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Criblage bioguidé et analyse CL-SMHR des toxines et d'autres métabolites d'intérêt chez les dinoflagellés *Gambierdiscus* et *Fukuyoa*

JURY

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1-BuOH: 1-butanol
Ach: acetylcholine
$AcONH_4$: ammonium acetate
Al_2O_3: alumina
ANSES: Agence Nationale de sécurité sanitaire de l'alimentation, de l'environnement et du travail
aq.: aqueous
BTX: brevetoxin
C_6H_{14}: hexane
CBA: cell-based assay
CCFHR: Center for Coastal Fisheries Habit Research laboratory
CCMP: Culture Collection of Marine Phytoplankton
CCVIEO: Culture Collection of Harmful Microalgae of IEO
CH_2Cl_2 (or **DCM**): dichloromethane
$CHCl_3$: chloroform
COP: cytolytic/oncotic pore
CTX: ciguatoxin
DAD: diode array detector
e.g.: *exempli gratia* (for example)
EFSA: European Food Safety Authority
ELISA: enzyme linked immunosorbent assays
ESI: electrospray ionization
Et_2O: diethyl ether
EtOAc: ethyl acetate
FAB: fast atom bombardment
FAO: Food and Agriculture Organization of the United Nations
FDA: U.S. Food and Drug Administration
FLD: fluorescence detector
GA: gambieric acid
GoM: Gulf of Mexico
H_2O: water
HAB: harmful algae bloom
HPLC: high performance liquid chromatography
HPLC-MS/MS: high performance liquid chromatography coupled to tandem mass spectrometry
HRMS: high resolution mass spectrometry
HTS: high throughput screening
i.e.: *id est* (that is)
i.p.: intraperitoneal
IAEA: International Atomic Energy Agency
IFREMER: Institut Français de Recherche pour l'Exploitation de la Mer
ILM: Institut Louis Malardé
IOC: Intergovernmental Oceanographic Commission of UNESCO
iNOS: inducible nitric oxide synthetase
IS: ionspray ionization
KU: Kochi University
LD_{50}: lethal dose 50
LOD: limit of detection
LOQ: limit of quantification
LRMS: low resolution mass spectrometry
LSU: large subunit
m/z: mass-to-charge ratio
MAb: monoclonal antibody
MBA: mouse bioassay
Me_2CO: acetone
MeCN (or **ACN**): acetonitrile
MeOH: methanol
MMS: Mer, Molécules et Santé Laboratory
MRM (or **SRM**): multiple reaction monitoring (or selective reaction monitoring)
MTX: maitotoxin
MS/MS (or **MSMS**): tandem mass spectrometry
MTT: 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
N2a: mouse neuroblastoma neuro-2a
NCMA: Provasoli – Guillard National Center for Marine Algae and Microbiota
NCX: sodium-calcium exchanger
NH_4OH: ammonium hydroxide
NHE: sodium-hydrogen exchanger
NO: nitric oxide
NOAA: National Oceanic and Atmospheric Administration
p.o.: *per os* (oral)
PAb: polyclonal antibody
PHYC: Phycotoxins laboratory
PTX: pectenotoxin
RBA: receptor binding assay
rDNA: ribosomal deoxyribonucleic acid

RT: retention time
RV: retention volume
SAR: structure-activity relationship
SEC: size-exclusion chromatography
SIM: single ion monitoring
SiO_2: silica
sp.: species (singular form)
spp.: species (plural form)
SPE: solid phase extraction
SSU: small subunit
TFA: trifluoroacetic acid
UHPLC: ultra performance liquid chromatography
UNESCO: United Nations Educational Scientific and Cultural Organization
VGSC: voltage-gated sodium channel
VNIO: Viet Nam National Institute of Oceanography
WHO: World Health Organization
YTX: yessotoxin

Résumé

L'intoxication de type ciguatéra est l'intoxication alimentaire non bactérienne la plus répandue dans le monde. Elle est causée par des toxines produites par les dinoflagellés *Gambierdiscus* et *Fukuyoa*. Les principales toxines responsables de la ciguatéra sont les ciguatoxines (CTXs), car elles se bioaccumulent dans la chair de poisson au travers de la chaîne alimentaire marine. Les maïtotoxines (MTXs) pourraient également avoir un rôle dans la ciguatéra, surtout si les viscères de poissons contaminés étaient consommés. En outre, ces dinoflagellés produisent d'autres composés présentant une structure de polyéthers cycliques (par exemple le gambierol, le gambieroxide, les acides gambieriques et la gambierone) et leur rôle dans la ciguatéra n'a pas été évalué.

Au cours des dernières décennies, on observe une incidence accrue de la ciguatéra dans l'océan Pacifique et dans la mer des Caraïbes (Friedman et al., 2017), ainsi que l'émergence de la ciguatéra dans les zones tempérées (par exemple les îles Canaries) (Rodríguez et al., 2017). Cette extension géographique du phénomène a nécessité la mise en place d'études portant sur la connaissance de l'impact de la ciguatéra dans le monde et est à l'origine de cette thèse. Le **chapitre 1** est consacré à l'état de l'art de l'ensemble des thèmes abordés dans cette thèse. Le chapitre est divisé en trois sections concernant : (i) l'intoxication de type ciguatéra; (ii) les dinoflagellés impliqués *Gambierdiscus* et *Fukuyoa* et (iii) les composés bioactifs produits par ces dinoflagellés, notamment les ciguatoxines (CTXs) et les maïtotoxines (MTXs). Les objectifs de cette thèse ont été les suivants: (i) évaluer la toxicité relative en termes de CTXs et de MTXs de différentes espèces de *Gambierdiscus* et *Fukuyoa* et (ii) identifier les molécules présentant des toxicités équivalentes aux ciguatoxines ou aux maïtotoxines dans les souches les plus toxiques.

Toxicité relative de différentes espèces de *Gambierdiscus* et *Fukuyoa*

Au cours des dix dernières années, des techniques de biologie moléculaire ont été développées pour l'identification de presque toutes les espèces et phylotypes de dinoflagellés appartenant aux genres *Gambierdiscus* et *Fukuyoa* décrits à ce jour (Lyu et al., 2017; Nishimura et al., 2016; Vandersea et al., 2012). Cependant, très peu d'études récentes ont évalué la toxicité relative entre ces espèces ou phylotypes (Chinain et al., 2010a; Holland et al., 2013; Lewis et al., 2016; Munday et al., 2017). L'une des raisons de ce manque d'information est que la taxonomie de ces organismes a subi plusieurs mises à jour, au cours de la dernière décennie, grâce au développement d'outils morphologiques et moléculaires plus avancés (**chapitre 1**, **section 2.2**). À ce jour, la souche la plus ciguatoxique appartient à l'espèce *G. polynesiensis* (TB-92, du Pacifique Sud) (Chinain et al., 2010a), la souche la plus maïtotoxique appartient au phylotype *Gambierdiscus* sp. ribotype 2 (CCMP1655, des Caraïbes) (Holland et al., 2013). La connaissance de la quantité et de la nature des toxines produites par une espèce ou un phylotype donné est essentielle pour le développement de stratégies d'évaluation du risque de la ciguatéra, basées sur la présence de ces dinoflagellés dans le milieu naturel. Cela a donc été une des premières tâches accomplies au cours de ce travail de thèse (**chapitre 2**). L'évaluation de la toxicité relative de différentes espèces ou phylotypes de *Gambierdiscus* et *Fukuyoa* a été réalisée en utilisant des essais fonctionnels (test neuro-2a et test hémolytique) (Litaker et al., 2017; Pisapia et al., 2017a). Des différences substantielles de toxicité parmi les espèces ou les phylotypes examinés ont été découvertes, confirmant les résultats reportés dans la littérature. La toxicité globale par cellule, évaluée grâce à ces méthodes, a montré que *G. excentricus* est l'espèce qui produit la plus grande quantité de CTXs par cellule parmi les espèces présents dans la région de l'Atlantique. Par ailleurs, cette étude corrobore les résultats précedemment publiés par Fraga et al. (2011). Ceci est particulièrement important car deux approches différentes ont été utilisées pour mettre en évidence la présence de CTXs : suppression de l'activité des MTXs par addition de SK&F 96365 dans des extraits bruts (Fraga et al., 2011) et séparation préalable des MTXs des CTXs par un partage liquide-liquide réalisé dans cette étude (Litaker et al., 2017; Pisapia

et al., 2017a). *Gambierdiscus excentricus* présente une toxicité de type CTX qui peut atteindre, pour certaines souches, de concentrations de l'ordre du picogramme de CTX3C équivalents (éq.) par cellule, c'est-à-dire du même ordre de grandeur que *G. polynesiensis* du Pacifique Sud (Chinain et al., 2010a). Toutes les autres espèces ou phylotypes testés ont présenté une toxicité de type CTX plus faible, voire quasi-inexistante, de l'ordre du femtogramme de CTX3C éq. par cellule. Toutes les souches ont également montré une toxicité de type MTX de l'ordre du picogramme de MTX éq. par cellule, *G. excentricus* étant encore une fois l'espèce la plus maïtotoxique. Bien que les cellules de *G. excentricus* se divisent plus lentement que toutes les autres espèces ou phylotypes de *Gambierdiscus*, les données de toxicité fournies dans cette thèse suggèrent que *G. excentricus* a une probabilité plus élevée d'être un contributeur majeur aux épisodes de ciguatéra signalés dans l'Atlantique. Néanmoins, des limites méthodologiques doivent être signalées. Les taux de croissance et la production de toxines obtenus en laboratoire ne peuvent pas refléter ce qui se produit dans le milieu naturel.

La répartition et l'abondance de *G. excentricus* devraient être surveillées sur le terrain pour l'évaluation du risque de ciguatéra. Des études de terrain peuvent être utilisées pour déterminer la répartition des espèces dans des zones spécifiques, mais l'analyse des toxines des échantillons d'algues, de poissons ou de coquillages reste difficile, en raison de problèmes de sensibilité et de manque de connaissances sur les toxines impliquées dans les cas d'intoxication. En outre, il ne peut pas être exclu que des composés, autres que les CTXs ou les MTXs, puissent également interférer avec les essais fonctionnels. Par conséquent, la caractérisation chimique des toxines impliquées et la détermination du profil toxinique est nécessaire pour mieux évaluer le risque de ciguatéra lié à la prolifération de *G. excentricus*.

À la recherche des composés bioactifs produits par *Gambierdiscus excentricus*

Les connaissances concernant la biodiversité de *Gambierdiscus* et *Fukuyoa* ont augmenté rapidement au cours de la dernière décennie, avec la description de plusieurs nouvelles espèces. Pourtant, la caractérisation des molécules appartenant à la famille des MTXs ou des CTXs produites par ces espèces n'augmente pas au même rythme, principalement en

raison de contraintes techniques liées aux difficultés de culture de ces microalgues en laboratoire et de la difficulté à mettre en place des méthodes de séparation et d'identification suffisamment performantes.

Après avoir identifié *G. excentricus* comme étant l'espèce la plus toxique de notre collection de cultures, l'objectif a été de décrire son profil toxinique. Pour cela, une stratégie simple de purification partielle, consistant en une étape de partage liquide-liquide, suivie par une chromatographie d'exclusion stérique, a été développée pour obtenir des fractions pré-purifiées dont la toxicité a été testée sur le modèle cellulaire neuro-2a ou par le test fonctionnel de radioligand-récepteur ($RBA_{(R)}$) (**chapitre 3**). Le criblage de la toxicité de ces fractions a permis la mise en évidence de la présence de composés présentant une activité de type CTX et MTX. Il est donc nécessaire d'isoler ces composés afin de déterminer leur structure moléculaire. Comme la concentration en MTXs a été estimée être environ 60 fois supérieure à celle des CTXs, il a donc été décidé de commencer l'isolement des molécules supposées appartenir à la famille des MTXs.

La structure de la maïtotoxine (MTX) a été élucidée par spectroscopie RMN et FAB. L'interprétation des spectres RMN et la connaissance des mécanismes de fragmentation en FAB est bien documentée dans la littérature. En revanche, il n'en est pas de même pour la spectrométrie de masse à haute résolution (SMHR), associée à une ionisation par électrospray (ESI). Il a donc fallu développer des méthodes d'analyse de la MTX utilisant la chromatographie liquide couplée à la spectrométrie de masse à haute résolution (SMHR). Les travaux de cette thèse constituent la première étude portant sur l'analyse de la maïtotoxine (MTX), en utilisant la spectrométrie de masse à haute résolution (SMHR), à la fois dans les modes d'acquisition d'ions négatifs et positifs. De plus, des spectres de fragmentation de la MTX ont été obtenus et des voies de fragmentation ont été proposées. L'attribution des ions fragments aidera à l'élucidation de la structure de congénères de MTX jusque-là non décrits.

Le résultat majeur de cette étude a été la découverte d'un nouveau congénère de MTXs, appelé maïtotoxine-4 (MTX4) (Pisapia et al., 2017b). Cette découverte a été possible grâce à la comparaison des spectres de masse de ce composé avec ceux de la MTX de référence. Comme il n'a pas été possible d'isoler la MTX4, en raison d'une quantité de biomasse de *G. excentricus* insuffisante, des études d'élucidation de structure n'ont pas

pu être menées à leur terme. Pourtant, l'appartenance de cette molécule à la famille des MTXs a pu être établie sur la base de plusieurs données obtenues : (i) une cytotoxicité élevée et une activité d'afflux d'ions Ca^{2+} positivement corrélées avec la teneur de MTX4 estimée par chromatographie liquide couplée à la spectrométrie de masse en tandem ; (ii) la similarité des spectres de masse entre la MTX4 et le composé de référence, la MTX, permettant l'attribution des anions bi-chargés moléculaires et de leurs adduits sodiées et di-sodiées (Pisapia et al., 2017b). La confirmation de la présence d'un composé appartenant à la famille des MTXs dans *G. excentricus* a par ailleurs pu être obtenue, en mode d'ionisation positive, par l'attribution des cations moléculaires bi-chargés et de leurs adduits ammonium (n = 1 à 3), par la présence de deux groupements sulfate et par la mise en evidence d'un fragment terminal commun avec la MTX (Pisapia et al., *in preparation*). Lorsqu'une quantité suffisante de MTX4 purifiée sera obtenue, des études portant sur l'interprétation complète des voies de fragmentation et la détermination de sa structure pourront être réalisées.

Un autre objectif de cette thèse a été d'évaluer la présence de la MTX4 et des MTXs décrites dans la littérature (MTX, MTX2 et MTX3) dans plusieurs espèces ou phylotypes de *Gambierdiscus* et *Fukuyoa,* en utilisant la chromatographie liquide couplée à la spectrométrie de masse basse résolution en mode tandem (en utilisant les données de la littérature pour cibler ces molécules). Ces études ont montré que la production de certains congénères de MTXs semble être spécifique d'une espèce donnée. En effet, la MTX n'a été détectée que dans l'espèce *G. australes*, en accord avec les données de la littérature. La MTX4, quant à elle, n'a été détectée que dans l'espèce *G. excentricus*, quelle que soit l'origine géographique des souches examinées (îles Canaries, Brésil, Caraïbes) (Pisapia et al., 2017b). Par conséquent, la MTX4 pourrait servir de biomarqueur pour mettre en évidence la présence de *G. excentricus*. La détection de la MTX4 dans des échantillons de terrain révélerait indirectement la présence de *G. excentricus* dans l'environnement, et aiderait à identifier les zones à risque pour la ciguatéra. Enfin, concernant les MTX2 et MTX3 aucun des chromatogrammes et spectres acquis n'a pu confirmer la présence de ces toxines dans l'ensemble des treize espèces/phylotypes étudiés.

Context and aims of the study

Ciguatera Fish Poisoning (CFP) is the most common non-bacterial food-borne intoxication worldwide. The principal toxins responsible for CFP are ciguatoxins (CTXs), which mainly bioaccumulate in fish flesh through the marine food chain. CFP has long been known as a highly localized phenomenon, limited to coastal areas and islands in tropical zones, i.e. between 35 °N and 35 °S latitude. Nevertheless, the geographical range of CFP patients have become widespread with increasing global seafood trade, seafood consumption and international tourism. A recent geographical extension of CFP endemicity to more temperate areas has also been reported. Numerous studies have been published on the etiology, epidemiology and clinical features of CFP, as well as on the associated toxins, their transfer, structures and pharmacological effects. These studies provide an extensive but still incomplete understanding of the ciguatera phenomenon. Recent studies have also highlighted a new eco-toxicological phenomenon involving the consumption of giant clams called Ciguatera Shellfish Poisoning (CSP).

The primary producers of ciguatera toxins are commonly assumed to be dinoflagellates of the genera *Gambierdiscus* and *Fukuyoa*, which often proliferate in damaged coral reef areas. Human exposure to CTXs occurs after a process of bioaccumulation along the marine trophic chain, mostly from dinoflagellates to herbivorous fish until carnivorous fish. Along with the biomagnification in the marine food web, algal CTXs are likely to be metabolized in fish, a process that may result in congeners that are more potent. To date, few congeners of CTXs have been described, mostly from fish caught in the Pacific area.

CTXs are a family of lipophilic toxins presenting a cyclic polyether structure. *Gambierdiscus* and *Fukuyoa* also produce other bioactive compounds with a cyclic polyether structure, e.g. maitotoxins (MTXs), gambierol, gambieric acids, gambieroxide and gambierone. To date, it is not fully understood if these compounds play a role in CFP events, but they are considered as compounds of interest for their pharmacological activities.

In addition to uncertainties regarding different toxins involved in CFP and the routes of bioaccumulation and biotransformation in the marine trophic chain, little is known about the degree to which toxicity varies

among species of the causative agents *Gambierdiscus* and *Fukuyoa*. One reason this has proven challenging is that the taxonomy has only recently been sufficiently resolved to examine species-specific toxicity (Fraga and Rodríguez, 2014; Fraga et al., 2011; Fraga et al., 2016; Kretzschmar et al., 2017; Litaker et al., 2009; Nishimura et al., 2014; Smith et al., 2016).

In France, CFP has long been known in the overseas islands, e.g. French Polynesia (Pacific Ocean), the Antilles (Caribbean Sea) and the Mascarene Islands (Indian Ocean). Over the last decades, increased incidences of CFP in the Pacific Ocean and the Caribbean Sea (Friedman et al., 2017), as well as the apparent spread of *Gambierdiscus* and *Fukuyoa* spp. into more temperate areas (Mediterranean Sea and North-Eastern Atlantic Ocean) (Aligizaki, 2008; Laza-Martinez et al., 2016; Rodríguez et al., 2017), necessitates additional efforts in the study of CFP worldwide.

