohammad-Noor et al., 2007). Additional strains of *Gambierdiscus* sp. from the Pacific Ocean were isolated from a sample taken in Indonesia, leading to the establishment of two clonal cultures, for which identification is currently ongoing (personal communication, S. Fraga).

The aim of the present study was to evaluate the CTX and MTX production by the different strains isolated from Canary Islands, Crete, Malaysia and Indonesia. The information relative to the production of CTXs by the different strains will help to evaluate the risk of CFP in the area from which they were isolated. Additionally the information relative to the MTX and CTX production will help to characterize the different species/strains of *Gambierdiscus* spp. in addition to morphology and genetics, especially for novel species (*Gambierdiscus* sp1 from Canary Islands and *Gambierdiscus* sp2 from Crete).

In the present study, the CTX- and MTX-like toxicity of the different *Gambierdiscus* spp. strains was assessed using the Neuroblastoma (Neuro-2a) cell-based assay (CBA).

Ciguatoxins (CTXs) are voltage-gated sodium (Na^+) channel activator toxins which are unlikely to induced cell death when exposed to Neuro-2a cells due to the diversity of Na^+ channel systems that may compensate the intracellular Na^+ increase and the ATP dependent Na^+/K^+ pump that may counteract intracellular Na^+ increase caused by CTXs. The ouabain (O) /veratridine (V) dependent Neuro-2a CBA for CTXs (Manger et al., 1995; Manger et al., 1993) takes advantage of the increased sensitivity of Neuro-2a cells to CTXs when pretreated with O/V. Ouabain blocks Na^+ influx through an inhibition of the ATP dependent Na^+/K^+ pump (Catterall, 1986) and V increases Na^+

permeability through a blockage of the voltage-gated Na^+ channel in a open position (Catterall and Nirenberg, 1973), leading to cell mortality according to the concentration tested. Exposure of Neuro-2a cells to CTXs in presence of O/V treatment increases cell mortality elicited by O/V treatment.

MTXs increase intracellular calcium (Ca^{2+}) through mechanisms that remain unclear at this time (Gusovsky and Daly, 1990). Although SK&F 96365 an inhibitor of the voltage-gated Ca^{2+}channel and of the receptor mediated Ca^{2+} entry (Merritt et al., 1990), was described as an inhibitor of the MTX-induced toxic effects (Soergel et al., 1992). This antagonistic effect was improved for the development of a Neuro-2a CBA specific for the detection of MTX in natural samples (Caillaud et al., 2010b).

Materials and Methods

Origin of Gambierdiscus *spp. strains*

Nine strains of *Gambierdiscus* spp. from Canary Islands (North Eastern Atlantic Ocean), Crete (Eastern Mediterranean Sea), Malaysia and Indonesia (South West Pacific Ocean) have been examined for their toxicity on the Neuro-2a CBA (See **Table 1** and **Figure 1**). Taxonomical identification of *Gambierdiscus* spp. specimens from the Canary Islands, Crete and Indonesia is currently ongoing.

Cultures of Gambierdiscus *spp. and preparation of microalgal crude extracts*

Gambierdiscus spp. strains were cultured in a 33 practical salinity unit (psu) modified ES medium (Provasoli, 1968) at 24ºC under a 12:12 light:dark regime with a photons flux rate of 80 µmol photons m^{-2} s^{-1} (QSL-2100 Radiometer, Biospherical instruments, San Diego, USA) and under permanent aeration. Cultures in the stationary growth phase (**Figure 2**) were harvested through filtration under gentle vacuum using GF/F filters (Whatman). Cell density of cultures is presented in **Table 1**. Filters were stored in absolute methanol at -20ºC until toxin extraction.

Toxin extraction procedure consisted in the sonication of filters during 30 minutes at 38% amplitude (Sonics Vibracell, Newton, USA) in an extraction volume (Ve) of absolute methanol proportional to total cell density with Ve in mL equivalent to 10 x 10^6 cells. Methanol was further recovered after 5 minutes centrifugation at 4 ºC at 600 g (Joan MR23i, Sant Herblain, France). This

procedure was repeated once with absolute methanol and twice with methanol:water (50:50, v:v). Supernatants were pooled and evaporated until dryness at 40 ºC (Büchi R-200 or Büchi Syncore, Flawil, Switzerland). Extracts were finally dissolved in absolute methanol and keep at -20 ºC until analysis.

Toxin standards

Pacific type 1 CTX (CTX1B) standard solution was purified from moray eel (*Lycodontis javanicus*) as described in Lewis et al. (1991) and was provided by R.J. Lewis (The Queensland University, Australia).

MTX standard solution was a generous gify from Pr T. Yasumoto (Japan Food Research Laboratory, Japan) extracted from a cultured strain of *G. toxicus* isolated from Gambier Island (French Polynesia) and purified according to the procedure described in Yokoyama et al. (1988).

Neuroblastoma cells maintenance and cytotoxicity assays for CTX- and MTX-like toxicity detection

Neuro-2a cells (ATCC, CCL131) were maintained in 10% foetal bovin serum (FBS) RPMI medium (Sigma) at 37ºC in a 5% CO2 humid atmosphere (Binder, Tuttlingen, Germany) as previously described in Cañete and Diogène (2008). For experiments, cells were inoculated in a 96-well microplate at a density of 35 000 cells per well and incubated 24h before cytotoxicity assays under the same conditions as described for cell maintenance.

In order to detect CTX-like toxicity in *Gambierdiscus* spp. crude extracts, Neuro-2a cells were treated with 0.1 mM ouabain (O) (Sigma-Aldrich, USA) and 0.01 mM veratridine (V) (Sigma-Aldrich, USA) previous to exposure to CTX standard solution or *Gambierdiscus* spp. extracts for 24 hours. The concentrations of O and V were set to allow a reduction of 20% cell viability as described before (Cañete and Diogène, 2008). Sensitivity of the Neuro-2a cells to the presence of CTX was calibrated each day of the experiment with a standard solution of CTX1B at 20 ng mL^{-1}.

Regarding the detection of the MTX-like toxicity in *Gambierdiscus* spp. crude extracts, Neuro-2a cells were treated with 30µM SK&F 96365 (Sigma-Aldrich, USA) during 30 minutes

previous to exposure to MTX standard solution and *Gambierdiscus* spp. extracts for 2.5 hours (Caillaud et al., 2010b). Sensitivity of Neuro-2a cells to MTX was calibrated each day of the experiment using a MTX standard solution at 2.5 µg mL^{-1}.

Cytotoxic effects measurement and analysis

After 24 hour exposure (for CTX-like toxicity evaluation) and 2.5 hour exposure (for MTX-like toxicity evaluation), cell viability was used as an endpoint to quantitatively assess cytotoxic effects. Cell viability was measured using the colorimetric [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium] MTT (Sigma-Aldrich, USA) method (Mosmann, 1983). Absorbance was read at 570 nm using an automated multi-well scanning spectrophotometer (Biotek, Synergy HT, Winooski, Vermont, USA). Absorbance values are further expressed in percentage of viability respect to its respective control (with and without O/V or SK&F 96365 treatment).

Results of cell viability were analyzed using the software Prism 4 (GraphPad, San Diego, California, USA). A dose-response curve fit with sigmoid regression curve (with variable slope) is determined for each experiment. The volume of extract or quantity of standard that inhibited 50% cell viability (IC_{50}) is estimated from the dose-response curve and is further used as a toxicological parameter for results analysis.

The content in CTX1B equivalents per cells of *Gambierdiscus* spp. (when differences between O/V treated and non-treated cells are significant) is quantitatively estimated by substituting the quantity of CTX1B responsible for 50% cell viability inhibition (IC_{50} of the CTX1B calibration curve with O/V treatment) for the number of *Gambierdiscus* spp. cells also responsible for 50% cell viability inhibition (IC_{50} of *Gambierdiscus* spp. crude extract) in both conditions (with and without O/V treatment). Since unspecific toxic effects are measured in crude extracts of *Gambierdiscus* spp. (toxic effects measured in absence of O/V treatment), the equivalent of CTX-like compounds per cells of *Gambierdiscus* spp. is finally estimated after subtraction of the CTX equivalents in presence of O/V treatment with the CTX equivalents in absence of O/V treatment (Lartigue et al., 2009).