The study of CFP was declared a priority in May 2013 at the Intergovernmental Oceanographic Commission of UNESCO (IOC-UNESCO), Intergovernmental Panel on Harmful Algal Blooms (HAB). In December 2015, an interagency initiative for the study of CFP was established with the participation of different partners, i.e. the Intergovernmental Oceanographic Commission of UNESCO (IOC), the International Atomic Energy Agency (IAEA), the Food and Agriculture Organization of the United Nations (FAO) and the World Health Organization (WHO) (Chinain et al., 2017). In October 2016, the leadership of the interagency initiative changed from the National Oceanic and Atmospheric Administration (NOAA) to the Institut Louis Malardé (ILM), the Institut Français de Recherche pour l'Exploitation de la Mer (IFREMER) and the IAEA (Chinain et al., 2017). The intergovernmental strategy points towards three main objectives: (i) to improve the organism detection and sampling; (ii) to improve toxin detection; (iii) to improve epidemiological data, reporting and risk assessment (Chinain et al., 2017).

The detection of the tropical genera *Gambierdiscus* and *Fukuyoa* in the North-Eastern Atlantic Ocean and in the Mediterranean Sea, and the confirmation of a subtropical range extension of CFP cases (Canary Islands and Madeira) (Otero et al., 2010; Rodríguez et al., 2017), along with a suspected case in Israel (Bentur and Spanier, 2007) and potential risk in Cameroun (Bienfang et al., 2008) are at the origin of my PhD thesis. This research took place primarily at the Phycotoxins Laboratory (IFREMER,

Nantes, France) and also, in part, at the Center for Coastal Fisheries Habit Research (CCFHR) Laboratory (NOAA, Beaufort, NC, USA), the Toxicology of Contaminants Unit Laboratory (ANSES, Fougères, France) and the Mer, Molécules et Santé (MMS) Laboratory (University of Nantes, Nantes, France). In the last few years, the Phycotoxins Laboratory of Ifremer has intensified its research on ciguatera, which has involved (i) the establishment of laboratory cultures of different strains of *Gambierdiscus* spp. and (ii) the introduction of methods of toxin detection and quantification (biological assays, LC-MS analyses in high and low resolution).

The main aims of the study are described as follows:

(i) to establish laboratory cultures of *Gambierdiscus* and *Fukuyoa* species isolated from different locations worldwide and to assess their relative CTX- and MTX-toxicity using functional bioassays (i.e. neuro-2a and erythrocyte lysis assays).

(ii) to pinpoint CTX and MTX candidates in the most toxic strains using an approach combining bioguided fractionation with liquid chromatography coupled to high resolution mass spectrometry.

Outline of the PhD thesis

Chapter 1 is devoted to the state of the art of the themes addressed in the PhD thesis. The chapter is divided into three sections with the following main contents: (i) ciguatera disease; (ii) the dinoflagellates involved, i.e. *Gambierdiscus* and *Fukuyoa* and (iii) the bioactive compounds produced by these dinoflagellates, focusing on ciguatoxins (CTXs) and maitotoxins (MTXs).

Chapter 2 concerns the evaluation of relative toxicity among different species of *Gambierdiscus* and *Fukuyoa*. Ciguatoxicity was evaluated using the neuro-2a assay, and maitotoxicity using the erythrocyte lysis assay. The chapter is divided into two sections. The first section corresponds to a scientific article published in 2017 in the journal Harmful Algae. The second section corresponds to a scientific article published in 2017 to the journal PLoS One. The first article focuses on strains isolated from the Pacific Ocean, the Mediterranean Sea and the North-Eastern Atlantic Ocean. The second article focuses on strains isolated from the Caribbean Sea and the Gulf of Mexico. The main finding of these two studies concerns the high toxicity exhibited by strains of the species *G. excentricus*.

Chapter 3 focuses on the bioguided fractionation of highly toxic strains of *G. excentricus*. Purification steps, toxicity screening and chemical analyses adopted will be addressed. The chapter is divided into three sections, corresponding to (i) an article published in 2017 in the journal Marine Drugs, (ii) an article in preparation for the journal Rapid Communication in Mass Spectrometry and (iii) preliminary results for a future study. The main finding of this chapter is the discovery of a novel MTX congener, maitotoxin-4 (MTX4), only produced by strains of *G. excentricus*.

Chapter 1: State of the art

1. Ciguatera poisoning

1.1. General introduction

Ciguatera Fish Poisoning (CFP) is a form of ichthyosarcotoxism (ichtus = fish, sarcos = flesh, toxikon = poison) caused by the ingestion of reef or carnivorous fish. The flesh of these fish accumulates neurotoxins known as ciguatoxins. Fish acquire their toxicity through their diet and it is not possible to distinguish a tainted or toxic fish from an untainted one by appearance, texture, smell or taste. More than 400 species of fish are potential vectors and other potential vectors are being proposed or identified, e.g. Ciguatera Shellfish Poisoning (CSP) (Laurent et al., 2012).

Ciguatera poisoning is a foodborne illness. Despite the fact that CFP is very often not recognized and is universally under-reported (Begier et al., 2006; Lewis, 2001), it is, nonetheless, the most common non-bacterial fish-related intoxication in the world. It has an annual incidence estimated between 50,000 and 100,000 people worldwide (Lehane and Lewis, 2000). This high incidence is due to the lack of health management, the high potency of the causative toxins and the fact that carnivorous fishes sometimes migrate over long distances, making the original source of the toxins difficult to identify.

CFP constitutes not only a serious threat for human health, but it also leads to considerable socio-economic consequences, representing an obstacle to fisheries and international tourism, especially in tropical and subtropical island communities (Bagnis, 1993; Glaziou and Legrand, 1994; Lewis and Ruff, 1993).

The term “ciguatera” was coined in 1866 by the ichthyologist Felipe Poey y Aloy to describe a neuro-digestive intoxication which frequently occurred in Cuba after the ingestion of *Cittarium pica* (also reported as *Livona* or *Turbo pica*), a gastropod mollusk known locally as “cigua” (Poey, 1866). The term was later extended to a similar clinical syndrome arising from fish in the region. The term "ciguatoxin" was first used in 1967 by Scheuer et al. (1967) to describe the most abundant toxin isolated from the flesh of a Javanese giant moray eel *Gymnothorax* (or *Lycodontis*) *javanicus*.

The ciguatera syndrome manifests itself mainly as gastrointestinal, neurological, and, in severe cases, cardiovascular symptoms. The

distinctive clinical sign of CFP is cold allodynia, i.e. the inversion of the perception of temperature, wherein a cold spot induces a burning sensation. The duration, severity and number of symptoms depend on the amount of ciguatoxins (CTXs) consumed, the type of fish ingested (herbivorous, carnivorous) and the location where the fish was caught. Poisoning can also cause long-term effects, e.g. asthenia and depression, which can last for months or even years in chronic and relapsing cases.

1.2. **Clinical picture**

Epidemiological characterization of ciguatera poisoning has been limited by the lack of laboratory tests able to confirm the presence of CTXs. In the absence of a human biomarker specifically attributed to CTXs, the diagnosis of ciguatera is based on a detailed anamnesis and the patient's recent fish-eating history. The clinical picture of ciguatera disease is complex since different systems can be affected (gastro-intestinal, neurological, musculoskeletal and cardiovascular) with number, severity and persistence of symptoms that may vary from case to case. As reviewed by Palafox and Buenconsejo-Lum (2001), three different phases of ciguatera can be distinguished with distinct patterns: acute ciguatera, chronic ciguatera and relapsing ciguatera.

1.2.1. Acute ciguatera

Acute ciguatera is defined as the clinical manifestations occurring within the first two weeks of exposure to CTXs (Palafox and Buenconsejo-Lum, 2001). Acute ciguatera generally manifests within 1-6 hours after ingestion of toxic fish (Ruff and Lewis, 1997). The syndrome generally involves multiple gastrointestinal (nausea, vomiting, abdominal pain, diarrhea), neurological (paresthesia in the extremities and oral region, dysesthesia, itching, myalgia, arthralgia, fatigue) and neuropsychiatric (hallucinations, giddiness, incoordination or ataxia) symptoms. In severe cases, cardiovascular symptoms (hypotension followed by irregular attacks of hypertension, bradycardia) may also occur (Friedman et al., 2008; Palafox and Buenconsejo-Lum, 2001). Cold allodynia, i.e. the intense and painful tingling, burning, or electric shock sensation in response to a cold stimulus (Palafox and Buenconsejo-Lum, 2001) is considered the distinctive sign of acute ciguatera intoxications (Château-Degat et al., 2007b), even if it is not always present. Allodynia is also documented in

severe cases of neurotoxic shellfish poisoning (NSP) caused by brevetoxins (Watkins et al., 2008).

The symptomatology may vary in different parts of the world. For example, Palafox and Buenconsejo-Lum (2001) reported that (i) gastro-intestinal symptoms are more common in the US Virgin Islands than in French Polynesia; (ii) the diarrheic component of ciguatera is almost absent in the Marshall Islands and (iii) itching is more prominent in Australia than in other regions. Friedman et al. (2008) reported that (i) in the Caribbean Sea neurologic symptoms generally become prominent after an acute phase of gastrointestinal symptoms (i.e. first 12 hours (Lawrence et al., 1980)); (ii) in the Pacific Ocean the neurological symptoms predominate (Nicholson and Lewis, 2006) and seem to be more severe, sometimes including coma (Bagnis et al., 1979); (iii) neuropsychiatric alterations seem to be specific to the Indian Ocean cases (Quod and Turquet, 1996). The variability of the clinical presentations of the disease can be attributed to different factors, e.g. different CTX congeners involved depending on the region (Pacific, Caribbean and Indian), the strains of *Gambierdiscus*/*Fukuyoa* implicated, the species of fish consumed (Kodama and Hokama, 1989; Morris et al., 1982; Vernoux and Lewis, 1997), the co-occurrence of other toxins (e.g. maitotoxin, okadaic acid, brevetoxin) (Hokama, 1988). In addition, the individual susceptibility of the affected patients is a significant factor in the variability of symptoms, and may be correlated to ethnic/genetic differences (Bagnis and Legrand, 1987; Kodama and Hokama, 1989), as well as age (Glaziou and Martin, 1993).

The severity of the symptoms depends on the amount of toxins ingested and the individual susceptibility. Symptoms are more severe when other parts of the fish than muscle (e.g. head, liver, gonad) are consumed (Château-Degat et al., 2007b; Ting et al., 1998; Tosteson, 1995). In severe cases, death may occur due to severe dehydration, cardiovascular shock or respiratory failure, resulting from paralysis of the respiratory musculature (Friedman et al., 2008). According to Ruff and Lewis (1997), the mortality rate due to ciguatera intoxication is low and is noted in only 0.1% of the reported cases. Notwithstanding, poisoning events due to the ingestion of shark tissues, which occurred in Madagascar (Indian Ocean) in 1993 and in 2013, constitute an exception to this general pattern for their unprecedented severity. In 1993, a poisoning outbreak was reported in Madagascar after the consumption of a single shark specimen, either a bull

shark of the species *Carcharhinus leucas* or a pigeye shark of the species *Carcharhinus amboinensis* (Boisier et al., 1995; Habermehl et al., 1994). This outbreak affected hundreds of people (200-500) and resulted in a mortality rate of 20% (Habermehl et al., 1994) or 30% (Boisier et al., 1995), depending on the report. Short thereafter, it was determined that the causative agents of the shark poisoning were not ciguatoxins (CTXs), but rather previously undescribed toxins, tentatively named carchatoxins (Boisier et al., 1995). Eventually, this poisoning case was not classified as a ciguatera fish poisoning (CFP) episode. In 2013, a novel poisoning outbreak was reported in Madagascar after the consumption of a bull shark specimen of the species *Carcharhinus leucas* (ANSES, 2015; Rabenjarison et al., 2016). This outbreak involved 124 people and resulted in a mortality rate of 9% (ANSES, 2015). A recent study conducted by Diogène et al. (2017) provided evidence of the involvement of CTXs in this poisoning event, using both toxicity evaluation (mouse bioassay and neuro-2a cell-based assay) and chemical analysis (high resolution mass spectrometry).

1.2.2. Chronic ciguatera

The chronicity of the symptoms sets ciguatera poisoning apart from other seafood intoxications with similar symptomatology (Kumar-Roiné et al., 2010) and makes it an intensely distressing illness with long-term debilitation. To date, rarely have studies been specifically designed to address chronic ciguatera (Baumann et al., 2010; Château-Degat et al., 2007a; Château-Degat et al., 2007b). Even less is known in the case of repeated exposures to sub-clinical doses of CTXs.

There is no real consensus on the period over which ciguatera symptoms are considered chronic. Palafox and Buenconsejo-Lum (2001) introduced the definition of chronic ciguatera as the clinical syndrome that persists beyond two weeks of the initial intoxication. Nevertheless, more recent studies conducted by Château-Degat et al. (2007a) and Château-Degat et al. (2007b) showed that no symptoms totally disappear between the onset and 15 days after exposure. A three months period of persistence of the symptoms has been proposed for attributing chronicity to this illness (Chung et al., 2016). Chung et al. (2016) reported that at least 20% of CFP victims conserve long-term manifestations (> 3 months after exposure).

Chronic ciguatera is very difficult to diagnose, often considered as an imaginary illness of hypochondriac subjects (Chung et al., 2016). Since the

patients affected by chronic ciguatera present visual disturbances (inability to discriminate the contrast between white, grey and black), Shoemaker et al. (2010) proposed the visual contrast sensitivity (VCS) test to aid the diagnosis.

The symptoms most frequently reported in chronic ciguatera are neurological symptoms comprising of musculoskeletal (myalgia, arthralgia) and neurocutaneous (itching) features (Kumar-Roiné et al., 2010). Itching is proposed by Château-Degat et al. (2007b) as the characteristic sign of the chronic stage of ciguatera disease because of its delayed appearance and its long duration. Neuropsychiatric symptoms such as asthenia, depression, anxiety and cognitive dysfunction are also observed in patients affected by chronic ciguatera, similar to patients affected by chronic fatigue syndrome (Pearn, 2001; Racciatti et al., 2001). These symptoms may be due to CTXs themselves or may be secondary to prolonged illness and the stress associated with being debilitated (Palafox and Buenconsejo-Lum, 2001). Terao et al. (1992) observed that repeated administration (either i.p. or oral) of low doses of CTX1B and CTX4C to mice induced swelling of cardiac muscle cells and endothelial lining cells in a similar manner to that seen after administration of a single high dose (Terao et al., 1991). Wang et al. (2017) provided evidence that repeated exposure to low doses of CTX1B, one of the main congeners of CTXs, causes emotional and cognitive dysfunctions in rats.

The long-term duration of symptoms may be due to the prolonged activation of voltage-gated sodium channels (VGSCs) elicited by CTXs (Lewis, 2006; Ting and Brown, 2001) and/or to a dysregulation of the immune system based on genetic control of the patient response (Lopez et al., 2016; Shoemaker et al., 2010).

Some questions still need to be answered. Epidemiological studies of chronic ciguatera are generally limited to isolated poisoning cases occurring in a given location at a given time. Multicentric studies (Pacific *vs* Caribbean *vs* Indian Ocean) would permit the exploration of the different effects of the CTX family on the chronic nature of CFP (Chung et al., 2016).

1.2.3. Relapsing ciguatera

Relapsing ciguatera is defined as the clinical syndrome that occurs only with specific induction triggers (Palafox and Buenconsejo-Lum, 2001). Recurrence of ciguatera symptoms has been reported in individuals who

previously suffered from CFP, even years after the first exposure (Bagnis, 1993; Lange et al., 1992). This relapsing phase or sensitization phenomenon generally occurs after eating a potentially ciguateric fish that did not produce symptoms in other individuals. Relapsing CFP may also occur when consuming certain other foodstuffs (e.g. pork, chicken, peanuts) or alcohol (Glaziou and Martin, 1993; Mines et al., 1997). The basis of recurrent attacks is unknown but it is generally presumed to be an immunologic reaction (Kumar-Roiné et al., 2010). The patients experience a new attack of acute ciguatera symptoms, generally more severe than the first one (Glaziou and Martin, 1993). Sensitization suggests that, during the first exposure, lipophilic CTXs may have been stored in adipose tissue. Increased lipid metabolism could cause CTXs to re-enter the blood stream and, consequently, the re-emergence of CFP symptoms (Barton et al., 1995). Brevetoxins (BTXs), cyclic polyether toxins having molecular structures similar to CTXs, have been shown to bind to plasma lipoproteins (Woofter and Ramsdell, 2007; Woofter et al., 2005). If this is the case for CTXs as well, toxins in the blood stream may be distributed by lipoproteins either to tissues for storage and/or biological action or to the liver for metabolism. This phenomenon would explain why these toxins are stored for so long in human body.

1.3. **Geographical distribution**

Ciguatera Fish Poisoning is endemic in tropical and subtropical areas of the Pacific Ocean (Bagnis et al., 1979; Chinain et al., 2010b; Rhodes et al., 2017a), the Caribbean Sea (Lawrence et al., 1980; Morris et al., 1982; Taylor, 1985; Tester et al., 2010) and the Indian Ocean (Hansen et al., 2001; Lebeau and Telmar, 1978; Quod et al., 1990; Quod and Turquet, 1996; Thomassin et al., 1992), with the most affected area being the Pacific Island Countries and Territories (PICTs) (Skinner et al., 2011). Poisoning cases have also been reported in more temperate areas (e.g. the United States, Canada and Europe) and are, for the most part, related to tourism (Bavastrelli et al., 2001; Glaizal et al., 2011; Winter, 2009) or to the importation of fish from endemic areas (de Haro et al., 2003; Geller et al., 1991; Puente et al., 2005).

Over the last decades, the geographical distribution of CFP appears to be expanding to new areas previously not considered endemic (**Figure 1**). CFP outbreaks have occurred in the North-Eastern Atlantic, i.e. Canary

Islands (Spain) and Madeira Islands (Portugal). In the Canary Islands, cases of CFP have been reported since 2004 after consumption of local amberjack fish (*Seriola* spp.) (Boada et al., 2010; Nuñez et al., 2012; Pérez-Arellano et al., 2005). Moreover, a recent survey conducted by Rodríguez et al. (2017) identified a large biodiversity of the causative dinoflagellate *Gambierdiscus* in this area, with the ubiquitous *G. australes*, *G. caribaeus* and *G. carolinianus* and the description of two new species, *G. excentricus* (Fraga et al., 2011) and *G. silvae* (Fraga and Rodríguez, 2014), and a new ribotype, *Gambierdiscus* sp. ribotype 3 (Rodríguez et al., 2017). In the Madeira Islands, cases of CFP have also been reported since 2008 after consumption of local fish (*Seriola* spp.) (Caires, 2010; Cunha, 2008; Gouveia et al., 2009). Otero et al. (2010) confirmed the presence of CTXs using UHPLC-MS/MS analysis. Still, in the North-Eastern Atlantic Ocean, the tropical coasts of Cameroon (West Africa) are also considered a potential hotspot for CFP. Although no CFP outbreaks have been reported in this area yet, the presence of ciguatoxic fish (i.e. barracuda, *Sphyraena barracuda* and snappers, *Lutjanus* spp.) was reported in 2008 (Bienfang et al., 2008). Toxicity of those fishes was estimated using two bioassays (i.e. the non-specific *Artemia* bioassay and the VGSC-specific neuro-2a assay). In addition, a tentative identification of the toxins involved was also performed using LC-MS analysis (Bienfang et al., 2008). Ennaffah and Chaira (2015) reported the presence of *Gambierdiscus* on the Southern Atlantic coasts of Morocco for the first time in 2015.

In the Mediterranean Sea, *Gambierdiscus* and *Fukuyoa* spp. have been observed in Crete Island (Greece) since 2003 (Aligizaki and Nikolaidis, 2008; Aligizaki, 2008) and in the island of Formentera (Balearic Islands) since 2016 (Laza-Martinez et al., 2016). Bentur and Spanier (2007) also reported a suspected case of CFP on the Eastern Mediterranean coast of Israel after the consumption of edible fish (*Siganus* spp.).

The detection of the tropical genera *Gambierdiscus* and *Fukuyoa* in the Atlantic Ocean and in the Mediterranean Sea, along with the confirmation of CFP cases in the Canary Islands and in Madeira and the suspected case in Israel along with the presence of ciguatoxic fish on Cameroon coasts, suggests additional efforts are needed to study CFP worldwide.

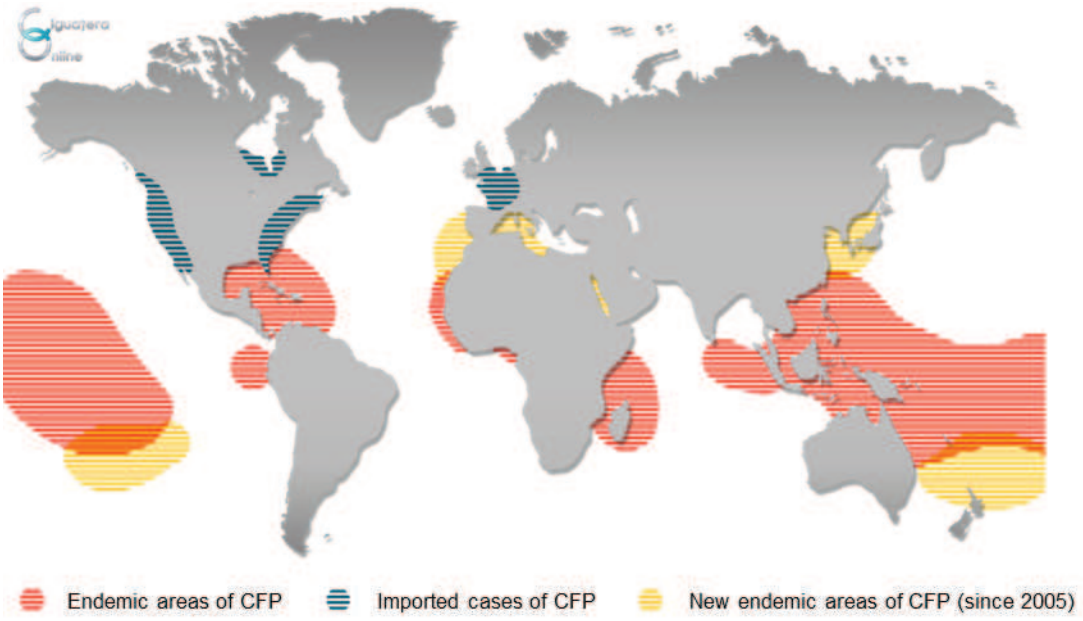

Figure 1. World distribution of ciguatera, from Ciguatera Online website (http://www.ciguatera-online.com), image credit: Louis Malardé Institute (Gatti, 2014).

A CFP outbreak, at a given time, in a certain location, and the presence of the toxin-producing *Gambierdiscus* and *Fukuyoa* spp. in seawater are difficult to correlate for several reasons. First, not all species and strains of *Gambierdiscus* and *Fukuyoa* are equally toxic; therefore, a bloom does not necessarily translate into a CFP episode. Moreover, fish are moving vectors, often covering long distances hampering an accurate spatial correlation. Finally, the bioaccumulation of toxins in fish flesh along the marine food chain is a long-term process. All these factors suggest that (i) the causative dinoflagellates are not necessarily found at the onset of an episode of ciguatera in the location from which the contaminated fish was caught and that (ii) the occurrence of a dinoflagellate bloom could represent a false alert if it is represented by non-toxic clones unable to trigger a CTX bioaccumulation. Therefore, monitoring programs and predictive models to prevent CFP are very difficult to establish. However, the occurrence of toxic (CTX-producing) blooms may be used as a bio-indicator of local ciguatera risk in a specific area. The establishment of laboratory cultures of *Gambierdiscus* and *Fukuyoa* strains, in parallel with advances in their taxonomic and phylogenetic characterization, is crucial for better assessment of CFP risk associated with a particular bloom. Significant progress has been made on understanding the intrinsic factors leading to CTX production, e.g. the genetic pattern of a given strain/species, and the

extrinsic factors, e.g. the environmental conditions favorable to proliferation and toxin production.

1.4. **Intoxication routes**

1.4.1. Ciguatera fish poisoning

Ciguatera poisoning mainly involves consumption of reef fish contaminated by ciguatoxins (CTXs). Studies conducted in late 1950s first hypothesized the transmission of ciguatera disease from an unspecified epiphytic benthic microorganism to humans through the food chain (Mills, 1956; Randall, 1958). After a thorough examination of the feeding behavior of ciguatoxic fish in the Pacific, Randall (1958) proposed the food chain theory. Natural or anthropogenic disturbances (e.g. cyclones, coral bleaching, dredging, and shipwrecks) in coral reef ecosystems provide denuded surfaces for macroalgae to grow on and serve as substrate for toxic epi-benthic microorganisms. Subsequently, the toxins produced by *Gambierdiscus*/*Fukuyoa* are biomagnified up through the marine food web, from herbivorous and detritus-feeding fish to carnivorous fish. Later, some studies corroborated Randall's hypothesis. Helfrich and Banner (1963) provided evidence for trophic transfer in the food web using laboratory-controlled feeding experiments of a non-toxic fish (*Acanhurus xanthopterus*) with ciguateric fish flesh (*Lutjanus bohar*). Banner et al. (1966) showed that a non-toxic diet dispensed over a period of 30 months to specimens of a ciguateric fish (*Lutjanus bohar*) was not enough to reduce their toxicity, suggesting a slow elimination of the toxins involved. Investigation of ciguatoxic fish and their benthic food sources in several French Polynesian islands provided evidence to identify a dinoflagellate as the causative organism of ciguatera. Yasumoto et al. (1977a) correlated the high toxicity of algae and detritus on dead coral samples collected from Gambier Islands with the presence of high quantities of a dinoflagellate, tentatively identified as *Diplopsalis* sp. nov. The dinoflagellate was also found in stomach contents of a toxic specimen of unicorn fish (*Naso unicornis*) from the same area. Isolation of this dinoflagellate from coral debris collected from Gambier Islands and organic extraction and fractionation permitted the evaluation of ciguatoxin activity (Yasumoto et al., 1977b). It was found to be similar to the ciguatoxin reference material isolated from the notably highly ciguatoxic moray eel

(*Gymnothorax javanicus*) (Scheuer et al., 1967). The causative organism was later renamed as a new species in a monospecific genus, *Gambierdiscus toxicus* (Adachi and Fukuyo, 1979). Bagnis et al. (1980) finally confirmed the involvement of the dinoflagellate *G. toxicus* into CFP examining the toxicity of both wild (biodetritus samples) and isolated laboratory-cultured cells from Gambier Islands. Since the initial designation of *G. toxicus*, a number of new species have been described within this genus and a new, closely related genus, *Fukuyoa*, has recently been distinguished from *Gambierdiscus* (Gómez et al., 2015). *Fukuyoa* may also be involved in the ciguatera phenomenon.

Gillespie et al. (1986) revealed high local variability in population densities of *G. toxicus* on the Queensland coast and highly variable toxin production. It was later discovered that only certain genetic strains produced ciguatoxins (Holmes et al., 1991; Lehane and Lewis, 2000). Although the dinoflagellate is present in Caribbean waters (Taylor, 1985) and in the Indian Ocean (Hansen et al., 2001), no algal ciguatoxin from these areas has yet been isolated.

A study conducted by Château-Degat et al. (2005) in French Polynesia showed that ciguatera outbreaks might occur as late as three months after the appearance of a *Gambierdiscus* bloom. This long time gap is likely to derive, at least in part, from the bioaccumulation of ciguatera toxins from epi-benthic microalgae up to the highest trophic levels of the marine food chain (**Figure 2**).

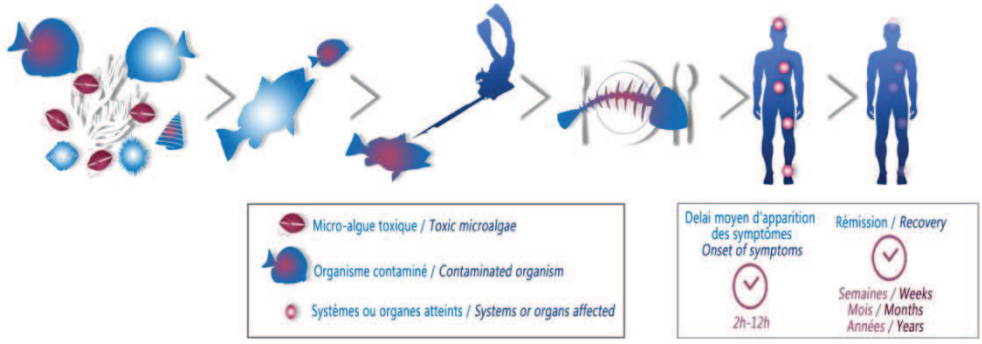

Figure 2. Intoxication route of ciguatera fish poisoning (Gatti, 2014), from Ciguatera Online website (http://www.ciguatera-online.com). Image credit: Institut Louis Malardé.

Along with bioaccumulation, Lewis and Holmes (1993) proposed that toxins (at least Pacific CTXs) undergo metabolization (e.g. oxidation) in fish, concomitant with an increase in toxicity along the marine trophic chain. Because of this process, it is commonly assumed that carnivorous predators at the top of the food web have the highest concentrations of toxins in their flesh. Ingestion of large carnivorous fish (barracuda, snappers, groupers, jacks and moray eels) has often been associated with severe cases of CFP, arising from high toxins loads through their diets (Pearn, 2001; Van Dolah et al., 2000). Positive relationship between fish size and CTX levels was found for four fish species in Japan (Oshiro et al., 2010). In contrast, a study conducted by Gaboriau et al. (2014) in French Polynesia concluded that fish size does not always matter, since their data show that small herbivorous fish could be as toxic as large predatory ones. Similarly, a recent study conducted by Kohli et al. (2017b) in Australia found no relationship between the length or the weight of specimens of the Spanish mackerel *Scomberomorus commerson* and CTX1B amounts in their flesh or liver. These studies indicate that the relationship between fish size and CTX levels may differ on a regional basis and/or according to species-specific toxin bioaccumulation and depuration rates.

Halstead (1978) suggested that more than 400 species of fish can be ciguatoxic, although this number may be too high (Juranovic and Park, 1991). Nevertheless, it is likely that CTXs move throughout the piscivorous food web resulting in numerous species accumulating CTXs. Some examples of CFP vectors described in literature are shown in **Figure 3**.

Recent studies conducted by the International Atomic Energy Agency (IAEA) research group (Monaco) have focused on the dynamics of the CTX transfer and bioaccumulation from the dinoflagellate to herbivorous reef fish in laboratory-controlled feeding conditions. The feeding experiments conducted by Ledreux et al. (2014) consisted of single and repeated (9 times, once every other day) oral exposures of the mullet *Mugil cephalus* (sediment feeding fish) to the most toxic *Gambierdiscus* strain known to date, *G. polynesiensis* TB-92 (French Polynesia). Initial results showed rapid absorption and distribution of CTXs in the blood stream of fish, but no retention in the flesh. A novel experimental set-up consisting of a continued feeding of the juvenile surgeonfish *Naso brevirostris* with TB-92 over a longer period (16 weeks) resulted in increasing accumulation of CTXs in fish flesh (Clausing et al., 2017).

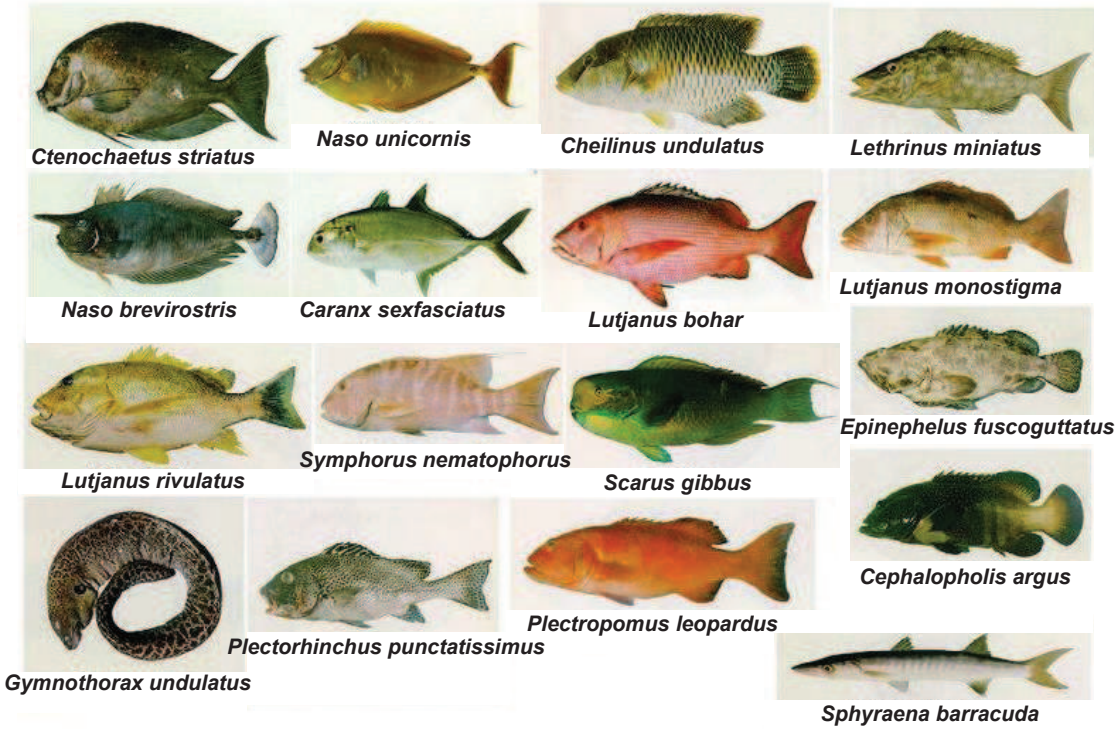

Figure 3. Examples of fish implicated in ciguatera fish poisoning. Photo credit: Yasuwo Fukuyo, UNESCO.

1.4.2. Other CTX vectors and sources - Ciguatera shellfish poisoning (CSP)

Although coral reef fish are considered as the main vectors of ciguatera, other vectors should not be neglected. This is the case of several marine invertebrates, such as giant clams (Laurent et al., 2012; Roué et al., 2013; Roué et al., 2016), crustaceans (Lewis et al., 1994b), gastropods (Gatti et al., 2015; Lonati et al., 2015; Yasumoto and Kanno, 1976) and echinoderms (Silva et al., 2015).

Among all non-fish vectors, most studies have focused on giant clams. In the 1960s, giant clams were already known to be involved in ciguatera cases (Bagnis, 1967; Banner, 1967). An epidemiological survey conducted in 1974 in Gambier Islands (French Polynesia) established that 4% of all cases of poisoning were due to consumption of giant clams (Bagnis, 1974), as reviewed by Laurent et al. (2012). Laurent et al. (2008) reported numerous cases of an atypical ciguatera-like syndrome due to consumption of giant clams (*Tridacna* spp.) in New Caledonia during the period 2001-2005. The symptomatology of this atypical syndrome includes the

characteristics of CFP associated with a rapid onset of additional symptoms (~ 2 hours after ingestion) such as the burning of mouth and throat followed by severe paralysis. Benthic cyanobacteria of the genus *Hydrocoleum* were proposed as causative agents, albeit with the involvement of a mixture of toxins, e.g. lipophilic CTX-like toxins and water-soluble paralyzing toxins (PSTs and/or anatoxin-a and homoanatoxin-a). Sea urchins have also been involved in human intoxications and have been suggested as potential vectors of the *Hydrocoleum* CTX-like toxins (Pawlowiez et al., 2013). Another study led by Kerbrat et al. (2010) in New Caledonia suggested the potential involvement of *Trichodesmium* spp., pelagic cyanobacteria morphologically and genetically very close to the genus *Hydrocoleum* (Abed et al., 2006). Chinain et al. (2010b) and Rongo and van Woesik (2011) also reported cases of intoxication due to consumption of giant clams in Raivavae Island (French Polynesia) and Rarotonga (Cook Islands). Later, extensive epidemiological surveys conducted during the period 2007-2010 in New Caledonia, Republic of Vanuatu and French Polynesia corroborated the previous findings and strengthened the link between benthic cyanobacteria and these intoxications (Laurent et al., 2012). The authors proposed the name Ciguatera Shellfish Poisoning (CSP) to designate this newly eco-toxicological phenomenon (**Figure 4**). Roué et al. (2016) provided evidence of the ability of giant clams to retain CTXs in their tissues, confirming the potential threat of these mollusks in areas where *Gambierdiscus* and *Fukuyoa* are endemic. LC-LRMS/MS analyses revealed the presence of algal CTXs in giant clam (*Tridacna maxima*) tissues after a 48 hours *ex-situ* exposure to the highly toxic strain *G. polynesiensis* TB-92.