For the determination of the presence of MTX-like compounds, the antagonistic effects of SK&F 96365 on the MTX-induced toxic effects is assess by the measurement of a doses ratio (DR)

above 1 (Caillaud et al., 2010b). DR is equivalent to the ratio of the IC_{50} with SK&F 96365 between the IC_{50} without SK&F 96365. When DR > 1, the content in MTX equivalents is quantitatively estimated by substituting the quantity of MTX responsible for 50% cell viability inhibition with SK&F 96365 treatment (IC_{50} of the MTX calibration curve with SK&F 96365 treatment) for the number of *Gambierdiscus* spp. cells also responsible for 50% cell viability inhibition with SK&F 96365 treatment (IC_{50} of *Gambierdiscus* spp. crude extract with SK&F 96365 treatment) (Caillaud et al., 2010b).

Unpaired t-test (comparison of two means) and ANOVA (comparison of three or more means) with a 95% confidence level were used to analyze significant differences between experiments.

Results

CTX-like toxicity

All extracts of strains of *Gambierdiscus* spp were toxic to Neuro-2a cells with and without pretreatment with O/V (**Table 2**). Toxic effects were significantly higher in the presence of O/V treatment for all strains from the Canary Island, Malaysia and Indonesia, thus indicating the production of CTX-like compounds by these strains (**Figure 3**). Estimations of the equivalents in CTX1B per cells are given in **Table 2**. Toxic effects elicited after exposure of Neuro-2a cells to *Gambierdiscus* sp2 from Crete (Strain KC81) in both experimental conditions (with and without O/V treatment) were not significantly different (t test, $p>0.05$). However Neuro-2a cells pretreated with O/V tend to be slightly more sensitive to *Gambierdiscus* sp2 crude extracts than non O/V treated cells **(Table 2)**, suggesting the production of a very low amount of CTX-like compounds by *Gambierdiscus* sp2.

MTX-like toxicity

All strains of *Gambierdiscus* spp were toxic to Neuro-2a cells after 2.5 hour exposure with and without SK&F 96365 pretreatment (**Table 3**), with toxic effects significantly different between both treatments (t test, $p<0.05$) (**Figure 4**). DRs calculated for *Gambierdiscus* spp from Canary Islands, Malaysia and Indonesia were > 1, suggesting the production of MTX-like compounds by these strains.

MTX-like equivalents produced by each strain are given in **Table 3**. DR<1 for *Gambierdiscus* sp2 suggested the non-production of MTX-like compounds by this strain (**Table 3**).

Discussion and Conclusions

Production of CTX-like compounds was identified for *Gambierdiscus* spp. from the Canary Islands, Malaysia and Indonesia, and non significant for *Gambierdiscus* sp2 from Crete (**Table 2**, **Figure 5**). Among the three strains from Canary Islands, strains VGO790 and VGO791 produce significantly higher levels of CTX-like compounds respect to strain VGO792 (ANOVA, $p<0.01$). No significant differences were identified among strains isolated from Malaysia, as well as among both strains from Indonesia.

A comparative analysis of CTX-like content between strains from the four distinct geographical origins, identified *Gambierdiscus* sp1 from Canary Islands as a significant higher CTX-like producer respect to *Gambierdiscus* sp2 and sp3 from Indonesia and Crete (ANOVA, $p<0.001$). The CTX-like content in strains VGO790 and VGO791 from Canary Island was significantly higher than the CTX-like content in *G. pacificus* from Malaysia (ANOVA, $p<0.001$). Although no significant differences were found between CTX-like contents in *Gambierdiscus* spp. from Indonesia, Malaysia and Crete (ANOVA, $p>0.05$).

Estimation of the CTX-like content in the nine strains of *Gambierdiscus* spp. of the present study ranged between 0.06 and 1.10 pg CTX1B eq. cell^{-1}. These values are in the same order of previously reported data on the CTX-like content in *Gambierdiscus* spp. Rhodes et al (2009) reported the production of 0.4 pg CTX3C eq. cell^{-1} (equivalent to 0.04 pg CTX1B cell^{-1}) by *G. australes* from the Cook Islands using the Neuro-2a CBA for CTXs. Chinain et al (2010a) reported Receptor Binding Assay (RBA) values for *G. toxicus*, *G. australes*, *G. pacificus*, *G. belizeanus* and *G. polynesiensis* from French Polynesia ranging from 0.017 and 11.9 pg CTX3C (equivalent to 0.0017 and 1.19 pg CTX1B eq cell^{-1}), *G. polynesiensis* being described as a potent CTXs producer. This result confirmed the suitability of the Neuro-2a CBA for the determination of CTXs in *Gambierdiscus* spp. crude extracts.

Production of CTXs by *Gambierdiscus* sp1. proposed *G. excentricus* supports the discussion relative to an emergent risk of CFP in Canary Islands (Caillaud et al., 2010a). As stated, CFP was first reported in 2005 after consumption of a 26-kg amberjack (*Seriola rivoliana*) caught in Canary Islands (Pérez-Arellano et al., 2005). Later on, in 2008 and 2009, other CFP events were reported in Canary Islands and confirmed by Boada et al.(2010). However, association between CFP in Canary Islands with its original CTXs producer would need further investigation. Still more strains of *Gambierdiscus* sp. belonging to a different species than the proposed *G. excentricus* have been recently isolated and will require further analysis respect to their CTXs production. In the present study, the CTX-like toxicity was confirmed in *G. pacificus* as previously described (Chinain et al., 2010a; Chinain et al., 1999) and identified in *Gambierdiscus* sp3 from Indonesia. Hence, Malaysia and Indonesia are both areas susceptible to suffer CFP. To the best of our knowledge, no data are currently available regarding the occurrence of CFP in Malaysia and Indonesia. However CFP was suspected in Sabah (Malaysia) since 2004 (N. Mohammad-Noor, personal communication). The fact that *Gambierdiscus* sp2 from Crete does not produce significant levels of CTXs is in accordance with epidemiological data that did not have recorded any CFP events in that area for the time being. Nevertheless, taking into consideration that only one strain of *Gambierdiscus* from Crete was isolated and considering that accumulation of CTXs in carnivorous fish may result of a long-term transfer along the food web it is not possible to exclude a possible risk of CFP in that area. Toxicity analysis of the different *Gambierdiscus* spp. have been realized using cell pellet obtained under *in vitro* conditions and analysis of natural populations of *Gambierdiscus* spp. would definitively help to understand the risk of CFP in the distinct areas examined in the study.

All the strains of *Gambierdiscus* spp from Canary Islands, Malaysia and Indonesia have been identified as MTX-like producers (**Table 3**, **Figure 6**). Among the three strains from the Canary Islands, strain VGO790 produces significantly higher MTX-like compounds than strains VGO791 and VGO792 (ANOVA, $P<0.001$). Production of MTX-like compounds by the different strains of *G. pacificus* from Malaysia was statistically similar ($p>0.05$). Similar production of MTX-like

compounds was observed in both strains from Indonesia. *Gambierdiscus* sp2 KC81 from Crete was the only strain lacking the production of MTX-like compounds.

Gambierdiscus sp1 from Canary Islands was a significant higher MTX-like producer respect to *Gambierdiscus* spp. from Malaysia and Indonesia (ANOVA, $p<0.05$). No significant differences in the MTX-like production was found between strains from Malaysia and Indonesia (ANOVA, $p>0.05$). To the best of our knowledge, *Gambierdiscus* sp2 from Crete is the first strain examined for its toxicity that does not produces MTX-like compounds under *in vitro* conditions. Nevertheless, no information exists in the literature to compare MTX-like content of the already described species of *Gambierdiscus* spp with the MTX-like content estimated in the nine strains of the present study with the SK&F 96365 based Neuro-2a assay.