Moreover, various forms of human-to-human transmission of ciguatera have been described as well: (i) across the placenta to the fœtus (Pearn et al., 1982), (ii) via milk to breastfed infants (Bagnis and Legrand, 1987; Blythe and de Sylva, 1990; Thoman, 1989) and (iii) via sexual intercourse (Geller et al., 1991; Lange et al., 1989).

The involvement of different species in CTX production and transfer of toxins in the marine food web and between humans makes ciguatera a complex phenomenon and requires an improved global strategy in risk assessment and management programs.

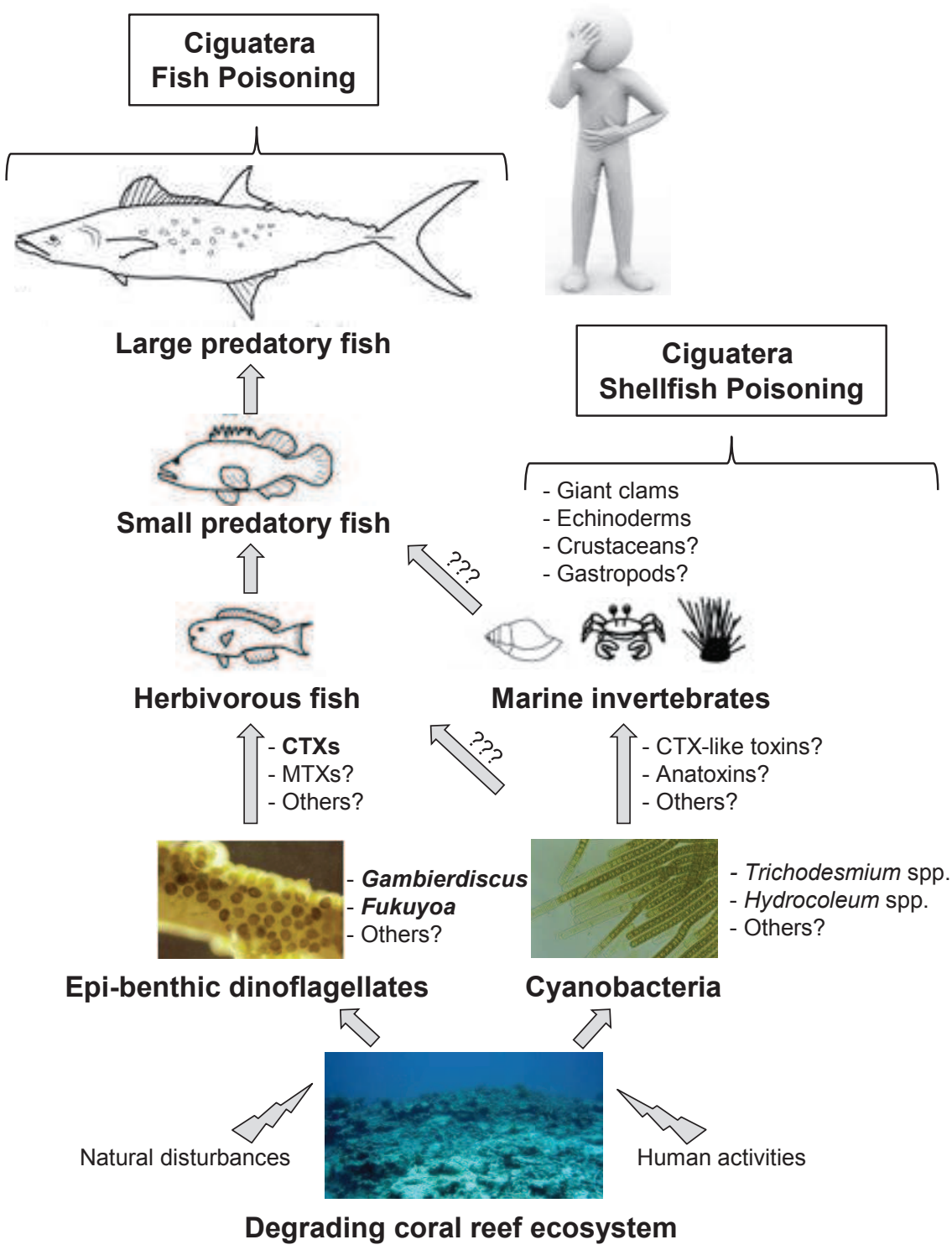

Figure 4. Schematic representation of the trophic pathways of ciguatera poisoning. Inspired by Laurent et al. (2012) and Heimann et al. (2001). ??? = hypothesized pathways.

1.5. **Treatments**

No specific therapy for ciguatera has been identified to date. Investigation of specific treatments is difficult because of (i) the relatively low incidence and irregularity of CFP occurrences, (ii) variability in the symptomatic profile of study participants (iii) lag-time between the intoxication and the treatment and (iv) the chemical diversity of the toxins involved (Kumar-Roiné et al., 2011). Other aspects which have hampered the research of specific treatments include: (i) the lack of resources for conducting such studies in regions where CFP is endemic and (ii) the absence of a clinical analytic test providing diagnostic precision (Kumar-Roiné et al., 2011). Only symptomatic treatments are currently available, and they can be divided into two groups: the traditional medicine and the conventional medicine.

1.5.1. Traditional medicine

Numerous traditional herbal remedies are employed to treat CFP, usually prepared by boiling, infusion or maceration of different parts (leaves, barks, roots or latex) of plants (Kumar-Roiné et al., 2011). The application of these herbal preparations can either be internal, as oral administration, external, in the form of a bath, to help calm the sensation of pruritus, or both (Bourdy et al., 1992; Laurent et al., 1993). In the Pacific, the most popular traditional treatment is an infusion of *Heliotropium foertherianum* leaves, formerly known as *Argusia argentea* (Kumar-Roiné et al., 2011). Its major bioactive constituent is rosmarinic acid (Rossi et al., 2012).

1.5.2. Conventional drugs

In case of acute ciguatera, intravenous hypertonic D-mannitol is currently considered the treatment of choice (Birinyi-Strachan et al., 2005a; Lewis et al., 1993; Mattei et al., 1999b; Mitchell, 2005; Purcell et al., 1999), although its efficacy still remains controversial (Palafox, 1992).

The mechanisms for mannitol reducing the symptoms of ciguatera are postulated to be either a reduction of axonal œdema and/or a scavenger effect (Palafox and Buenconsejo-Lum, 2001). Success of the therapy is generally associated with early administration, i.e. within 48 hours of poisoning (Mines et al., 1997). The choice of other treatments depends on

the symptoms displayed and the severity of each case and may include: (i) antispasmodic, antiemetic and/or anti-diarrhea drugs for gastro-intestinal disorders, (ii) antihistaminic drugs for pruritus and (iii) vitamin complex (B_1, B_6, B_{12}), colchicine, acetylsalicylic acid and calcium gluconate injections for neurological symptoms (Kumar-Roiné et al., 2010). In severe cases, cardiovascular stimulants are administered to counter shock or collapse. In case of hydro-electrolytic diseases, hospitalization needs to be considered, especially if the patient is a high-risk individual (e.g. child, aged person, pregnant woman) (Friedman et al., 2017).

For chronic ciguatera, non-steroidal anti-inflammatory agents and antidepressants are used for joint pain and depression, respectively. Oral administration of cholestyramine, a non-absorbable anion-binding resin, has also shown efficacy against several chronic symptoms (Shoemaker, 2000; Shoemaker et al., 2010).

2. The dinoflagellate genera *Fukuyoa* and *Gambierdiscus*

2.1. General introduction

Compared to planktonic microalgae, epiphytic and benthic harmful species have a relatively short history of study. Indeed, the study of such organisms started in the mid-1970s when a benthic dinoflagellate, tentatively identified as *Diplopsalis* sp. (Yasumoto et al., 1977b) and later described as a new genus and a new species *Gambierdiscus toxicus* (Adachi and Fukuyo, 1979), was found to be the causative agent of ciguatera fish poisoning (CFP). Since then, many other species and phylotypes of *Gambierdiscus* and the genus *Fukuyoa*, closely related to *Gambierdiscus*, have been described.

Gambierdiscus and *Fukuyoa* are thecate dinoflagellates (Gonyaulacales, Dinophyceae), unicellular eukaryotic organisms that are autotrophic, i.e. photosynthetic. They behave as epiphytic and benthic microorganisms, which mean that they need a surface on which they can attach in order to proliferate, e.g. macroalgae or rocky sediments. Free-swimming forms in the water column have also been reported, demonstrating that they are not obligate epiphytes, at least not over the complete life cycle (Bomber, 1987; Yasumoto et al., 1977b). They possess trichocysts and mucocysts, internal organelles related to extrusion and mucus production, respectively (Durand-Clément and Couté, 1991). They produce several classes of cyclic polyether compounds, e.g. ciguatoxins (CTXs) and maitotoxins (MTXs). As *Gambierdiscus*, and potentially *Fukuyoa*, are the primary producers of CTXs, they are considered the causative agents of ciguatera fish poisoning. Nevertheless, other toxin-producing benthic/epiphytic dinoflagellates usually co-occur with *Gambierdiscus* and *Fukuyoa* (e.g. *Amphidinium*, *Coolia*, *Ostreopsis* and *Prorocentrum*) and could have a potential role in the diversity of ciguatera symptoms. Moreover, the involvement of cyanobacteria producing CTX-like compounds is suspected as well (Laurent et al., 2008).

This section of **chapter 1** is a brief review on the taxonomy and ecophysiology of the dinoflagellate genera *Gambierdiscus* and *Fukuyoa* to put their role in CFP in context.

2.2. **Taxonomy**

Initially, the genus *Gambierdiscus* included two morphotypes: globular and lenticular (or discoid). According to this morphological feature, the lineage *Gambierdiscus* has been split into two genera: the original name reserved for the species with lenticular shapes, whereas a new genus *Fukuyoa* (gen. nov.) applies to the globular species (Gómez et al., 2015) (**Figure 5**, ventral view). Other morphological differences concern the number, the size, the shape and the arrangement of thecal plates and include the following: (i) in *Fukuyoa* spp. the largest plate is 1', while in *Gambierdiscus* spp. the largest plate is 2'; (ii) the sulcus of *Fukuyoa* consists of seven plates rather than six plates (iii) and plate 2'''' invades the sulcus in *Fukuyoa* as for other genera such as *Alexandrium* and *Goniodoma* (Gómez et al., 2015). The separation of the two genera is supported by phylogenetic studies (**Figure 8**).

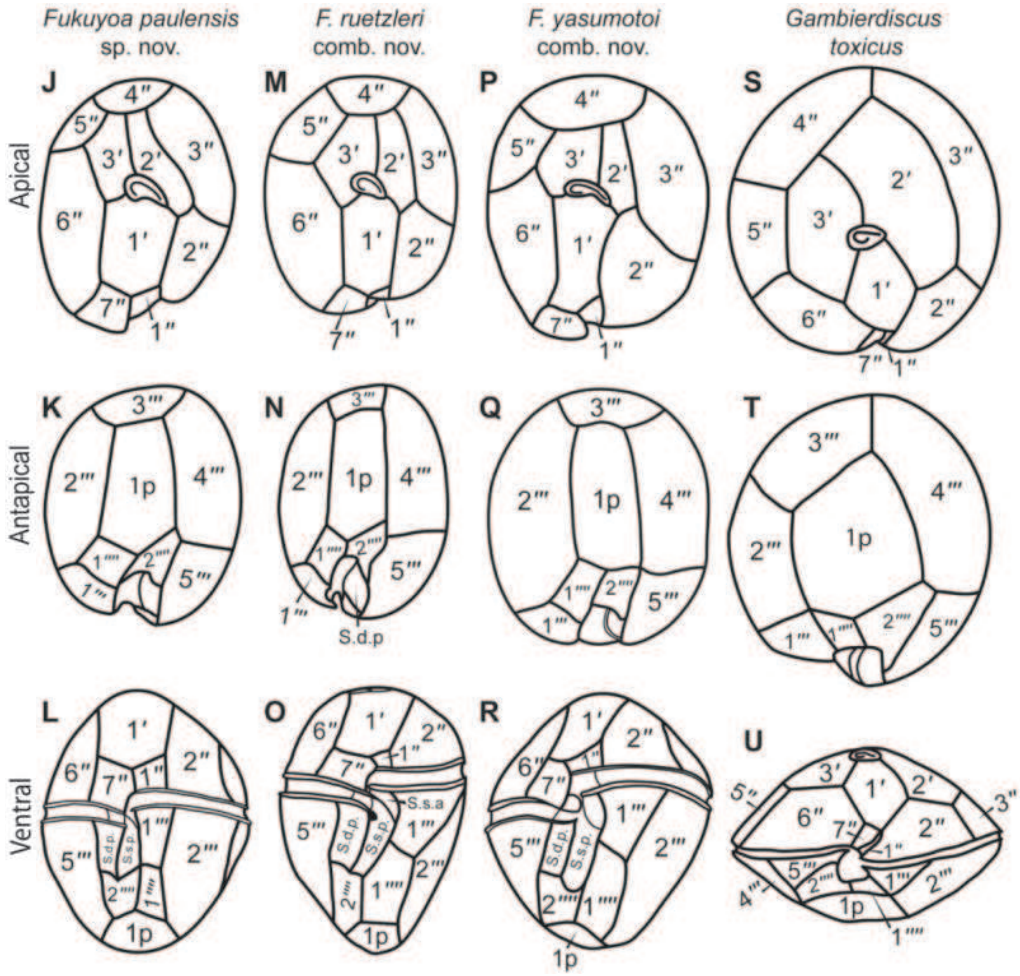

Figure 5. Schematic representation (apical, antapical and ventral view) of *F. paulensis* (**J**-**K**-**L**), *F. ruetzleri* (**M**-**N**-**O**); *F. yasumotoi* (**P**-**Q**-**R**) and *G. toxicus* (**S**-**T**-**U**) (Gómez et al., 2015).

To date, fifteen species and seven phylotypes of *Gambierdiscus* and three species and one phylotype of *Fukuyoa* have been described and are listed in **Table 1**.

Table 1. List of all *Gambierdiscus* and *Fukuyoa* species and phylotypes known with references.

Species / Phylotype	References
F. paulensis	Gómez et al. (2015)
F. ruetzleri [(a)]	Gómez et al. (2015)
F. yasumotoi [(b)]	Gómez et al. (2015)
Fukuyoa cf. *yasumotoi* [(c)]	Kretzschmar et al. (2017)
G. australes	Chinain et al. (1999a); Richlen et al. (2008); Litaker et al. (2009)
G. balechii [(d)]	Fraga et al. (2016); Dai et al. (2017)
G. belizeanus	Faust (1995); Richlen et al. (2008); Litaker et al. (2009)
G. caribaeus	Litaker et al. (2009)
Gambierdiscus cf. *caribaeus* (Korean isolate GCJJ1)	Jeong et al. (2012); Berdalet et al. (2017)
G. carolinianus	Litaker et al. (2009)
G. carpenteri	Litaker et al. (2009)
G. cheloniae	Smith et al. (2016)
G. excentricus	Fraga et al. (2011)
G. honu [(e)]	Rhodes et al. (2017b)
G. lapillus	(Kretzschmar et al., 2017)
G. pacificus	Chinain et al. (1999a); Richlen et al. (2008); Litaker et al. (2009)
G. polynesiensis	Chinain et al. (1999a); Richlen et al. (2008); Litaker et al. (2009)
G. scabrosus [(f)]	Nishimura et al. (2014)
G. silvae [(g)]	Fraga and Rodríguez (2014)
Gambierdiscus sp. ribotype 2	Litaker et al. (2010)
Gambierdiscus sp. ribotype 3	Rodríguez et al. (2017)
Gambierdiscus sp. type 2	Kuno et al. (2010)
Gambierdiscus sp. type 3	Nishimura et al. (2013)
Gambierdiscus sp. type 4	Xu et al. (2014)
Gambierdiscus sp. type 5	Xu et al. (2014)
G. toxicus	Adachi and Fukuyo (1979); Chinain et al. (1997); Chinain et al. (1999a); Richlen et al. (2008); Litaker et al. (2009)

[(a)] formerly designated as *G. ruetzleri* (Litaker et al., 2009)
[(b)] formerly designated as *G. yasumotoi* (Holmes, 1998; Litaker et al., 2009; Richlen et al., 2008)
[(c)] formerly designated as *Gambierdiscus* cf. *yasumotoi* (Nishimura et al., 2013)
[(d)] formerly designated as *Gambierdiscus* sp. type 6 (Xu et al., 2014)
[(e)] formerly designated as *Gambierdiscus* sp. (CAWD242, 233 and 250 isolates) (Rhodes et al., 2017a; Rhodes et al., 2017d; Smith et al., 2016)
[(f)] formerly designated as *Gambierdiscus* sp. type 1 (Kuno et al., 2010; Nishimura et al., 2013; Xu et al., 2014)
[(g)] formerly designated as *Gambierdiscus* sp. ribotype 1 (Litaker et al., 2010)

It should be noted that the nomenclature of the thecal plates is not consistent throughout the studies describing different species of those dinoflagellates; i.e. Litaker et al. (2009) used the traditional Kofoid tabulation system, while Fraga et al. (2011) used a modified system described by Besada et al. (1982). As previously suggested (Litaker et al., 2009), ongoing taxonomic studies being conducted by Tunin-Ley et al. (2017) also indicate that the biological diversity within these genera could be much higher than seen to date. Preliminary results of these studies

showed that a strain isolated from Cau Island, Viet Nam (Roeder et al., 2010; The et al., 2008) and strains recently isolated from Indian Ocean do not belong to any of the species already described and form different phylogenetic clades, indicating the existence of potentially new species (Tunin-Ley et al., 2017).

Since toxin production is not consistent among different species/phylotypes, the assessment of *Gambierdiscus* and *Fukuyoa* biodiversity and abundance in field samples is a useful tool for risk assessment purposes. Vandersea et al. (2012) developed rapid, specific and cost-effective qPCR assays for the identification and enumeration of different species/phylotypes found in the Caribbean, i.e. four *Gambierdiscus* species (*G. belizeanus*, *G. caribaeus*, *G. carpenteri*, *G. carolinianus*), one *Fukuyoa* species (*F. ruetzleri*) and one *Gambierdiscus* phylotype (*Gambierdiscus* sp. ribotype 2). Similarly, Nishimura et al. (2016) developed a qPCR assay for the Japanese species *G. australes*, *G. scabrosus*, *Gambierdiscus* sp. type 2, *Gambierdiscus* sp. type 3, and *Fukuyoa* cf. *yasumotoi*. Lyu et al. (2017) developed a restriction fragment length polymorphism (RFLP) assay based on the hypervariable D_1-D_2 region of the large subunit ribosomal RNA gene (LSU rDNA). This assay is rapid and cost-effective, requires basic molecular laboratory capabilities (PCR amplification and gel electrophoresis) and allows identification of 12 *Gambierdiscus* species (*G. australes*, *G. belizeanus*, *G. caribaeus*, *G. carolinianus*, *G. carpenteri*, *G. cheloniae*, *G. excentricus*, *G. pacificus*, *G. polynesiensis*, *G. scabrosus*, *G. silvae*, *G. toxicus*), one *Gambierdiscus* phylotype (*Gambierdiscus* sp. ribotype 2) and three *Fukuyoa* species (*F. paulensis*, *F. ruetzleri* and *F. yasumotoi*).

A historical review on what it is known so far on the taxonomy of these dinoflagellates is proposed in the following sections.

2.2.1. The genus *Gambierdiscus*

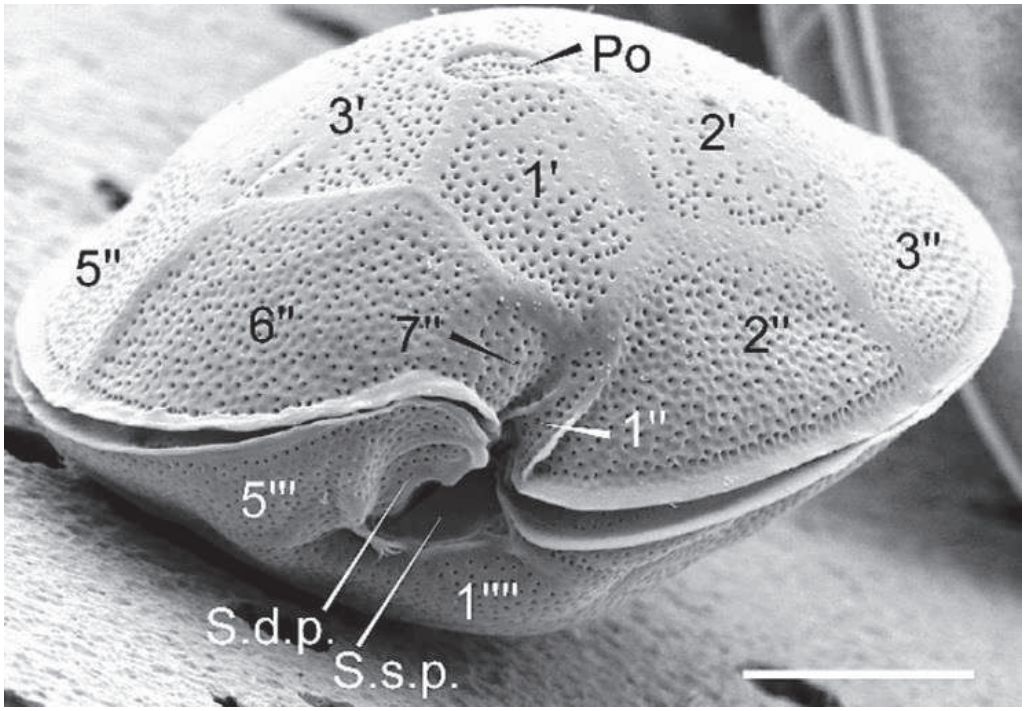

Figure 6. SEM micrograph of *Gambierdiscus toxicus* GTT-91 (ventral view) using SEM microscopy (Litaker et al., 2009). Scale bar: 20 µm.

The first report of *Gambierdiscus* (although referred as *Goniodoma* sp.) in the world dated from 1948 near the coast of Boavista Island (Cabo Verde archipelago) (Silva, 1956). After a tentative attribution to the genus *Diplopsalis* (Yasumoto et al., 1977b), the dinoflagellate *Gambierdiscus* (Adachi and Fukuyo, 1979) was described in the mid-1970s as a mono-specific genus, *G. toxicus*, using live and preserved materials collected in the Gambier Islands, located at the southeast terminus of the Tuamotu Archipelago in French Polynesia. The original species described by Adachi & Fukuyo in 1979 includes the following morphological features: "*Gambierdiscus* cells are large (length: 24-60 µm, transdiameter: 42-140 µm, dorsoventral diameter: 45-150 µm), anterior-posteriorly compressed, armoured, with a theca composed by a total of 33 plates, a fishhook-shaped apical pore, a deep hollow sulcus and a circular narrow deep cingulum, without antapical spine nor apical horn" (Adachi and Fukuyo, 1979) (**Figure 6**).

For almost two decades, *G. toxicus* has been considered the only existing species within this genus. Nevertheless, early studies already highlighted several differences among isolates, consisting of: (i) morphological features (cell size and shape), both in field samples

(Adachi and Fukuyo, 1979) and in laboratory cultures (Chinain et al., 1997; Durand-Clément, 1986, 1987), (ii) growth rates (Bomber et al., 1989b; Chinain et al., 1997; Sperr and Doucette, 1996), (iii) isozyme electrophoretic profiles (Chinain et al., 1997), and (iv) toxin production (Bomber et al., 1989b; Holmes et al., 1990; Holmes et al., 1991; Sperr and Doucette, 1996). Such heterogeneity among isolates suggested that *G. toxicus* was used to designate different species presenting genetic diversity. Early genetic studies also supported this hypothesis (Babinchak et al., 1994; Chinain et al., 1997).

Following the identification of *G. toxicus*, five additional *Gambierdiscus* species were described in the mid-1990s based on detailed morphological examination, mainly thecal plate architecture. *G. belizeanus* was collected from sand material collected from a shallow lagoon in the barrier reef off Belize (Central America) (Faust, 1995). *G. yasumotoi* (Holmes, 1998) was collected from macroalgae samples collected from fringing coral reef surrounding the Singapore island of Pulau Hantu (Singapore, South China Sea). *G. australes* (from Rairua in Raivavae Island, Australes Archipelago, South Pacific), *G. pacificus* (from Otepa in Hao Island, Tuamotu Archipelago, South Pacific), and *G. polynesiensis* (from both Mataura in Tubuai Island, Australes Archipelago and Avatoru in Rangiroa Island, Tuamotu Archipelago, South Pacific) were collected from red calcareous macroalgae in French Polynesia (Chinain et al., 1999a). Chinain et al. (1999a) also provided molecular analysis (D_8-D_{10} domain of large subunit (LSU) rDNA gene sequences) and biochemical data to support the establishment of the three latter species. In order to solve the taxonomic uncertainties linked to the inappropriate attribution of *Gambierdiscus* strains to the species *G. toxicus*, Tester et al. (2006) proposed that the strain described by Adachi and Fukuyo (1979) is the lectotype for this species and that the strain GTT-91 (**Figure 6**) described in Chinain et al. (1999a) is the epitype.

In the last decade, the concept of species within a dinoflagellate genus evolved in parallel with improved microscopic techniques and the advances in molecular phylogenies. The introduction of scanning electron microscopy (SEM) and genetic approaches in species identification permitted to unveil the existence of cryptic species, i.e. species presenting the indistinguishable morphological features but different genetic patterns, and pseudo-cryptic species, i.e. species that can be differentiated only on very

subtle morphological features (Simon et al., 2009). Another limitation of the traditional “morphological species concept” is that morphology can change according to life stages and is heavily influenced by external conditions (Bravo et al., 2014; Fraga et al., 2011; Simon et al., 2009). Now, it is widely accepted that morphological criteria alone are typically not enough for the establishment of a new species and that phylogenetic analyses are required.

The separation of *G. toxicus* (Adachi and Fukuyo, 1979) into a total of six species (Chinain et al., 1999a; Faust, 1995; Holmes, 1998) was further supported by phylogenetic analyses using both large subunit (LSU) rDNA (D_8-D_{10} domains) and small subunit (SSU) rDNA gene sequences (Richlen et al., 2008). Litaker et al. (2009) did an extensive review of *Gambierdiscus* taxonomy using both morphological (SEM) and more accurate phylogenetic criteria, i.e. D_1-D_3 domains of LSU rDNA in addition to D_8-D_{10} LSU rDNA and SSU gene sequences for all described species. The study also revised the genus and described four new species: *G. caribaeus* (Carrie Bow Cay, Belize, Central America), *G. carolinianus* (Cape Fear, North Carolina, USA), *G. carpenteri* and *G. ruetzleri* (South Water Cay, Belize, Central America).

More recent studies involved the description of the following species: *G. excentricus* (Fraga et al., 2011) and *G. silvae* (Fraga and Rodríguez, 2014), isolated from Canary Islands; *G. scabrosus* (Nishimura et al., 2014), from Japan; *G. balechii* (Dai et al., 2017; Fraga et al., 2016), from Celebes Sea, Indonesia; *G. lapillus* (Kretzschmar et al., 2017) from the Great Barrier Reef (Australia); *G. cheloniae* (Smith et al., 2016) from Rarotonga (Cook Islands) and *G. honu* from Rarotonga (Cook Islands) and from North Meyer Island (Kermadec Islands). *G. excentricus* has recently also been found in the Caribbean Sea (Litaker, personal communication) and in Brazil (Nascimento et al., 2015); *G. silvae* has recently been isolated from Brazil (Nascimento, 2017).

Some studies also introduced phylotypes, i.e. strains which are genetically distinct from all the other known species but whose morphological description has not yet been completed. This is the case of *Gambierdiscus* cf. *yasumotoi* (Nishimura et al., 2013), *Gambierdiscus* cf. *caribaeus* (Berdalet et al., 2017; Jeong et al., 2012), *Gambierdiscus* sp. ribotype 1, 2 (Litaker et al., 2010) and 3 (Rodríguez et al., 2017), *Gambierdiscus* sp. type 1 to 6 (Kuno et al., 2010; Nishimura et al., 2013; Xu

et al., 2014) and *Gambierdiscus* sp. (isolates CAWD242, 233 and 250) (Rhodes et al., 2017a; Rhodes et al., 2017d; Smith et al., 2016). Among the *Gambierdiscus* clades initially characterized on the basis of molecular data, four of these, i.e. *Gambierdiscus* sp. ribotype 1 (Litaker et al., 2010), *Gambierdiscus* sp. type 1 (Nishimura et al., 2013), *Gambierdiscus* sp. (isolates CAWD242, 233 and 250) (Rhodes et al., 2017a; Rhodes et al., 2017d; Smith et al., 2016) and *Gambierdiscus* sp. type 6 (Xu et al., 2014) were recently described as the new species *G. silvae* (Fraga and Rodríguez, 2014), *G. scabrosus* (Nishimura et al., 2014), *G. honu* (Rhodes et al., 2017b) and *G. balechii* (Dai et al., 2017), respectively. The others are pending their confirmation as valid taxonomic species, their examination being dependent on the availability of living specimens from the field and cultures. A recent study conducted by Gómez et al. (2015) transferred the species *G. yasumotoi* (Holmes, 1998; Litaker et al., 2009; Richlen et al., 2008) and *G. ruetzleri* (Litaker et al., 2009) into the genus *Fukuyoa*. The phylotype *Gambierdiscus* cf. *yasumotoi* (Nishimura et al., 2013) has been reclassified into *Fukuyoa* cf. *yasumotoi* (Kretzschmar et al., 2017) accordingly.

2.2.2. The genus *Fukuyoa*

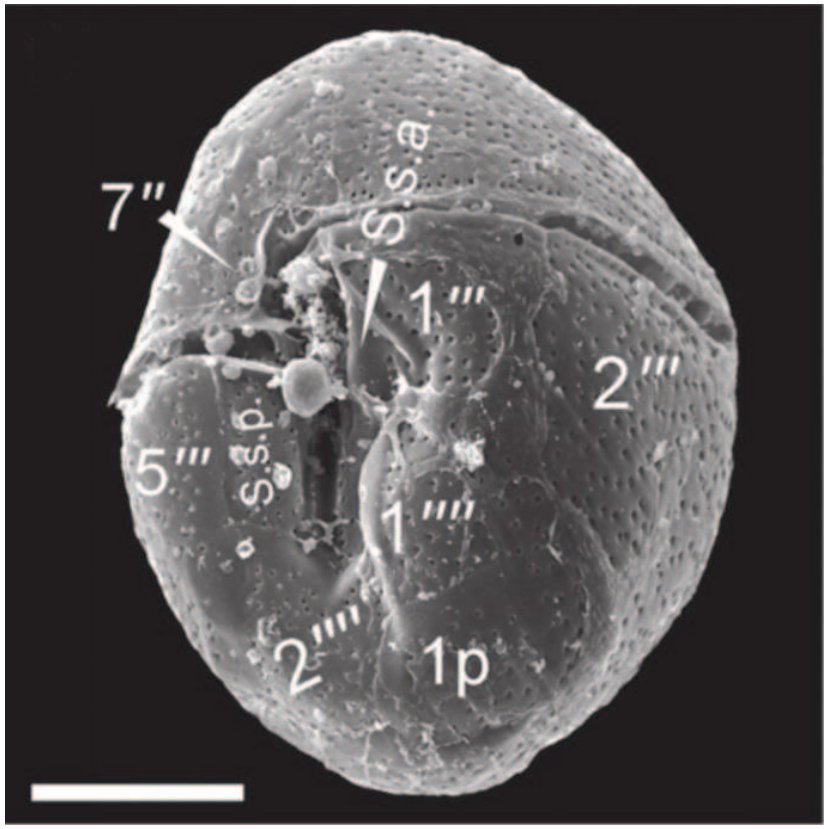

Figure 7. SEM micrograph of *Fukuyoa paulensis* (ventral view) (Gómez et al., 2015). Scale bar: 10 µm.

The dinoflagellate genus *Fukuyoa* has recently been described by Gómez et al. (2015) (**Figure 7**). The authors named a new species isolated from Brazil *F. paulensis* and they transferred two globular species of *Gambierdiscus* into the *Fukuyoa* genus, i.e. *F. yasumotoi* and *F. ruetzleri*. Accordingly, the phylotype *Gambierdiscus* cf. *yasumotoi* (Nishimura et al., 2013) was also reclassified as *Fukuyoa* cf. *yasumotoi* (Kretzschmar et al., 2017).

The distinction of *Fukuyoa* from *Gambierdiscus* is based on both morphological and genetic data. The two genera show considerable differences in cell shape (lenticular for *Gambierdiscus*, globular for *Fukuyoa*) and plate arrangement (morphology and pattern) (**Figure 5**) and form two well-separated phylogenetic clades when analyzing SSU and LSU rDNA sequences (Gómez et al., 2015), as shown in **Figure 8** (Kretzschmar et al., 2017). *F. paulensis* has recently been found in the Island of Formentera (Balearic Islands, Western Mediterranean Sea) (Laza-Martinez et al., 2016).

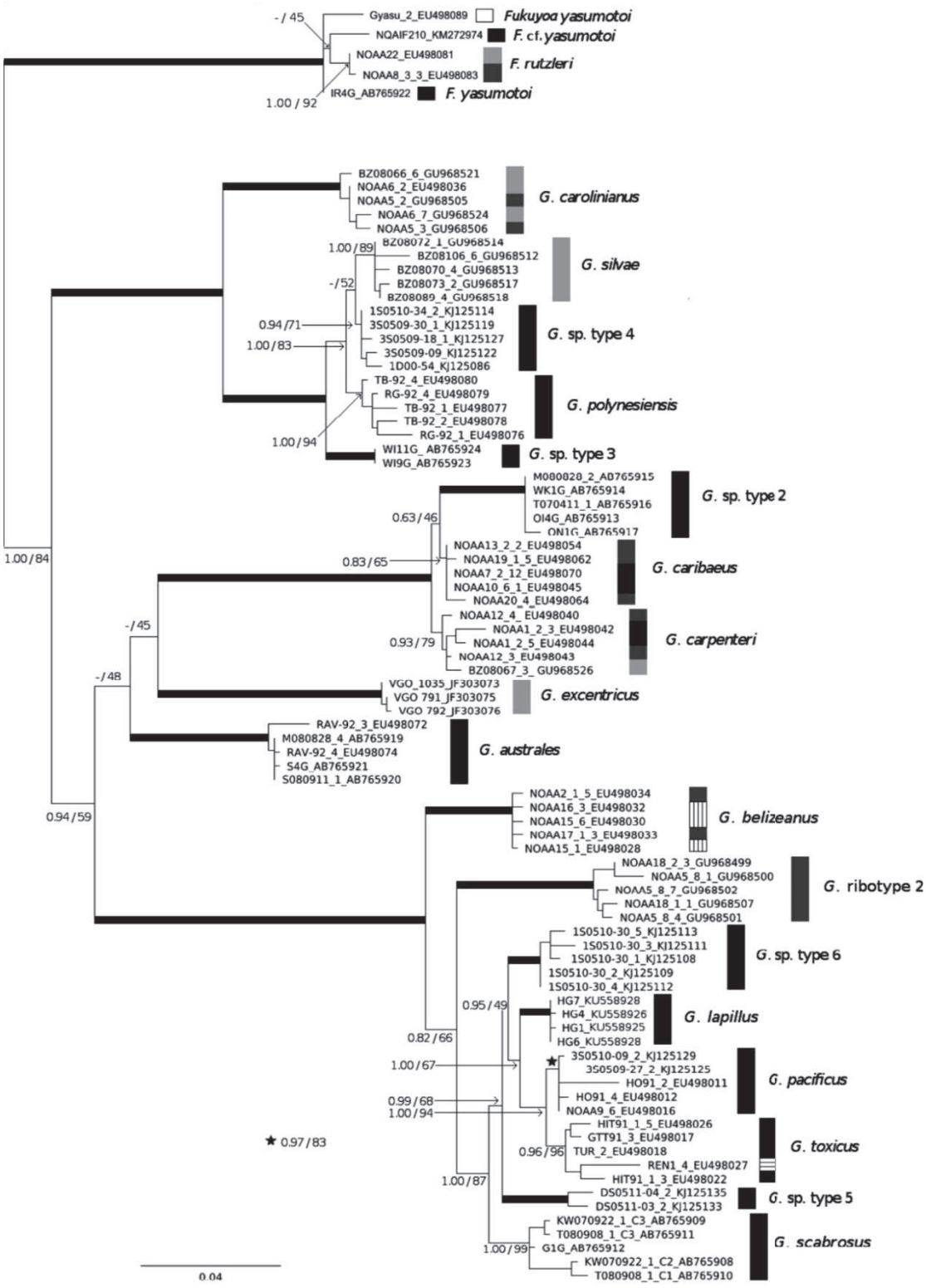

Figure 8. Phylogenetic tree of *Fukuyoa* and *Gambierdiscus* species/phylotypes based on D_8-D_{10} domains of large subunit (LSU) rDNA gene sequences (Kretzschmar et al., 2017). The geographical origin of the isolates is coded as follows: Black = Pacific Ocean; dark grey = Caribbean Ocean; light grey = Atlantic Ocean; white = South China Sea; white with black horizontal stripes = Indian Ocean. Nota bene: *Gambierdiscus* sp. type 6 has been assigned to *G. balechii* (Dai et al., 2017). *F. paulensis*, *Gambierdiscus* cf. *caribaeus* (Korean isolate GCJJ1), *G. cheloniae*, *G. honu* and *Gambierdiscus* sp. ribotype 3 are not included in the phylogenetic tree.