Presence of MTX was initially identified in the viscera of herbivorous fish (Yasumoto et al., 1976) however it has never been reported to induce seafood intoxication in humans probably due to its low oral potency and low capacity for accumulation in fish tissue (Yasumoto et al., 1976). Hence, the identification of the production of MTX in *Gambierdiscus* spp may have little value for seafood risk assessment. However these data help characterize the toxic potency of the different species of the genus *Gambierdiscus*. Still, the non MTX-like production by *Gambierdiscus* sp2 from Crete and the high MTX production by *Gambierdiscus* sp1 (proposed *G. excentricus*) from the Canary Islands might be used as additional chemotaxonomical character to differentiate these two distinct novel species.

In the present study, the Neuro-2a CBA for CTX and MTX was suitable for the identification of the production of CTX- and MTX-like compounds in crude extracts of the different strains of *Gambierdiscus* spp. However to definitively characterize toxin profile in *Gambierdiscus* spp, chemical analysis would be required to definitively identify the CTXs and MTX congeners responsible for the CTX- and MTX-like toxicity.

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Table 1: *Gambierdiscus* spp. strains analyzed in the study: origin and cell density of the culture analyzed for toxin production * novel species proposed *G. excentricus* (S. Fraga, unpublished data).

	Strain	Origin
Gambierdiscus sp1	VGO790 VGO791 VGO792	Canary Island, Spain
Gambierdiscus sp2	KC81	Crete, Greece
Gambierdiscus sp3	VGO917 VGO920	Indonesia
G. pacificus	GDSA01 GPSi G10DC	Sabah, Malasia

Table 2: CTX-like toxicity in *Gambierdiscus* spp.

Origen	Species	Strain	IC_{50} $^{O/V-}$ ±SD (cells eq. mL^{-1})		IC_{50} $^{O/V+}$ ±SD (cells eq. mL^{-1})		p value (t test)	pg CTX1B eq $cell^{-1}$ ±SD	
Canary Islands	*G. excentricus*	VGO790	2,11	0,26	0,87	0,10	0,00	1,10	0,19
		VGO791	1,60	0,28	0,65	0,23	0,01	1,05	0,18
		VGO792	4,58	0,86	2,35	0,77	0,00	0,37	0,17
Crete	*Gambierdiscus* sp2	KC81	4362,23	1145,33	2537,33	953,81	0,10	-	-
Indonesia	*Gambierdiscus* sp3	VGO917	91,65	6,53	43,57	10,12	0,00	0,08	0,01
		VGO920	76,63	15,23	26,50	4,89	0,01	0,06	0,01
Malaysia	*G. pacificus*	GDSA01	59,89	22,43	17,63	4,95	0,03	0,14	0,04
		GPSi	50,40	20,45	14,03	4,78	0,04	0,18	0,01
		G10DC	174,10	35,48	71,07	20,36	0,01	0,08	0,09

Table 3: MTX-like toxicity in *Gambierdiscus* spp.

Origen	Species	Strain	$IC_{50}^{SK\&F-}$ ±SD (cells eq. mL^{-1})		$IC_{50}^{SK\&F+}$ ±SD (cells eq. mL^{-1})		p value (t test)	DR	ng MTX-1 eq $cell^{-1}$ ±SD	
Canary Islands	*G. excentricus*	VGO790	7,73	0,64	28,81	5,97	0,00	3,73	1,38	0,31
		VGO791	14,40	0,33	68,99	24,88	0,02	4,79	0,60	0,24
		VGO792	19,78	3,62	71,51	19,43	0,01	3,62	0,48	0,16
Crete	*Gambierdiscus* sp2	KC81	27780,4	2173,2	23460,2	1154,1	0,04	0,84	0,00	0,00
Indonesia	*Gambierdiscus* sp3	VGO917	100,38	5,30	361,86	17,82	<0.0001	3,60	0,09	0,05
		VGO920	100,56	11,97	340,46	135,14	0,04	3,39	0,11	0,04
Malaysia	*G. pacificus*	GDSA01	88,36	8,57	328,36	80,67	0,01	3,72	0,11	0,03
		GPSi	130,28	23,37	372,73	111,99	0,02	2,86	0,09	0,02
		G10DC	761,23	68,31	1922,04	110,25	<0.0001	2,52	0,02	0,01

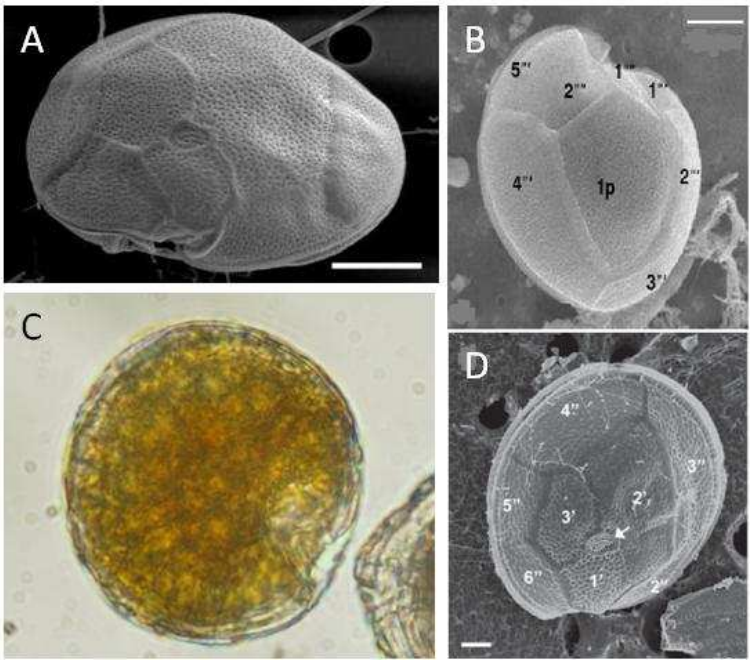

Figure 1: SEM micrographs of *Gambierdiscus* sp1 (proposed novel species *G. excentricus*) from the Canary Islands (scale bar =20µm) (Aligizaki et al., 2008) (A) and *Gambierdiscus* sp2 from Crete (scale bar =20µm) (Aligizaki and Nikolaidis, 2008) (B), light micrograph of *Gambierdiscus* sp3 from Indonesia (X400, Nikon Eclipse TE 2000-5) (C), and SEM micrograph of *G. pacificus* from Malaysia (scale bar = 10µm) (Mohammad-Noor et al., 2007).

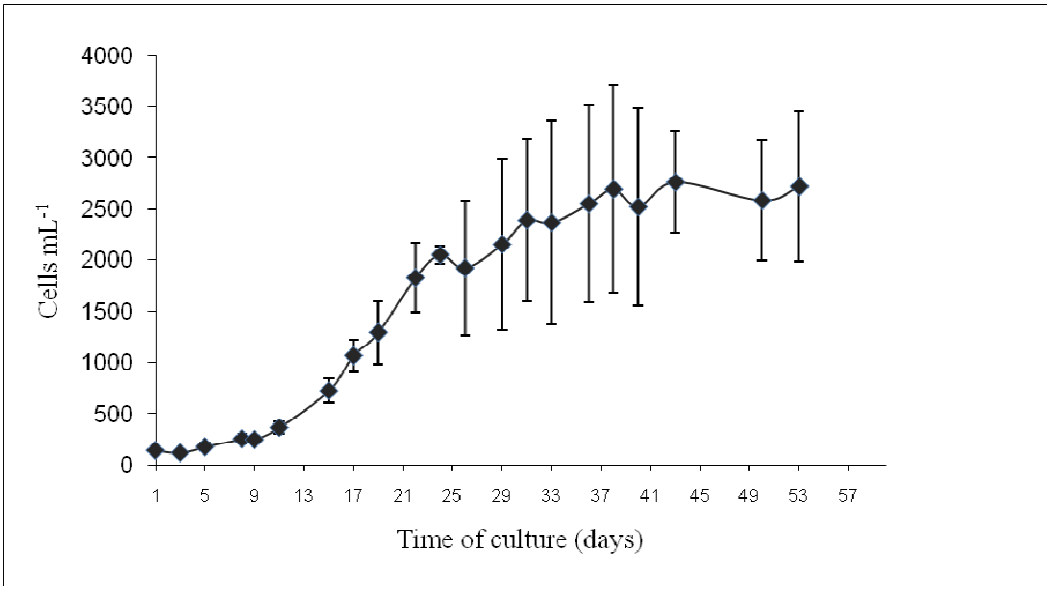

Figure 2: *Gambierdiscus pacificus* (Strain GDSA01) culture density according to the time of culture, exponential and stationary growth phases being respectively from day 9 to 22, and from day 22 to 43.