2.3. **Ecophysiology**

2.3.1. Life cycle

Van Dolah et al. (1995) studied the circadian regulation of cell division in *Gambierdiscus* sp. ribotype 2 CCMP1655 from Martinique, Caribbean Sea (formerly designated as MQ2 strain). The authors observed that cell division occurs only in the dark period, more precisely during a 3h-window beginning 6h after the onset of the dark phase. A further study conducted on another *Gambierdiscus* strain (*G. balechii* VGO917) by Bravo et al. (2014) corroborated this finding. Phased division during the dark period was previously reported for several other dinoflagellate species (Chisholm, 1981). Van Dolah et al. (1995) also showed that a CDC2-like kinase, a cell-cycle regulatory protein, is constitutively expressed throughout the cell cycles in *Gambierdiscus*, like in higher eukaryotes, and that its activation occurs during mitosis. However, there is also information on one strain that short periods of darkness (photoperiod: 18/6 h light/dark) may still be sufficient for growth and toxin production (Yokoyama et al., 1988).

A first attempt to describe the life cycle of *Gambierdiscus* was made by Hokama et al. (1996). The authors based their proposition on microscopic observations of an unnamed strain originating from Waianae Boat Harbor, both in natural environment and in laboratory cultures. The following phases have been described: “(i) a *motile free-swimming phase* at the beginning of the culture, characterized by the presence of light brown respiratory pigments; (ii) a *pre-cyst phase*, occurring after 2-3 weeks, in which pigments darken, cell walls begin to thicken, cells become immobile and a mucoid sheath begins to be secreted; (iii) a *cyst phase,* characterized by a strong adherence of cells to the petri dish bottom, a dark centrally located pigment and a thick mucoid sheath; (iv) a *secondary cyst phase* presenting thinner cell wall; (iv) a *mitotic phase* in which cysts divide via ecdysis, i.e. extension from the thick mucoid wall (about 20 divisions per cyst) and (v) an *undefined phase* of unknown conditions that lasts for 4-6 months before the cycle restarts”. Unfortunately, a species name was never assigned to this strain.

More recently, Bravo et al. (2014) also studied the life cycle of *Gambierdiscus*. The different cell-cycle stages in cultures of *G. balechii* VGO917 (Manado, Indonesia, Celebes Sea, SW Pacific Ocean) were monitored using nuclear staining and are represented in **Figure 9**. The

authors observed high variability in nuclear and cell shapes, the presence of gamete pairs and putative planozygotes, demonstrating that sexuality occurs in cultures of *Gambierdiscus*, as previously suggested by Taylor (1979). According to these findings, the life cycle of *Gambierdiscus* likely involves both asexual and sexual processes, as described for other dinoflagellates (Figueroa et al., 2006; Gribble et al., 2009; Tillmann and Hoppenrath, 2013; von Stosch, 1973). Still, it is unclear at what stage mitosis and meiosis occur (Berdalet et al., 2017).

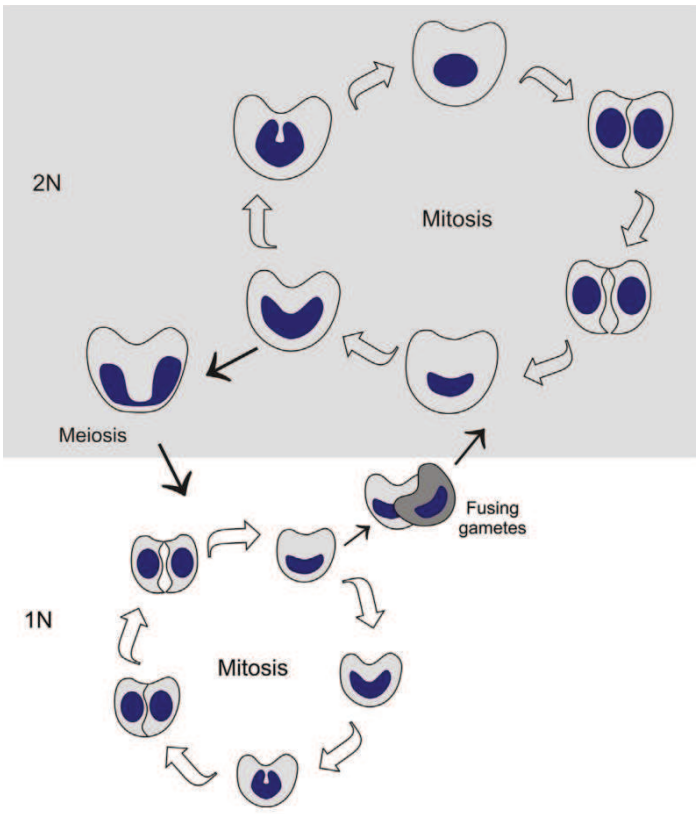

Figure 9. Schematic drawing of proposed life cycle of *Gambierdiscus*. The strain used in this study was *G. balechii* VGO917 (Manado, Indonesia, Celebes Sea, SW Pacific Ocean) (Bravo et al., 2014).

It is currently unknown if toxin production is associated with a particular phase of the life cycle. Since toxins are considered secondary metabolites, toxin production is expected to occur when cells are not involved in mitosis or meiosis processes. Laboratory studies seem to corroborate this hypothesis, as more toxin production has been associated to late exponential growth phase and stationary phase (Caillaud et al., 2011; Chinain et al., 2010a; Holland et al., 2013; Lartigue et al., 2009).

2.3.2. Environmental factors

Proliferation of *Gambierdiscus*/*Fukuyoa* populations, as for many other marine organisms, depends on a combination of several environmental factors. Two major categories of such environmental factors can be distinguished: biotic and abiotic factors. Biotic factors involve the living components of an ecosystem that influence the proliferation of a given organism of that ecosystem. Abiotic factors involve non-living components of an ecosystem, e.g. regimes of temperature, salinity, irradiance, nutrient availability, presence of rocky sediments in the marine environment.

2.3.2.1. Biotic factors

In the case of the dinoflagellate genera *Gambierdiscus* and *Fukuyoa*, biotic factors essentially involve macro-algae. Indeed, these genera are epi-benthic and are found on many substrates including macro-algae, algal turfs, sea grasses and coral rubble (Parsons and Preskitt, 2007; Rains and Parsons, 2015) but they can also be found in near bottom plankton as shown using moored screens (Tester et al., 2014). A recent survey showed that algal turfs appear to be very suitable substrates as support for *Gambierdiscus*, even when compared to macrophytes (Leaw et al., 2016).

Saint Martin et al. (1988) demonstrated a general preference for algal over inorganic substrates. Preferences for certain algal hosts have been reported (Cruz-Rivera and Villareal, 2006; Parsons et al., 2011), although the nature of this preference is unknown. Some studies suggested that the preference is a function of surface area (Bomber et al., 1989a), class of algae (Rhodophytes (Taylor, 1979; Yasumoto et al., 1979; Yasumoto et al., 1980), Chlorophytes (Bomber et al., 1989a; Carlson et al., 1984)), algal structure (Taylor and Gustavson, 1983), stimulatory or inhibitory compounds (Bomber et al., 1989a; Carlson et al., 1984; Carlson and Tindall, 1985), or the presence of specific chelators in algal extracts/exudates (Bomber et al., 1989a). One study also reported that gambieric acid-A, an antifungal compound produced by *Gambierdiscus* itself, may act as a stimulatory compound for its own growth (Sakamoto et al., 1996). With respect to macro-algal species, contradictory results have been reported in laboratory-based experiments. For example, *Dictyota* is a preferred host in the Caribbean (Carlson and Tindall, 1985), but not in the Pacific (Parsons et al., 2011). Extracts of *Halymenia formosa* are reported to stimulate growth (Asuncion et al., 1994), while Grzebyk et al. (1994)

reported opposite results. Similarly, Grzebyk et al. (1994) concluded that *Portieria hornemannii* stimulated growth, whereas Parsons et al. (2011) found that *Gambierdiscus* cells in culture did not even attach to *P. hornemannii*, with most cells dying within five days.

2.3.2.2. Abiotic factors

The impact of abiotic factors, e.g. temperature, salinity and irradiance, on a marine organism can be evaluated in laboratory cultures comparing growth rates at different levels of each parameter. This is the case of single factor experiments, which permit to establish an optimal range of such parameters, as well as the minimum and maximum values (= tolerance). The interaction between two or more factors on the growth responses can be highlighted using factorial-designed experiments (Collins et al., 2009).

Several studies focused on the influence of abiotic factors on *Gambierdiscus*/*Fukuyoa* proliferation (Bomber et al., 1988a; Kibler et al., 2012; Morton et al., 1992; Tawong et al., 2016; Xu et al., 2016; Yoshimatsu et al., 2016; Yoshimatsu et al., 2014). All in all, the epi-benthic genera *Gambierdiscus* and *Fukuyoa* are slow-growing organisms compared to most planktonic dinoflagellates, with maximum growth rates generally lower than 0.5 divisions day^{-1} (Chinain et al., 2010a; Kibler et al., 2012; Kibler et al., 2015; Lartigue et al., 2009; Lehane and Lewis, 2000; Xu et al., 2016) (**Table 2**).

The environmental conditions favorable to the proliferation of these genera are the following: shallow water habitat (< 50 m), annual water temperatures between 21 °C and 31 °C (with optimum between 25-29 °C), abundant substrates (e.g. macrophytes, algal turfs or rocky sediments), low to moderate turbulence, high and stable salinities, actual or attenuated incident light levels (< 10%), and sufficient or elevated nutrient inputs, some of which can be obtained directly from the macrophyte hosts (Litaker et al., 2010).

Table 2. Maximum growth rates (μ_{max}, divisions day^{-1}) for *Gambierdiscus* and *Fukuyoa* species/phylotypes measured in various studies using either direct cell counts or relative changes in chlorophyll *a* (Chl *a*) fluorescence.

	Maximum growth rates (μ_{max}, divisions day^{-1})			
Species / Phylotype	**Direct cell counts**	**Ref.**	**Chl *a* fluorescence**	**Ref.**
F. ruetzleri			0.30 – 0.53	[1]
			0.50	[2]
G. australes	0.12 – 0.19	[3]	0.26 – 0.36	[4, 5]
			0.43	[6]
G. belizeanus	0.14	[3]	0.21	[1]
			0.29	[2]
			0.27 – 0.41	[7]
G. caribaeus	0.10 – 0.20	[8]	0.28 – 0.46	[1]
	0.16 – 0.24	[9]	0.48	[2]
			0.25 – 0.44	[7]
G. cf. *caribaeus*			0.29	[10]
G. carolinianus			0.23 – 0.51	[1]
			0.25 – 0.48	[7]
			0.46	[2]
G. carpenteri			0.19 – 0.29	[7]
			0.55	[6]
G. pacificus	0.18 – 0.21	[3]	0.30 – 0.42	[7]
			0.36	[6]
			0.29	[2]
G. polynesiensis	0.13 – 0.17	[3]		
Gambierdiscus sp. ribotype 2	0.14 – 0.19	[8]	0.17	[2]
			0.28	[1]
G. scabrosus			0.29 – 0.40	[4, 5]
Gambierdiscus sp. type 2			0.22 – 0.24	[4, 5]
Gambierdiscus sp. type 3			0.37 – 0.40	[4, 5]
Gambierdiscus sp. type 4			0.28 – 0.34	[7]
Gambierdiscus sp. type 5			0.27	[7]
G. silvae			0.18 – 0.21	[7]
G. toxicus	0.16 – 0.19	[3]		

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Early studies on abiotic factors were conducted by Bomber et al. (1988a) and Morton et al. (1992) using monoclonal cultures of *Gambierdiscus*. At that time, the taxonomy was unresolved and morphological and phylogenetic data for the strains examined were not provided. Therefore, it is uncertain whether the growth differences among strains resulted from intra- or inter-species variability. Indeed, strains were all designated as "*Gambierdiscus toxicus*" or "*Gambierdiscus* sp.", and could actually belong to (i) a species/phylotype of the genus *Fukuyoa* (**Table 1**), (ii) a species/phylotype of the genus *Gambierdiscus* (**Table 1**) or

even (iii) a yet undescribed *Gambierdiscus* or *Fukuyoa* species. The effects of abiotic factors on *Gambierdiscus*/*Fukuyoa* growth are not well known across the multiple species in these genera, particularly with respect to species-specific responses. Only few recent studies provided species-specific growth data (Chinain et al., 2010a; Kibler et al., 2012; Tawong et al., 2016; Xu et al., 2016; Yoshimatsu et al., 2016; Yoshimatsu et al., 2014). Overall, *G. caribaeus*, *G. carpenteri* and *G. pacificus*, appear to be more tolerant than other species tested, i.e. maintain positive growth over a broader range of temperature, salinity and light intensity levels, consistent with their broad geographic distribution (Berdalet et al., 2017; Litaker et al., 2010).

Temperature. With respect to temperature, *Gambierdiscus* generally achieves maximum growth at 25-31 °C and cannot tolerate temperatures below ~ 15-21 °C or over ~ 31-34 °C under experimental culture conditions (Kibler et al., 2012; Parsons et al., 2010; Tester et al., 2010; Xu et al., 2016; Yoshimatsu et al., 2014). Only one study reported survival of *Gambierdiscus* at 10 °C for at least three months (Ishikawa et al., 2011). Field surveys generally agree with laboratory studies, with *Gambierdiscus* populations and ciguatera incidence primarily reported from areas with a temperature range of 25-30 °C (Chinain et al., 1999b; Tester et al., 2010). Notwithstanding, strains of *G silvae*, *G. excentricus* and *G. australes* were isolated in the Canary Islands from an area exposed to the dominant trade winds and with seawater temperatures of 18-26 °C (Boada et al., 2010). Still, recent surveys recorded the presence of *Gambierdiscus* cells at extreme temperatures, lower and higher than what was previously reported. For example, in the North-Eastern Atlantic, *G. australes* was isolated from Isla Graciosa (Canary Islands) with a seawater temperature below 16 °C (Fraga, 2017). In the Pacific, *Gambierdiscus* was found at temperatures as low as 14 °C in Korea (*Gambierdiscus* cf. *caribaeus*) (Jeong et al., 2012) and even at ~ 11 °C in Japan (Ishikawa et al., 2011). Similarly, recent surveys reported the presence of *G. caribaeus* in the Gulf of Thailand and the Andaman Sea (Tawong et al., 2015; Tawong et al., 2016), where temperatures can exceed 33 °C (Krishnan et al., 2011), or even in the Red Sea and Arabian Gulf (Catania, 2012; Saburova et al., 2013), where temperatures can reach or exceed 35 °C (Llewellyn, 2010). A further study conducted by Tawong et al. (2016) confirmed the tolerance of *G. caribaeus* to such high temperatures in laboratory, in accordance with previous

findings in the Caribbean (Kibler et al., 2012; Tester et al., 2010). Little is known about the influence of temperature on toxin production. Bomber et al. (1988a) observed that *Gambierdiscus* spp. cells were more toxic at 27 °C rather than 21 °C (mouse bioassay). Holland et al. (2013) found that hemolytic activity of *G. caribaeus* increased slightly at higher temperatures (31 °C), while hemolytic activity of *G. carolinianus* remained constant, suggesting that growth temperature is not a significant factor in modulating the inter-isolate and interspecific maitotoxin (MTX) production.

In a context of climate change, studies concerning temperature optima and tolerances for growth of toxin-producing organisms are important to better evaluate risk assessment on a global scale as they provide insights to predict their distribution, abundance and seasonality (Kibler et al., 2017). High seawater temperatures have been associated to coral bleaching events (Gleeson and Strong, 1995; Goreau and Hayes, 1994), which, in turn, may lead to an increased available surface area for macroscopic algal growth and therefore also for toxic dinoflagellates (Lehane and Lewis, 2000). Tester et al. (2010) described a positive correlation between seawater temperature and abundance of different *Gambierdiscus* species in the Caribbean. Hales et al. (1999) found a strong positive correlation between the annual incidence of ciguatera and local warming of surface seawater during El Niño conditions in New Zealand. Similarly, Château-Degat et al. (2005) conducted a survey on a period from 1993 to 2001 in French Polynesia and found that an increase in seawater temperature promoted *Gambierdiscus* proliferation with a time-lag of 13-17 months and that ciguatera cases might be observed 3 months after the appearance of the bloom. Gingold et al. (2014) conducted a review in the United States from 2001 to 2011 and also correlated ciguatera cases reports with higher seawater temperature and increased tropical storm frequency, with a similar time-lag (18 months). In recent years, the presence of *Gambierdiscus* and *Fukuyoa* spp. has been observed in temperate areas previously not considered endemic: (i) in the North-Eastern Atlantic Ocean, in the Canary Islands, Spain (*G. excentricus*, *G. silvae*) (Fraga and Rodríguez, 2014; Fraga et al., 2011) and (ii) in the Mediterranean Sea, in Crete Island, Greece (*G. carolinianus*) (Aligizaki and Nikolaidis, 2008; Aligizaki, 2008) and in the island of Formentera, Balearic Islands (*F. paulensis*) (Laza-Martinez et al., 2016). It is not possible to determine whether *Gambierdiscus* and *Fukuyoa* are truly spreading to new areas as a

consequence of global warming or they were already present and found only recently because they were searched for. Nevertheless, taken together, all these findings suggest that climate change could contribute to an increase of the CFP burden (Berdalet et al., 2017; Kibler et al., 2015; Rodríguez et al., 2017).

Salinity. With regard to salinity, *Gambierdiscus* and *Fukuyoa* generally prefer high, stable salinities of 28-35 (Parsons et al., 2012). Nevertheless, *Gambierdiscus* cells have been reported from areas where salinity levels occasionally are outside of this range, such as near river outlets (Delgado et al., 2006) or enclosed water bodies (Saburova et al., 2013). Moreover, recent studies showed that some species maintain growth over a much wider salinity range (15-41) under laboratory culture conditions (Kibler et al., 2012; Yoshimatsu et al., 2014).