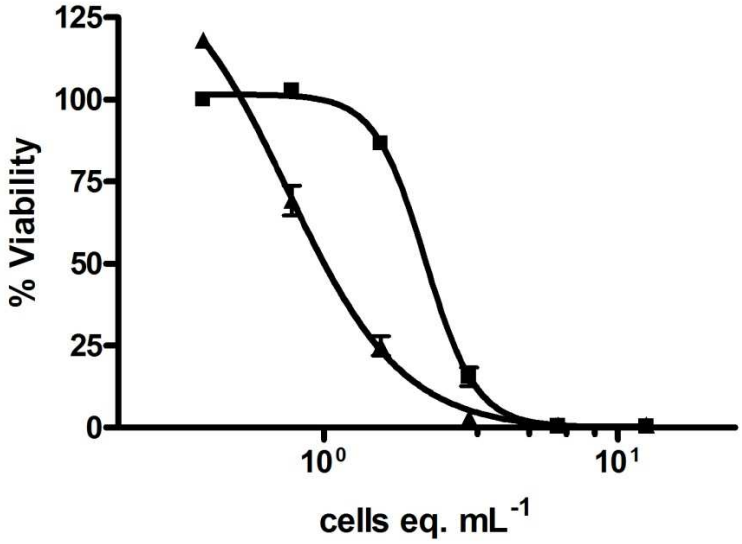

Figure 3: Dose-response curves of Neuro-2a cells exposed 24 hours to *Gambierdiscus* sp1. VGO790 with (▲) and without (■) ouabain/veratridine pretreatment.

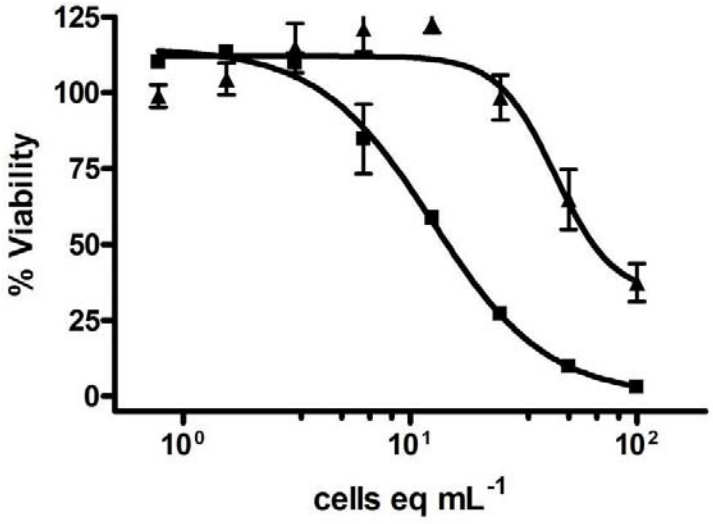

Figure 4: Dose-response curves of Neuro-2a cells exposed 2.5 hours to *Gambierdiscus excentricus* strainVGO791 with (▲) and without (■) SK&F 96365 pretreatment.

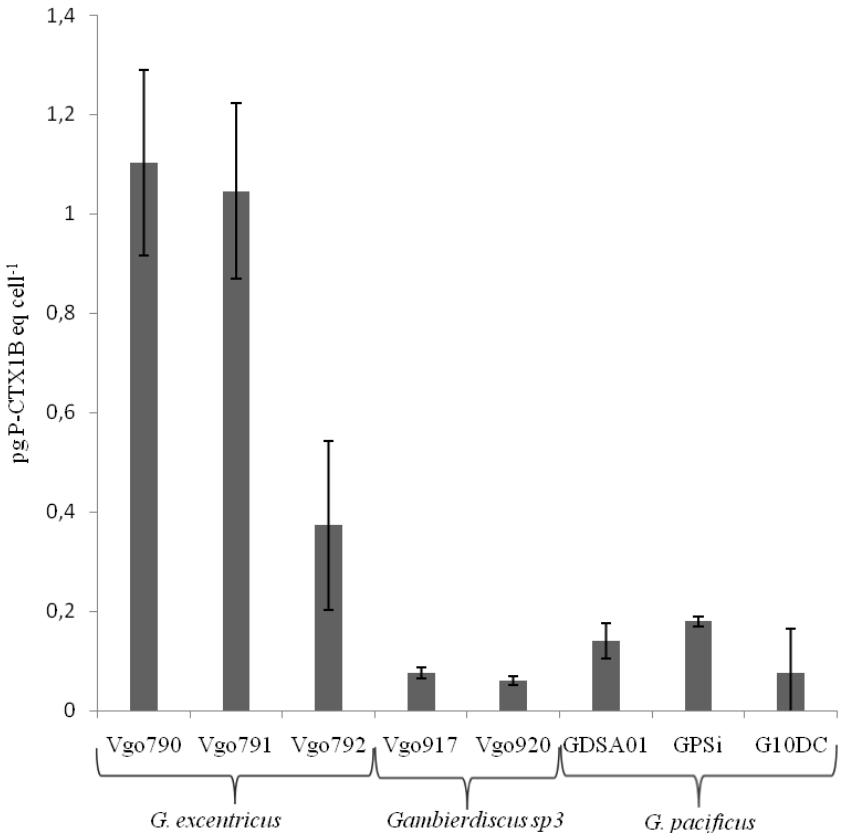

Figure 5: pg CTX1B equivalents per cells in *Gambierdiscus* spp.

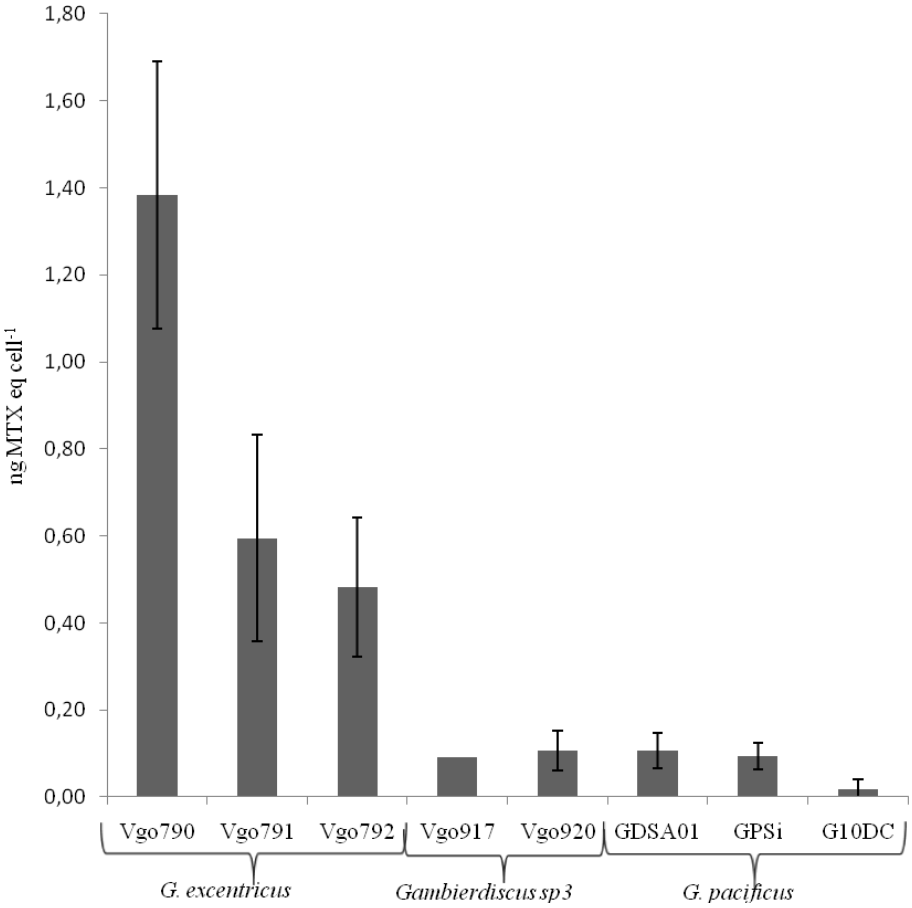

Figure 6: ng MTX equivalents per cell in *Gambierdiscus* spp.