Oceanic waters in areas where ciguatera is endemic are restricted to salinities of 34-38, thus oceanic salinity should sustain maximum growth of most *Gambierdiscus*/*Fukuyoa* species (Chinain et al., 1999b; Tester et al., 2013). In contrast with the stable or narrow range of salinity in oceanic regions, some coastal locations such as estuaries and bays are affected by freshwater inputs from precipitation and from land, e.g. some coastal zones in the Gulf of Mexico (Yáñez-Arancibia and Day, 2004). At these locations, hyposaline conditions (salinity <14) may occur, hampering *Gambierdiscus*/*Fukuyoa* proliferation. Nevertheless, some species/phylotypes may have good chance of survival, as they showed broader tolerance at extremely low salinities under laboratory culture conditions, e.g. *G. caribaeus*, *G. carpenteri* (Xu et al., 2016). Contrary to estuaries and bays, restricted water bodies in tropical and subtropical areas, such as tropical lagoons (Vega-Cendejas and de Santillana, 2004), undergo high evaporation, poor circulation, and low freshwater input. At these locations, hypersaline conditions (salinity even > 40) are not favorable to most *Gambierdiscus*/*Fukuyoa* species. Nevertheless, according to laboratory experiments conducted by Xu et al. (2016), *G. belizeanus*, *G. caribaeus*, and *G. carpenteri* may be able to survive at these extremely high salinities.

Within the context of climate change, precipitation is predicted to become less frequent but more intense, followed by longer dry periods (Hallegraeff, 2010). This new pattern is expected to favor dinoflagellate growth due to increases in water stratification and the availability of

nutrients (Doblin et al., 2006; Hallegraeff, 2010). Nevertheless, it is difficult to predict the response of epi-benthic dinoflagellates to these dramatic salinity changes as it also depends on the response of the macrophyte hosts (Xu et al., 2016). The only study that provides some insights on this aspect was conducted by Kibler et al. (2012). The authors found that growth responses of *Gambierdiscus*/*Fukuyoa* to instantaneous salinity decreases were species-dependent and included: no effect, slowed growth, or mortality. In addition, little is known about the impact of salinity conditions on toxin production. The only study which provides some insights on this aspect was conducted by Roeder et al. (2010). Roeder et al. (2010) showed that toxin profile of *Gambierdiscus* sp. Viet Nam was affected by the salinity conditions used for culture in laboratory, i.e. amounts of CTX4A and 2,3-Dihydro-2,3-dihydroxyCTX3C were roughly stable throughout the three salinity conditions tested (30, 33, 35), while CTX3C sensibly decreased at the highest salinity tested (35). Most culture systems allow for air exchange: thus, evaporation of culture medium could play a role in experimental design. The studies reporting on salinity in culture did not mention a control of salinity over the duration of the experiments.

Irradiance. Concerning irradiance, early studies estimated the upper threshold for maximum growth of *Gambierdiscus* spp. to be approximately 10% of full sunlight (Bomber et al., 1988a; Morton et al., 1992), which agrees well with the general irradiance requirement for dinoflagellates (Guillard and Keller, 1984). In agreement with these studies, Kibler et al. (2012) found that maxima growth rates in laboratory were achieved at ~ 2.5-10% of surface irradiance for the eight species/phylotypes examined (*F. ruetzleri*, *G. australes*, *G. belizeanus*, *G. caribaeus*, *G. carolinianus*, *G. carpenteri*, *G. pacificus* and *Gambierdiscus* sp. ribotype 2). Interestingly, the authors showed that the strains examined could maintain positive growth at very low irradiance levels, between 6 and 17 µmol photons m^{-2} s^{-1} (0.2-0.7% of ambient surface irradiances), which would allow *Gambierdiscus*/*Fukuyoa* cells to survive at depths >100 m in oligotrophic Caribbean waters (Kibler et al., 2012). Similarly, Yoshimatsu et al. (2016) showed that the Japanese strains examined (*G. australes*, *G. scabrosus*, *Gambierdiscus* spp. type 2 and type 3) maintained growth at low irradiances (10 µmol photons m^{-2} s^{-1}, i.e. maximum projected depth of approximately 87 m). Still, field ecological surveys highlighted the presence

of *Gambierdiscus* in habitats exposed to high irradiance levels, close to surface sunlight, e.g. in the shallow waters of 1-5 m (Tindall and Morton, 1998), on sparse macroalgae and bright sand flats (Bomber et al., 1989a), on drifting seaweed (Bomber et al., 1988b; Fraga et al., 2011), or floating detritus (Faust, 2009). Possible explanations of those field observations involve: (i) the finely-branched and three-dimensional structure of host macroalgae, which may provide shade surfaces and prevent exposure of *Gambierdiscus*/*Fukuyoa* cells to strong incident light (Nakahara et al., 1996; Villareal and Morton, 2002); (ii) an increased production of mucus as a self-shading strategy to face high irradiance levels, as shown for other epi-benthic dinoflagellates, e.g. *Ostreopsis* (Heil et al., 1993). Recent studies also highlighted some species-specific responses to irradiance levels in laboratory cultures. Kibler et al. (2012) found that *G. carolinianus* and *G. pacificus* were least adapted to high irradiance and experienced mortality at ~ 300 µmol photons m^{-2} s^{-1}. Conversely, a recent study conducted by Xu et al. (2016) on cultures of eight species/phylotypes (*G. belizeanus*, *G. caribaeus*, *G. carolinianus*, *G. carpenteri*, *G. pacificus*, *G. silvae*, *Gambierdiscus* sp. type 4 and *Gambierdiscus* sp. type 5) highlighted broader tolerance to high light intensity than what was previously reported, i.e. until 400 µmol photons m^{-2} s^{-1}.

3. Cyclic polyether compounds produced by *Gambierdiscus* and *Fukuyoa*

3.1. General introduction

The dinoflagellate genera *Gambierdiscus* and *Fukuyoa* produce different families of secondary metabolites with a cyclic polyether structure: ciguatoxins (CTXs), maitotoxins (MTXs), gambieric acids, gambierol, gambieroxide and gambierone (**Figure 10**). Except for gambieroxide, for which biological activity has not been evaluated yet, these compounds all show biological activity. While CTXs have been implicated in ciguatera poisoning, it is not fully understood if the other compounds could also play a role in ciguatera events. In any case, most of them are considered as compounds of interest for their bioactivity and potential therapeutical application. A comprehensive review on what it is known so far on the cyclic polyether compounds produced by *Gambierdiscus* and *Fukuyoa* is proposed in the following sections. **Table 7** summarizes the procedures of extraction and purification of these compounds from microalgae.

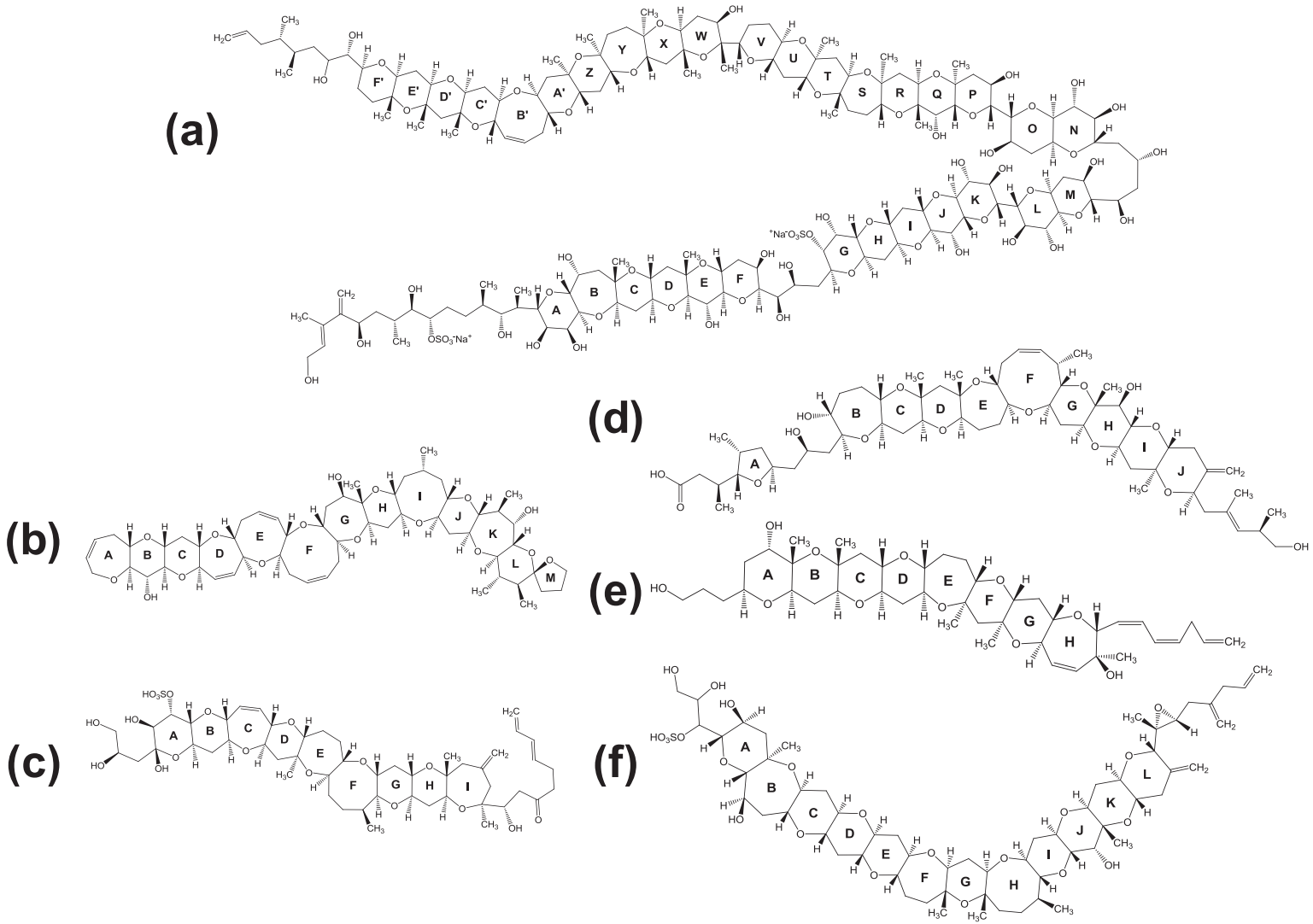

Figure 10. Cyclic polyether compounds produced by *Gambierdiscus* and *Fukuyoa*: **(a)** maitotoxin (MTX), **(b)** Pacific ciguatoxin-3C (CTX3C), **(c)** gambierone, **(d)** gambieric acid A (GA A), **(e)** gambierol, **(f)** gambieroxide.

3.2. **Ciguatoxins**

3.2.1. Background

Ciguatoxins (CTXs, **Table 3**) are cyclic polyether compounds with a rigid structure, consisting of 13 or 14 transfused rings fused by ether bonds. These toxins are lipid-soluble and heat resistant, so they are not degraded by cooking processes (Abraham et al., 2012). CTXs are potent toxins, with an LD_{50} in mice (i.p.) equivalent to 0.25, 2.3, 0.9 µg kg^{-1} for CTX1B, 52-*Epi*-54-DeoxyCTX (CTX2), and 54-DeoxyCTX (CTX3), respectively (Lewis et al., 1991). CTXs mainly act as voltage-gated sodium channel (VGSC) activators, leading to numerous dysfunctions at the cellular and physiological level of most excitable cells (Yamaoka et al., 2011). CTXs are responsible for ciguatera fish poisoning (CFP) events: they are produced by the dinoflagellates *Gambierdiscus* and *Fukuyoa* and they gradually bioaccumulate in fish flesh through the marine food chain, from herbivorous grazers to carnivorous predators (Lewis and Holmes, 1993). Along with this bioaccumulation, the algal CTXs undergo metabolization in fish (Ikehara et al., 2017), resulting in more toxic congeners, at least in the Pacific area (Chinain et al., 2010b). As an example, the dominant CTX extracted from fish, CTX1B, arises from the acid-catalyzed spiro-isomerization and oxidative metabolization of the algal precursor CTX4A (Lewis and Holmes, 1993). CTXs are grouped into three sub-families according to their geographical origin, i.e. Pacific, Caribbean, and Indian CTXs (Caillaud et al., 2010a) (**Table 3**, **Table 4**, **Table 5**). In the Pacific area, several CTX congeners have been isolated, from both fish and dinoflagellate species (Satake et al., 1998; Satake et al., 1997a; Satake et al., 1993b; Yasumoto, 2001). To avoid confusion, the term "ciguatoxin" and its abbreviation "CTX" are used, throughout the present manuscript, to designate any congener of this family of toxins, whereas the first congener described by Scheuer et al. (1967) is designated as CTX1B. In the Caribbean area, few congeners are known to date and have been extracted from fish. Only the two dominant congeners, C-CTX1 and its C_{56}-epimer C-CTX2, have been structurally elucidated (Lewis et al., 1998). In the Indian Ocean, none of the six congeners found in fish has been structurally characterized (Diogène et al., 2017). No algal precursors of Caribbean or Indian CTXs from *Gambierdiscus*/*Fukuyoa* strains are known to date.

Table 3. List of all the ciguatoxin congeners that have been structurally characterized by NMR studies. Alternative names found in literature are given as well. Relevant information such as elemental formula, exact mono-isotopic mass, CAS number and references of isolation and structural characterization are provided. Nota bene: the other CTX congeners that have not been structurally elucidated are presented in **Table 4** and **Table 5**.

Compound name	Alternative names	Elemental formula	Exact mono-isotopic mass	Ref.	CAS number
Pacific ciguatoxins - CTX1B congeners					
CTX1B	CTX, CTX-1, P-CTX-1	$C_{60}H_{86}O_{19}$	1110.5763	[1-5]	11050-21-8
52-*Epi*-54-deoxyCTX	CTX2, CTX-2, P-CTX-2, 52-*Epi*CTX3, CTX2A2	$C_{60}H_{86}O_{18}$	1094.5814	[6, 7]	142185-85-1
54-DeoxyCTX	CTX3, CTX-3, 54-DeoxyCTX1B, P-CTX-3, CTX2B2	$C_{60}H_{86}O_{18}$	1094.5814	[6, 7]	139641-09-6
52-*Epi*CTX		$C_{60}H_{86}O_{19}$	1110.5763	[8, 9]	189013-49-8
54-*Epi*CTX		$C_{60}H_{86}O_{19}$	1110.5763	[8, 9]	287732-40-5
52,54-*Diepi*CTX	52,54-*Epi*CTX	$C_{60}H_{86}O_{19}$	1110.5763	[8, 9]	287732-42-7
54-Deoxy-50-hydroxyCTX	50-HydroxyCTX3	$C_{60}H_{86}O_{19}$	1110.5763	[8, 9]	263336-54-5
7-OxoCTX	6,7-Dihydro-7-oxoCTX	$C_{60}H_{86}O_{20}$	1126.5712	[8, 9]	263336-55-6
6,7-Dihydro-7-hydroxyCTX	7-HydroxyCTX	$C_{60}H_{88}O_{20}$	1128.5869	[8, 9]	263336-56-7
3,4-Dihydro-3-hydroxy-7-oxoCTX	3,4,6,7-Tetrahydro-3-hydroxy-7-oxoCTX, 3-Hydroxy-7-oxoCTX	$C_{60}H_{88}O_{21}$	1144.5818	[10]	263336-57-8
3,4-Dihydro-4-hydroxy-7-oxoCTX	3,4,6,7-Tetrahydro-4-hydroxy-7-oxoCTX, 4-Hydroxy-7-oxoCTX	$C_{60}H_{88}O_{21}$	1144.5818	[8, 9]	287732-85-8
CTX4B	52-*Epi*CTX4A, 1,2,54-Trideoxy-1,2-didehydroCTX, P-CTX-4B, GTX-4B, GT4*b*	$C_{60}H_{84}O_{16}$	1060.5759	[2, 3, 11]	123676-76-6
CTX4A	52-*Epi*CTX4B, scaritoxin (SG1), GTX-4A, P-CTX-4A	$C_{60}H_{84}O_{16}$	1060.5759	[11, 12]	66231-73-0
M-*Seco*CTX4A (or 4B)	M-*Seco*-P-CTX-4A (or 4B)	$C_{60}H_{86}O_{17}$	1078.5865	[8, 9]	287412-00-4
Pacific ciguatoxins - CTX3C congeners					
CTX3C	P-CTX-3C, GTX-3C, 1,2,3,4-Nor-E-homo-GTX-4B	$C_{57}H_{82}O_{16}$	1022.5603	[13]	148471-85-6
51-HydroxyCTX3C	CTX2C1	$C_{57}H_{82}O_{17}$	1038.5552	[14]	263336-59-0
2,3-Dihydro-2,3-dihydroxyCTX3C	2,3-DihydroxyCTX3C, CTX2A1	$C_{57}H_{84}O_{18}$	1056.5658	[14]	263336-62-5
49-*Epi*CTX3C	CTX3B, P-CTX-3B	$C_{57}H_{82}O_{16}$	1022.5603	[8, 9]	263336-58-9
2,3-Dihydro-2-hydroxyCTX3C	2-HydroxyCTX3C	$C_{57}H_{84}O_{17}$	1040.5709	[8, 9]	287732-78-9
2,3-Dihydro-3-hydroxyCTX3C		$C_{57}H_{84}O_{17}$	1040.5709	[10]	263336-60-3
51-Hydroxy-2-oxoCTX3C	2,3-Dihydro-51-hydroxy-2-oxoCTX3C	$C_{57}H_{82}O_{18}$	1054.5501	[8, 9]	287732-77-8
51-Hydroxy-3-oxoCTX3C	2,3-Dihydro-51-hydroxy-3-oxoCTX3C	$C_{57}H_{82}O_{18}$	1054.5501	[10]	263336-61-4
2,3-Dihydro-2,3,51-trihydroxyCTX3C	2,3,51-TrihydroxyCTX3C	$C_{57}H_{84}O_{19}$	1072.5607	[8, 9]	263336-63-6
A-*Seco*-2,3-dihydro-51-hydroxyCTX3C	A-*Seco*-51-hidroxyCTX3C	$C_{57}H_{86}O_{18}$	1058.5814	[8, 9]	263336-64-7
M-*Seco*CTX3C (or 3B)	49,52-Deepoxy-49,52-dihydroxyCTX3C	$C_{57}H_{84}O_{17}$	1040.5709	[8, 9]	287411-98-7
M-*Seco*-49-O-methylCTX3C	M-*Seco*CTX3C methyl acetal	$C_{58}H_{86}O_{17}$	1054.5865	[8, 15]	287411-99-8
M-*Seco*-2,3-dihydro-2-hydroxy-49-O-methylCTX3C	M-*Seco*-2-hydroxy-49-methoxyCTX3C	$C_{58}H_{88}O_{18}$	1072.5971	[9]	374624-43-8

Compound name	Alternative names	Elemental formula	Exact mono-isotopic mass	Ref.	CAS number
	Caribbean ciguatoxins				
C-CTX1		$C_{62}H_{92}O_{19}$	1140.6233	[16, 17]	193363-37-0
C-CTX2	56-*Epi*C-CTX1	$C_{62}H_{92}O_{19}$	1140.6233	[17]	193363-38-1

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3.2.2. Pacific ciguatoxins

Pacific CTXs can be divided into two subfamilies presenting two structural differences in the cyclic polyether backbone: (i) the E-ring is 7-membered in CTX1B congeners and 8-membered in CTX3C congeners and (ii) CTX1B congeners present a 4-carbon side chain on the A ring, absent in CTX3C congeners (**Figure 11**).