Article 4

Monitoring of dissolved ciguatoxin and maitotoxin using solid-phase adsorption toxin tracking devices:
Application to *Gambierdiscus pacificus* in culture.

Published in *Harmful Algae* 10 (2011), 433-446

RESUMEN DE LA PUBLICACIÓN

La capacidad de la resina HP20 (DIAON®) para adsorber patrones de ciguatoxina tipo 1 del Pacifico (CTX-1) y maitotoxinas (MTXs) disueltas en agua de mar ha sido evaluada en condiciones *in vitro* para verificar la aplicabilidad de los SPATT (Solid Phase Toxin Tracking) o fase solida de adsorción de toxina como método de muestreo pasivo de toxinas disueltas relacionadas con la ciguatera. El ensayo celular con células de neuroblastoma Neuro-2a con tratamiento previo con ouabaina/veratridina y SK&F 96365 fue respectivamente utilizado para una detección especifica de toxicidad relacionada con la presencia de CTXs y MTXs. Los porcentajes de recuperación de CTX-1 por la resina HP20 han sido respectivamente estimados a 90.9 (±6.4), 85.5 (±13.2) y 89 (±11.3) % después de 24, 72 and 120h de exposición de la resina a la CTX-1 dissuelta. El porcentaje de recuperación de la MTX fue estimado a 66.2 (11.9) % después de 72h de exposición de la resina a la MTX dissuelta.

Esta aproximación metodológica se aplicó a la detección de CTXs y MTXs disueltas en el medio de cultivo de la dinoflagelada *Gambierdiscus pacificus*, con adicional cuantificación e identificación de los diferentes derivados de CTXs retenidos por la resina por análisis con LC-MS. Ambos métodos celulares y analiticos han permitido identificar y cuantificar CTXs disueltas en el medio de cultivo desde el día 16 hasta el día 47 de cultivo, los derivados 51-hydroxyCTX-3C and 2,3-dihydroxyCTX-3C siendo identificados de forma preliminar por LC-MS como los más abundantes. Adicionalmente se comparó la toxicidad de tipo CTX y MTX evaluada en la resina y en las células de *G. pacificus* con el método celular para investigar posible diferencias fisiológicas en la producción de toxinas según las diferentes fases de crecimiento del cultivo.

Los resultados presentados confirman la eficacia de los SPATT, mediante le uso de la resina HP20, para detectar CTXs y MTXs dissueltas en condiciones *in vitro*. El uso de los

SPATT se puede considerar como una aproximación útil para seguir la presencia de CTXs y MTXs en zonas ciguatericas y para incrementar el conocimiento sobre la produccíon de CTXs y MTXs por las especies del genero *Gambierdiscus*.

Article 5

Evidence of okadaic acid production in a cultured strain of the marine dinoflagellate *Prorocentrum rhathymum* from Malasia.

Published in *Toxicon* 55 (2010), 633-637

RESUMEN DE LA PUBLICACIÓN

Prorocentrum rhathymum es una dinoflagelada epibentónica previamente descrita como una especie tóxica pero cuyas toxinas nunca habían sido identificadas. Con el objetivo de verificar la posible producción de toxinas de tipo diarréico (ácido okadaico (AO) y sus derivados) por esta especie, hemos acoplado el fraccionamiento por cromatografía de un extracto de esta dinoflagelada a varios sistemas de detección de toxinas en las diferentes fracciones: un ensayo toxicológico con células de neuroblastoma (Neuro-2a), un ensayo enzimático de inhibición de las proteínas fosfatasas tipo 2 y una análisis por cromatografía liquida acoplada a un sistema de detección por espectrometría de masa en tándem (LC-MS/MS) para confirmar la identidad de los compuestos tóxicos sobre cultivos celulares y inhibidores de las proteínas fosfatasas. Esta aproximación metodológica ha permitido identificar por primera vez la producción de pequeñas cuantidades de AO y de un posible isómero de AO por *P. rhathymum*. Otras fracciones tóxicas sobre las células de neuroblastoma y con un efecto activador de las proteínas fosfatasas han podido ser identificas pero la identidad de estos nuevos compuestos bioactivos queda para resolver.

3.3.

CHAPTER III

APPLICATION OF CELL-BASED ASSAY TO TOXIN DETECTION IN NATURAL SAMPLES:

FISH SAMPLES

CIGUATERA FISH POISONING

3.3.1. Results and Discussion

Ciguatera Fish Poisoning (CFP) is usually restricted to tropical and subtropical areas of the Atlantic including the Caribbean, the Indian and Pacific Oceans. However recent reports suggested a possible geographical expansion of CFP to more temperate waters of Europe, especially in the Eastern Atlantic Ocean (**Article6**). European legislation provides no indication about a reference analysis method for CTXs, and does not establish maximum permited levels of CTXs in fishery products. However critical analysis and availability of methodologies for CTX determination is required for a rapid response to suspected CFP cases and to conduct sound CFP risk analysis. In this third Chapter of the thesis, a bibliographic review on ciguatera (**Article 6**) provides comprehensive analyses regarding the current methodological approaches for CTXs determination and critical discussion regarding their applicability for CFP management. In light of the onset of ciguatera in Europe, strategies for CFP risk analysis was proposed to confront this potential crisis (**Article 6**). Additionally, the suitability of the Neuro-CBA for the determination of CTXs in fish samples caught in the Canary Islands was verified (**Article 7**) and its applicability for CFP risk analysis was supported by results of the analysis of various ciguatera-suspected fish samples (**Article 7**).

The review article on ciguatera (**Article 6**) was introduced by a brief revision of the broad aspects associated to Ciguatera Fish Poisoning, providing general knowledge about the complexity associated to CFP and showing the necessity for the determination of CTXs in natural samples (fish and microalgae).

The review emphasized the necessity for effective extraction and clean-up procedures previous to CTXs analysis and presented detailed description of a selection of various protocols for CTXs recovery from natural samples. The applicability according to the nature of samples (either fish or microalgae) was examined taking into consideration the elimination of the interferences of biological matrices (especially fish samples) and the expected toxin

profile (especially for microalgal samples). The grade of purity of extracts required was shown to be dependent of the method of CTXs analysis applied. The time of preparation of extracts was another factor which was addressed with regard to its suitability for routine monitoring purposes while guaranteeing good CTXs recovery.

The high diversity, structural complexity and toxicity of CTX congeners present at trace levels in different matrices, were described as parameters that may difficult the development of reliable methods for CTXs determination. The limited availability of CTXs standards was underlined as an important limitation upon methods development, calibration or validation. Current methodologies for CTX determination i.e the mouse bioassay for CTXs, the *in vitro* Neuro-2a CBA for CTXs, the Receptor Binding Assay (RBA), immunoassays and instrumental analytical approaches were reviewed (**Article 6**). For each method, the review contemplated the phases of their development, their principles and the description of the methodology, examples of application and a critical discussion on their suitability as a screening tool for CTXs. All these methods presented good correlation and show good results for the determination of CTXs at levels that may cause CFP in humans. However we emphasized the necessity to consider the frame of application when selecting CTXs determination methods (rapid screening versus accurate and confirmatory methods), e.g for food safety assessment, research or diagnostic purposes. One should differentiate between methods that provide results on toxicity (e.g the Neuro-2a CBA) from methods that provide quantification of a specific compound (e.g LC-MS/MS). Actually no rapid and cost-effective individual tests to be used by fishermen and consumers for checking the safety of fishing products exist. However the Neuro-2a CBA and RBA have arisen as promising techniques for high-througput screening of CTX-containing fish samples. LC-MS/MS analysis is the most probable candidate to become the confirmatory method for CTXs once certified material is available, a standard procedure validated and regulatory limits of detection guaranteed.

Two important set of results were considerd in order to evaluate the possible onset of ciguatera in Europe. The presence of CTXs containing fish in the Eastern Atlantic Ocean, especially in theCanary Islands and Madeira [134, 135, 136], as well as the recent descriptions of the marine dinoflagellate *Gambierdiscus* spp. in the Canary Islands and Crete [29, 30, 38], constitute evidence of a possible onset of ciguatera in Europe. This evidence has open discussion on the impact of global warming to the distribution of HAB species and its impact on human health. It was suggested the importance of monitoring sea surface temperature in addition to the abundance and toxicity of *Gambierdiscus* spp. population for assessing a possible onset of ciguatera in Europe in relation to global warming. Furthermore, considering the current state of CFP in Europe and the lack of specification by European regulation regarding a reference analysis for CTXs and regulatory limits of CTXs in fish, the review advised a risk analysis approach to face CFP and establish recommendations when dealing with CFP and public safety. Three issues were addressed in the process of risk analysis: i) risk assessment which should consider the identification of the hazard, ii) risk management which should propose actions aiming at reducing CFP risk and iii) risk communication which should modulate population behavior and improve communication with competent agencies linked to food safety and medical care. In that sense, understanding the distribution of *Gambierdiscus* spp, evaluating CFP toxins in fish and microalgae should allow a better prediction of CFP in Europe. Although availability of a validated method for CTX determination would definitively accelerate CFP risk analysis.

The suitability of the Neuro-2a CBA for CTXs was verified for the determination of CTXs in thirteen ciguatera-suspected fish caught in the Canary Islands and belonging to the genus *Seriola* and *Acanthocybium* (**Article 7**). Extraction procedure of CTXs in fish samples was performed according to the protocol described in Lewis [150] for the determination of

CTXs using the mouse bioassay for CTXs. A limit of exposure of tissue equivalent (TE) to Neuro-2a cells was estimated according to unspecific toxic effects measured without ouabain/veratridine treatment of Neuro-2a cells and was set at 20 mg mL^{-1}. Above this limit, the measure of toxic effects is likely to be related to the interferences of the biological matrices of fish tissue. Although this value may be decreased by applying additional purification steps of the extracts for CTX recovery. Under the limit of TE exposure established, the limit of quantification (LOQ) of the method was estimated according to the response of Neuro-2a cells exposed to non toxic fish sample spiked with CTX1B and was estimated at 0.0096 ng CTX1B g TE^{-1}. According to the proposed safety limit of 0.01 ng CTX1B g TE-1 which is not expected to induce CFP, the Neuro-2a CBA is suitable for the determination of CTX content susceptible to induce CFP.

Analysis of the thirteen fish samples allowed identifying four CTX-containing fish samples with a content of CTX1B equivalents exceeding the safety limits proposed and ranging between 0.058 (±0.012) and 6.231 (±0.713) ng CTX1B equivalents g TE^{-1}. Among those three CTX-containing fish samples, three of them were previously implicated during CFP events and the last one was immobilized before commercialization. Those results confirmed the suitability of the Neuro-2a CBA for CTXs determination i) for the confirmation of the diagnostic of ciguatera and ii) as a preventive protection of the consumer against CFP.

Additionally to toxin analysis in fish, species identity of the different ciguatera-suspected fish of the genus *Seriola* was genetically assessed using mitochondrial Cyt B gene sequence analysis and reveal differences of speciation between the genetic and morphological diagnostic. All CTX-containing fish samples were morphologically identified as *S. fasciata* and genetically identified as *S. dumerilii* and *S. rivoliana*. This result evidenced the need for unequivocal species identification in order to definitively understand the source of the disease in Canary Islands. The genus *Seriola* has been incriminated during CFP events in our study as

well as in previous CFP records in the Canary Islands and Madeira. Although further confirmation of the suitability of the mitochondrial Cyt B gene for unequivocal species discrimination among the genus *Seriola* would definitively help confirm the identity of ciguatera risk species.

Results of the present study suggested the need for the establishment of preliminary monitoring programs for a better prediction of the risk of CFP in Canary Islands. Although efforts towards the implementation of the Neuro-2a CBA as a screening tool for CTXs in fish have to be potentiated additionally to a systematic confirmation of fish species identity.

3.3.2. Publications

Article 6

REVIEW

Update on the methodologies available for ciguatoxin determination. A perspective for facing up the onset of ciguatera in Europe.

Published in *Marine Drugs* (2010), 8, 1838-1907

RESUMEN DE LA PUBLICACIÓN

La ciguatera es una forma de ictiosarcotoxismo debido al consumo de pescado contaminado por ciguatoxinas (CTXs). El diagnóstico de la ciguatera se basa principalmente en la identificación de sus síntomas característicos pero la confirmación de un caso de ciguatera consiste en identificar la presencia de CTXs en la sangre de los afectados o en restos de comida. En este sentido, la disponibilidad de métodos de detección de CTXs es una necesidad requerida para poder confirmar el diagnóstico de ciguatera y prevenir posibles casos de intoxicación. De hecho la eficacia de los métodos de detección no depende solamente de su especificidad y sensibilidad para los diferentes derivados de CTXs, pero también de la eficacia de los protocolos de extracción y purificación de las CTXs y de la disponibilidad en patrones de CTXs.

En una primera parte del manuscrito, describimos los numerosos aspectos relacionados con la ciguatera como el origen de la ciguatera, las dinoflageladas del genero *Gambierdiscus* productoras de CTXs, los datos de epidemiología y sintomatología, la diversidad estructural de las CTXs, su toxicidad y mecanismo de acción. En una segunda parte del manuscrito, presentamos una selección de protocolos publicados para la preparación de muestras de microalgas y pescados para la determinación de CTXs. Estos protocolos han sido seleccionados en función del tipo de metodología elegido para la detección de las CTXs. A continuación, se revisan las diferentes metodologías actualmente en uso para la determinación de CTXs en muestras de pescado y de microalgas. Estos incluyen los ensayos toxicológicos con animales e *in vitro* con los cultivos de células de neuroblastoma (Neuro-2a), un ensayo farmacológico de competición por receptor (Receptor Binding Assay), ensayos inmunológicos y métodos analíticos. Para cada método, se revisan las metodologías utilizadas al igual que su aplicabilidad como método de detección en rutina de las CTXs.

En una tercera parte del manuscrito, discutimos de una posible extensión geográfica de la ciguatera normalmente confinada en las zonas más tropicales y sub tropicales del Océano Índico, Pacífico y Atlántico incluyendo el Mar Caribe hacia las aguas más templadas de Europa (entendemos como Europa su definición a nivel geográfico y no político). Nuestra argumentación se basa en hechos recientes que describen varios casos de intoxicación por ciguatera descritos después del consumo de pescado capturado en las Islas Canarias en 2004 y a Madeira en 2007 y en 2008. Además de estos datos epidemiológicos, se dispone de datos que confirman la presencia de *Gambierdiscus* spp.. En 2004 se aisló por primera vez la dinoflagelada *Gambierdiscus* spp. en las Islas Canarias y en el Mediterráneo (Creta) en el 2008. Abordamos una posible contribución del cambio global en la distribución de las especies de dinoflageladas productoras de toxinas y de la aparición de pescados contaminados con CTXs. Frente a estos primeros datos y a la identificación de carencias en la regulación europea respecto a la presencia de CTXs en pescado, proponemos estrategias para permitir una mejor predicción del riesgo de ciguatera a nivel Europeo. Para definir estas estrategias, describimos diferentes acciones para analizar el riesgo de ciguatera incluyendo la evaluación, la gestión y la comunicación del riesgo. Destacamos como necesidad la validación de un método de referencia para una identificación inequívoca de las CTXs y con elevada sensibilidad, de manera a mejorar la evaluación del riesgo de ciguatera.

Article 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.

Submitted in *Food Additives and Contaminants*

RESUMEN DE LA PUBLICACIÓN

El ensayo celular con células de neuroblastoma (Neuro-2a) especifico para CTXs ha sido aplicado a la detección de compuestos de tipo CTXs en muestras de pescado procedentes de las Islas Canarias. Un límite máximo de carga en tejido equivalente (TE) al que se puede exponer las células Neuro-2a fue establecido de manera a evitar posibles interferencias de las matrices en la detección de CTXs. Éstefue estimado en 20 mg TE mL^{-1}. Una muestra no tóxica dopada con patrón de ciguatoxina tipo 1 del Pacifico (CTX1B) ha permitido estimar el límite de cuantificación del método a 0.0096 ppb CTX1B. De las trece muestras de pescado procedentes de las Islas Canarias y examinadas por su contenido en ciguatoxinas (CTXs), se identificaron cuatro muestras tóxicas de las cuales tres fueron implicadas en intoxicaciones por ciguatera en 2008 y 2009, con un contenido en CTX1B estimado entre 0.058 (±0.012) and 6.231 (±0.713) ng CTX1B eq. g^{-1} de tejido de pescado. La elevada sensibilidad y especificidad del método demuestra su capacidad para detectar niveles mínimos de contaminación en CTX1B del Pacifico susceptibles de inducir intoxicaciones en humanos.

Adicionalmente al análisis del contenido en CTXs en las diferentes muestras de pescado, se analizó las diferentes muestras por secuenciación de ADN con el fin de poder confirmar la identidad de las especies con riesgo de ciguatera en las Islas Canarias. El análisis reveló diferencias de identidad entre el diagnóstico morfológico y genético.

Los resultados de los análisis de CTXs y del estudio genético presentados en este estudio contribuyen a la evaluación de riesgo de ciguatera en las Islas Canarias.

3.4.

GENERAL DISCUSSION AND PERSPECTIVES

The suitability of the Neuro-2a CBA for toxin detection in natural samples was confirmed in the present work as previously reported in the literature for microalgal and fishery samples [103, 107, 115, 117, 118, 119, 124, 125, 128]. The use of the Neuro-2a CBA specific for CTXs and MTXs (**Article 2**) as well as the use of chromatographic fractioning of microalgal samples for the elimination of biological matrix interferences (**Article 1**), have definitively contributed to the characterization of toxin profiles in *Gambierdiscus* spp. from different origins and *P. rhathymum*, and for the discrimination between CTX- and non CTX-containing fish from the Canary Islands. In their respective context, the characterization of toxin profiles in *Gambierdiscus* spp. and *P. rhathymum* may have taxonomical, phylogenetical (**Article 3** and **Article 5**) or physiological (**Article 4**) implications as discussed in the Chapter II of the present study. Additionally, the combination of chromatographic fractioning of microalgal extracts with various detection methods, e.g CBAs, biochemical assay and LC-MS analysis is likely to identify the production of new toxins or new bioactive compounds (**Article 5**), a strategy that may contribute for the identification of new molecules which may be of interest in the field of the pharmacology. In a food safety context, results of the Neuro-2a CBA reported in Chapters II and III strongly contribute for ciguatera risk assessment in the Canary Islands (**Article 6**). Results of the present work confirmed CBAs as powerful tools for marine toxins detection in natural samples in complement to chemical and biochemical methods. The contribution of the present work for ciguatera risk assessment in the Canary Islands and for the development of alternatives methods that may reduce animal testing in toxicological studies is discussed below.

Ciguatera risk assessment in the Canary Islands

According to the principles of seafood risk analysis described in Fazil [6] (**Figure 9**), hazard identification is the first stage in risk assessment. Hazard identification is an initial step to clearly define the hazard and that contributes to confirm that the hazard exists.

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

As described in the **Article 6** of the present work, various factors have to be addressed for the assessment of CFP risk at different levels. We have proposed three levels: food, social and environmental. In our study, CFP risk assessment was addressed through the identification of the hazard of CFP in the Canary Islands according to the following evidence:

- The identification of CTX-containing fish samples caught in the Canary Islands. Our results consisting on the identification of CTX-like activity in fish confirmed the diagnostic of various cases of CFP established according to clinical observation. Our results therefore contributed to the epidemiological approach of CFP in the Canary Islands and may be used to establish preventive actions to protect consumers (**Article 7**).

- The diagnostic of CTX presence in fish samples belonging to the genus *Seriola* spp. in our study and as previously described [134, 135]. Our results present evidence of CTX activity in four fish, confirming the genus *Seriola* spp. as a potential CFP vector in the Canary Islands (**Article 7**). Genetic analysis of the *Seriola* species confirms *S. dumerilii* as a particular species at risk.
- The CTX-like production in a clonal culture of *G. excentricus*, a novel species recently isolated from the Canary Islands. This result confirms *G. excentricus* as a possible CFP causative agent in the Canary Islands (**Article 3**).

All these results strongly confirmed the existence of the hazard of CFP in the Canary Islands. Quantifications given by the Neuro-2a CBA of CTX1B equivalents in fish samples implicated during CFP events (**Article 7**) have contributed to risk characterization of CFP intoxication in the Canary Islands as it may allow identifying the human threshold susceptibility to CTX (**Figure 10**) in that area. This value may be usefull for identifying how much CTXs in fish is required to cause illness (**Figure 10**) and it will contribute to CFP risk management for the definition of safety levels of CTXs in fish from the Canary Islands.

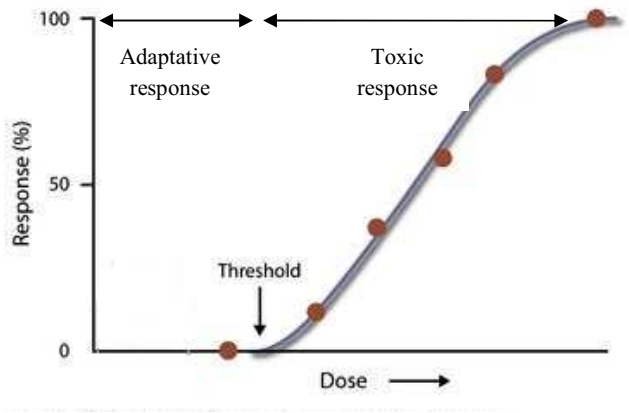

Figure 10: Dose-response curve : threshold approach in toxicology. Adapted from Johns Hopkins Bloomberg School of Public Health, available at http://ocw.jhsph.edu.

In our study, the lowest CTX content in fish implicated during CFP was estimated to 0.103 ng CTX1B equivalents g TE^{-1}. This value is supported by other studies in a closely area. A content as low as 0.08 ng C-CTX-1 equivalents g TE^{-1} (equivalent to 0.008 ng CTX1B) was reported in a fish sample implicated during CFP in Madeira, which is close to the Canary Islands [135]. The need for unequivocal quantification of the CTX content using LC-MS/MS analysis would be required for the estimation of the human threshold susceptibility to CTX in fish from the Canary Islands. For that purpose, availability of reference or certified CTX standard material will be needed.

The evidence of CFP hazard in the Canary Islands should encourage the implementation of preliminary monitoring programs for the management of specific fishery products (e.g. *Seriola* spp.) and that would contribute to better assess the risk of CFP in the area. As described in **Article 6**, toxin content in fish, identification of fish species, toxicity and dynamic of populations of *Gambierdiscus* spp, environmental data, are factors that should be addressed during monitoring programs of CFP. The present work encourages the use of the Neuro-2a CBA for the screening of the presence of CTXs in fish caught in the Canary Islands and in natural populations of *Gambierdiscus* spp. for assessing the risk of CFP. On the other hand, our results do not match the results of the CiguaCheck kit implemented by the Canary Islands government (**Article7**). This kit is a method not yet validated, and our results would suggest it as a dubious management strategy for CFP. The use of SPATT was shown suitable for tracking dissolved CTXs in the laboratory (**Article 4**) and its implementation for the monitoring of the presence of CTXs producing populations of *Gambierdiscus in situ* should be also evaluated.

Having the evidence of CFP hazard in Canary Islands, and being aware that it is also important not to magnify the risk perception associated with the consumption of fishery products, risk analysis for CFP in the Canary Islands is a need. Efforts should be implemented

in order to assure an extensive risk assessment of CFP in the Canary Islands. Taking into consideration that legislation on ciguatera toxins is precarious and does not establish an official method nor maximum permitted levels, the establishment of a validated reference method for CTX determination in fish would definitively improve CFP risk assessment.

CBAs as a powerful alternative testing strategy to living animals for marine toxins detection.

Cell cultures in the field of marine toxins have been widely used for the detection and elucidation of the mechanism of action of marine toxins [70, 122] . Their application has been extended for the determination of the level of toxicity of natural samples, especially for seafood risk assessment [22]. As little is known about repeated exposure of consumers to marine toxins, chronic toxicity evaluation of marine toxins is another field of application for cell cultures that should be explored.

The 3Rs rule principle described by William Russel and Rex Burch [151] defined as an alternative method to living animal testing any method that contributes for the Replacement (replace the use of animal), Reduction (use fewer animals), Refinement (cause least harm to animal) of animal testing. As described in the introduction of the present work, the European Union supports the development of alternative or complementary approaches to the mouse bioassay for marine toxins detection in fishery products according to the 3Rs rule principle. Although the implementation of such approach should provide an equivalent level of protection of human health in relation to official mehtods [23]. Ideally, analytical methods which could allow accurate estimation of toxins in a single procedure would provide the best mean for consumer protection and the most rational and economical tool in the management of risk posed by the presence of toxins in fishery products [22]. However the information obtained by analytical methods may not always provide an insight into the toxicity of the sample [152]. The increased number of toxins and derivatives and emerging toxins suggested

the need for the screening of marine toxins using functional approaches. Functional assays may include the use of biochemical assays (e.g the PP2A for OA and DTX1) or CBAs. Biochemical assays will mainly identify the compounds for which it was developped and very rarely, compounds that would also interact with the same principle of the assay (e.g. inhibition or actrivation of enzyme activity). Cell-based assays may be useful with a wider scope as these will recognize the potential toxicity of the toxins and other compounds, with the intended purpose, sometimes achieved, to mimick toxicological assays with animals. Functional assays are unlikely to identify single analystes in a given sample. In light of this evidence, the best initial approach for the detection of a wide range of marine toxins for food safety would be the use of a combination of functional assays, e.g CBAs, with chemical approaches in order to provide information regarding the toxicity and toxins profil in a given sample [152]. This approach was described in various studies, such as in Humpage et al. [153] for the detection of paralytic toxins in cyanobacterial blooms, in Cañete et al. [109] for the determination of DSP toxins in mussel samples, or in Espiña et al. [154] for the determination of palytoxin in *Ostreopsis* and mussel samples. In the field of ciguatera testing, Dickey [128]proposed the combination of the Neuro-2a CBA for CTXs for the screening of CTX-like toxicity in fish with confirmation of the identity of CTX congeners using LC-MS approach.

In the present work, numerous aspects have been addressed for the implementation of CBA as a screening tool for marine toxins detection in natural samples, e.g the sensitivity and specificity of CBA for marine toxins, the resistance to biological matrices and the accuracy of cell response for the detection and quantification of toxins. Table 2 described general aspects required for the implementation of CBAs for marine toxins detection, with regard to the actual knowledge provided in the present work and in the literature, and the requirements and perspectives identified for further work.

In the field of chemicals and especially for cosmetic and medicine, some CBAs have been now validated for acute toxicity testing [155]. This evidence suggests the need to increase efforts for the validation processes of CBAs that have shown great potential for marine toxins detection. The Neuro-2a CBA has been described as one of the best established and valuable tool for the screening of neurotoxins in natural samples [22]. Ledreux et al [107] proposed an experimental design for the specific detection of neurotoxins i.e CTX, PbTX, STX and for PLTX based on the use of the Neuro-2a CBA. Although the Neuro-2a CBA for CTX is actually one of the CBA most widely used for CTX toxicity screening which was found accurate in routine screening of CTXs compounds as described in the present work and in the literature [128]. The Neuro-2a CBA for CTXs is a promising candidate for validation processes as a screening tool of CTXs for ciguatera risk assessment.

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

Aspect to be adressed	Actual knowledge	Requirements, Perspectives
Interferences of biological matrices	CBAs support relative high content of biological matrices according to appropriate purification procedures [106, 109, 137]	Improve sample preparation procedure according to the nature of sample, time of preparation for routine monitoring purposes, standardization of the procedure
Sensitivity of CBA to marine toxins	Sensitivity of CBA demonstrated for major toxins implicated during foodborne intoxication [103, 108]	Improve experimental conditions for an increase sensitivity to marine toxins and a reduce time of exposure to CBA: e.g cell model, experimental design, endpoint for toxicity assessment
Specificity of CBA for one group of marine toxins	The use of specific agonist/antagonist of the route of action of marine toxins allowed the obtention of CBA specific for one group of toxins, e.g neurotoxins, PLTX, MTX.	Encourage studies for the elucidation of the mechanism of action of toxins. Increase the number of CBA specific for one group of toxins to a wider range of toxins using agonist/antagonists of toxins Explore the field of toxicogenomic and toxicoproteomic [156] as a new approach for the specificity of the response of CBA to marine toxins : identify specific marker of toxicity (gene transcription, proteins expression) in response to the exposure of cells to a particular group of toxins [157, 158]
Suitability of CBA for the quantification of marine toxins	The use of stable cell lines for CBA decreases the variability inter-experiments compared with the use of primary cell lines. The microplate format of CBA allows the generation of replicates and favors statistical treatment of results. Calibration of cells with standard solutions allowed repeatable and reproducible quantifications of toxins in natural samples	Decrease possible factors of variability between experiments, e.g the use of chemically defined serum to replace foetal bovin serum in culture medium.

IV. CONCLUSIONS

1- The elimination of biological matrices and the use of CBAs specific for one group of toxins improve the application of CBA for marine toxins detection in natural samples. Chromatographic fractioning combined with CBA is suitable for the elimination of the interferences of biological matrices, for the detection of marine toxins embedded at trace levels in natural sample and may serve as a bioguided purification procedure of toxins or bioactive compounds. The use of SK&F 96365, an antagonist of the MTX-induced toxic effects, was suitable for the settlement of a Neuro-2a CBA specific for the detection and quantification of MTX in *Gambierdiscus* spp.

2- The Neuro-2a CBAs specific for CTX and MTX were suitable for the detection and quantification of CTX- and MTX-like compounds in crude extracts of the microalgae *Gambierdiscus* spp. *Gambierdiscus excentricus* proposed novel species from the Canary Islands was identified as a high CTX and MTX producer species. *Gambierdiscus* sp. (strain KC81) from Crete was found not toxic.

3- The HP20 DIAON® resin was suitable for the recovery of dissolved CTX1B and MTX, and was suitable for the study of the CTXs and MTXs dissolved in the culture medium of *G. pacificus*. The Neuro-2a CBA specific for CTX and MTX were suitable for the detection and quantification of CTX and MTX tracked by the HP20 Diaion® resin.

4- The chromatographic fractioning combined with CBA, PP2A and LC-MS analysis approach was useful to investigate HAB suspicious species, and allowed identifying for the first time *P. rhathymum* from Malaysia as a producer of OA.

5- The Neuro-2a CBA for CTX allowed the detection and quantification of CTX-like compounds in various fish samples from the Canary Islands. *Seriola dumerilii* was identified as CFP risk fish species in the Canary Islands. The identification of a CFP hazard in the Canary Islands in the present study is a contribution to CFP risk assessment and is supporting the hypothesis of a possible onset of ciguatera in Europe.

6- Cell-based assay was found as a powerful toxicological approach for the 3R rule principle in the field of ciguatera risk assessment.