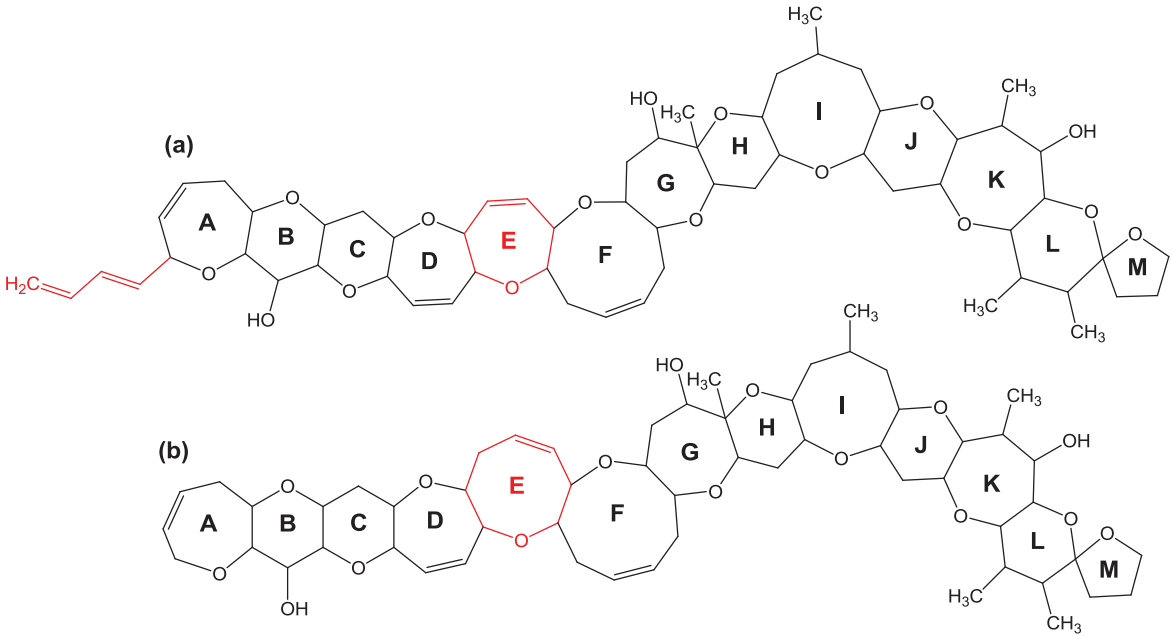

Figure 11. Backbone structures of Pacific CTXs: **(a) CTX4A/B**, representative of CTX1B congeners and **(b) CTX3C**, representative of CTX3C congeners. The two main structural differences (highlighted in red) involve: (i) the size of the E-ring (7-membered in CTX1B congeners, 8-membered in CTX3C congeners) and (ii) the presence of a 4-carbon side chain on the ring A in CTX1B congeners, absent in CTX3C congeners.

3.2.2.1. CTX1B congeners

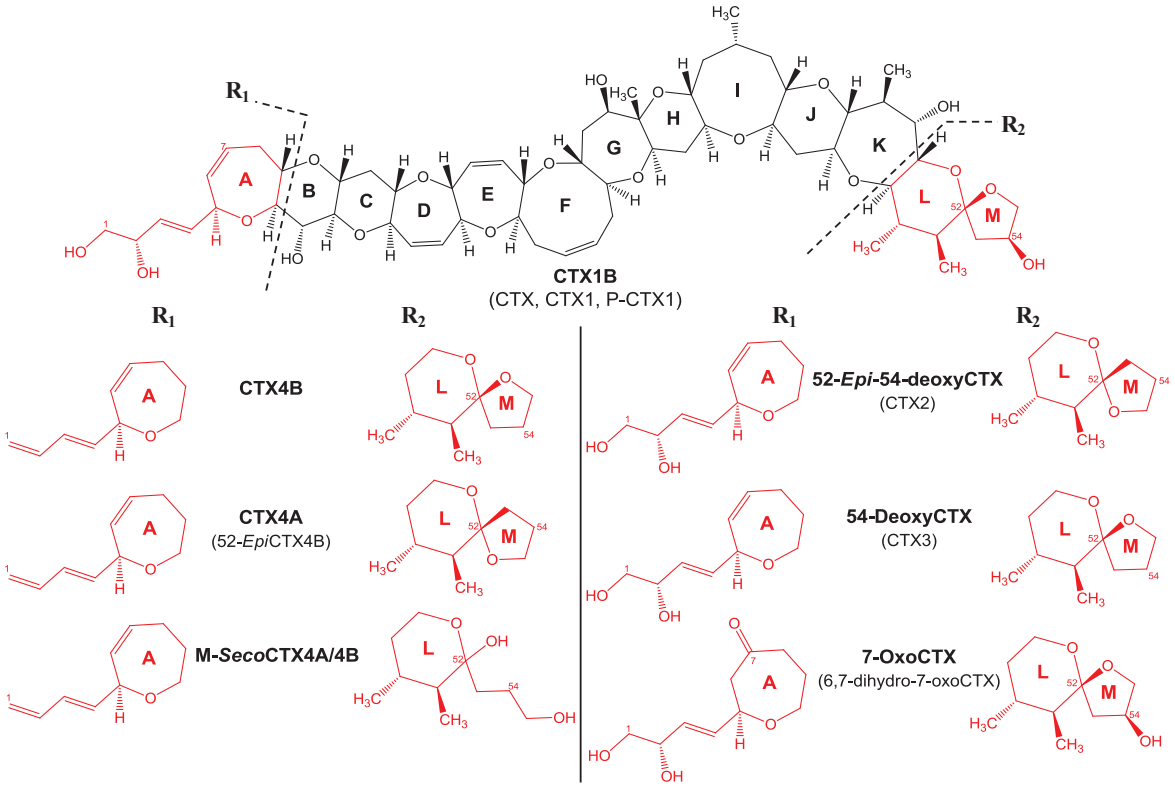

Figure 12. Structure of Pacific CTX1B (Murata et al., 1992; Murata et al., 1990; Suzuki et al., 1991) and some examples of CTX1B congeners.

Ciguatoxin (**CTX1B**, $C_{60}H_{86}O_{19}$, M = 1110.5763 Da) was the name given to the major toxin present in the flesh of the moray eel *Gymnothorax* (or *Lycodontis*) *javanicus* collected from Tuamotu Archipelago and Tahiti Island (French Polynesia) (Scheuer et al., 1967). Several years later, other studies identified CTX1B as the major toxin isolated from ciguateric fish from the Pacific area (Legrand et al., 1992; Lewis and Sellin, 1992; Lewis et al., 1991; Murata et al., 1990; Yogi et al., 2011; Yogi et al., 2014). The study conducted by Nukina et al. (1984) was a first attempt for structural elucidation of CTX1B using 600 MHz ^{1}H nuclear magnetic resonance (NMR). Complete structure, including assignment of stereochemistry (except at C_2) was elucidated few years later by further NMR studies (Murata et al., 1990; Murata et al., 1989) (**Figure 12**).

Around the same time, Murata et al. (1989) and Murata et al. (1990) isolated **CTX4B** (or 1,2,54-Trideoxy-1,2-didehydroCTX, GTX-4B, GT4*b*, $C_{60}H_{84}O_{16}$, M = 1060.5759 Da) from *Gambierdiscus* sp. (strain name not provided, origin: Gambier Islands, French Polynesia), a compound structurally similar to CTX1B. Extensive comparison between NMR spectra of CTX1B and CTX4B, as well as the enantioselective synthesis of the AB

ring of CTX4B, led to the elucidation of the absolute stereochemistry of both molecules (Suzuki et al., 1991), as further supported by ^{13}C NMR studies (Murata et al., 1992). The structural features specific to CTX4B, compared to CTX1B, are described as follows: (i) CTX4B does not present the three hydroxyl groups at C_1, C_2 and C_{54} and (ii) CTX4B presents a double bond on the left side chain on ring A in position C_1-C_2 (**Figure 12**). The discovery of a toxin (CTX4B) in microalgae with a chemical structure closely related to the major toxin isolated from fish (CTX1B) was the first evidence of *Gambierdiscus* spp. being the primary producer of the causative toxins of ciguatera poisoning and was at the origin of the theory of oxidative modification of CTXs during the food chain transmissions (Lewis and Holmes, 1993). Lewis et al. (1991) isolated two congeners of CTX1B from moray eel viscera, **CTX2** and **CTX3** (both $C_{60}H_{86}O_{18}$, M = 1094.5814 Da), being less polar and less potent (i.p. LD_{50}s in mice 2.3 and 0.9 µg kg^{-1}, respectively) than CTX1B (0.25 µg kg^{-1}). NMR studies and positive mode FAB MS/MS of $[M+H]^+$ revealed that CTX2 and CTX3 are diastereoisomers at C_{52} and both differ from CTX1B by the absence of one hydroxyl group at C_{54} (Lewis et al., 1991) (**Figure 12**). Consequently, CTX3 is also named 54-DeoxyCTX and CTX2 is also named 52-*Epi*-54-deoxyCTX (Yasumoto, 2001; Yasumoto et al., 2000).

The discovery of **CTX4A** is linked to an epidemiological survey of ciguatera intoxication conducted by Bagnis (1974) in the Gambier Islands. The authors observed that the most frequently implicated fish in CFP were parrotfish (Scaridae). CTX4A ($C_{60}H_{84}O_{16}$, M = 1060.5759 Da) was first isolated under the name of scaritoxin (SG-1 or ST-1) from the flesh of the ciguateric parrotfish *Scarus gibbus* (Gambier Islands, French Polynesia) (Chungue et al., 1977) and from the flesh and the viscera of *Scarus sordidus* (Tarawa Atoll, Republic of Kiribati) (Joh and Scheuer, 1986). Several years later, Satake et al. (1997a) isolated CTX4A both from *Gambierdiscus* sp. RGI-1 (Rangiroa Atoll, French Polynesia) and the parrotfish *Scarus gibbus*, giving the first evidence of the trophic transfer of CTXs from microalgae to fish. CTX4A presents the same chemical structure as CTX4B but different stereochemistry at C_{52} (i.e. 52-*Epi*CTX4B) (**Figure 12**). The comparison of the molecular structures of CTX4A/B, isolated from microalgae and found in herbivorous fish, with the major CTX congeners isolated from carnivorous fish (CTX1B, 52-*Epi*-54-DeoxyCTX and 54-DeoxyCTX) also corroborated the metabolization pathway

throughout the marine trophic chain proposed by Lewis and Holmes (1993) (**Figure 13**). Indeed, in acidic conditions (e.g. in the stomach of fish), CTX4A can be easily converted in CTX4B via acid-catalyzed spiro-isomerization, a reaction which is typical of spiroketals (Deslongchamps et al., 1981; Perron and Albizati, 1989). Subsequently, several hydroxyl-derivatives of CTX4A/B, including CTX1B, 52-*Epi*-54-DeoxyCTX and 54-DeoxyCTX, can be generated via oxidative metabolism in fish (e.g. in the liver).

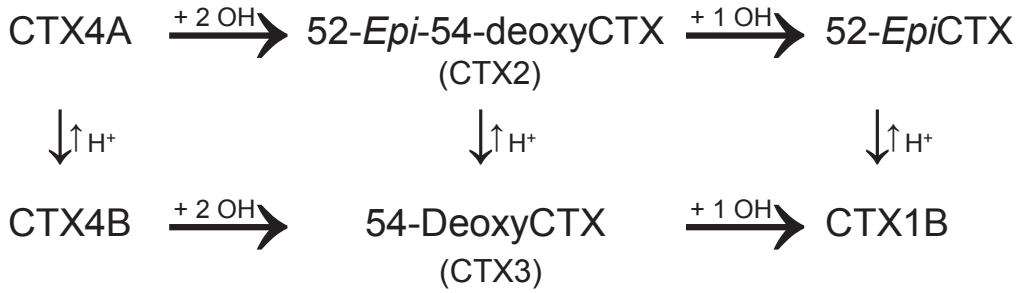

Figure 13. Scheme of the CTX metabolization pathway through the marine trophic chain originally proposed by Lewis and Holmes (1993).

Several **other congeners of CTX1B** were elucidated by Yasumoto et al. (2000) and Yasumoto (2001) using Fast-Atom Bombardment Tandem Mass Spectrometry (FAB-MS/MS) and NMR techniques, i.e. M-*Seco*CTX4A (or 4B), 52-*Epi*CTX, 54-*Epi*CTX, 52,54-*Diepi*CTX, 54-Deoxy-50-hydroxyCTX, 7-OxoCTX, 6,7-Dihydro-7-hydroxyCTX and 3,4-Dihydro-4-hydroxy-7-oxoCTX (**Table 3**). The authors stated that those congeners were found either in fish (no information about fish species examined) or in *Gambierdiscus* spp. (no information about species/strain examined), but they did not specify which congener derives from which matrix, except for M-*Seco* congeners, found in *Gambierdiscus* spp. (Yasumoto, 2001). The Dictionary of Marine Natural Products (Blunt and Munro, 2008) also reports another CTX1B congener, 3,4-Dihydro-3-hydroxy-7-oxoCTX ($C_{60}H_{88}O_{21}$, M = 1144.5818), isolated from fish. M-*Seco*CTX4A methyl acetal ($[M+Na]^+$ = 1115 *m/z*) was also found in *Gambierdiscus* sp. RGI-1 (Rangiroa Atoll, French Polynesia) in trace amounts using LC-MS analysis (Yogi et al., 2011). The authors suggested that M-*Seco* methyl acetals could be analytical artifacts because of the use of methanol as extraction and storage solvent (Yogi et al., 2011).

3.2.2.2. CTX3C congeners

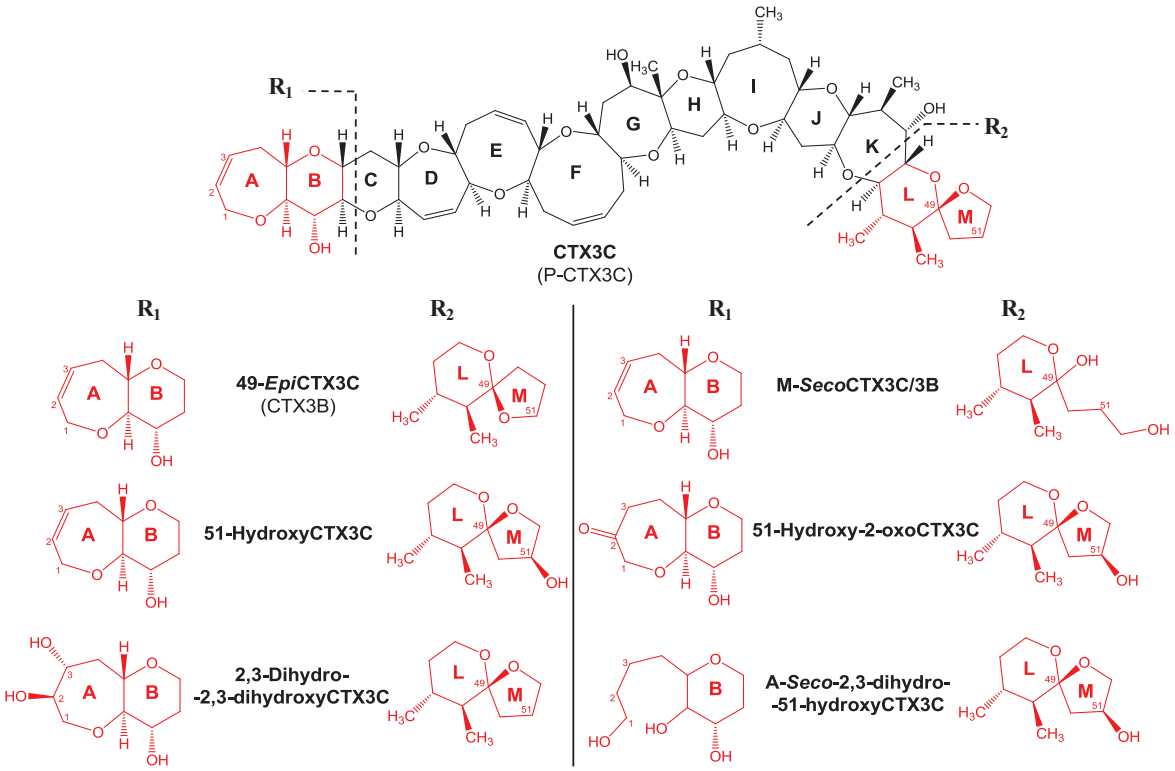

Figure 14. Structure of Pacific CTX3C (Satake et al., 1993b) and some examples of CTX3C congeners.

Ciguatoxin-3C (**CTX3C**, $C_{57}H_{82}O_{16}$, M = 1022.5603 Da) was isolated from *Gambierdiscus* sp. RGI-1 (Rangiroa Atoll, Tuamotu Archipelago, French Polynesia) and was structurally elucidated using NMR techniques (Satake et al., 1993b) (**Figure 14**). CTX3C potency was assessed using the mouse bioassay: intraperitoneal (i.p.) LD_{50} in mice was 1.3 µg kg^{-1}. CTX3C was also found in moray eels as minor toxin: this finding also corroborates the trophic transfer of CTXs from microalgae to fish.

Two more-polar congeners of CTX3C, **51-HydroxyCTX3C** (or CTX2C1, $C_{57}H_{82}O_{17}$, M = 1038.5552 Da) and **2,3-Dihydro-2,3-dihydroxyCTX3C** (or CTX2A1, or 2,3-DihydroxyCTX3C, $C_{57}H_{84}O_{18}$, M = 1056.5658 Da) were isolated from the viscera of the moray eel *Gymnothorax* (or *Lycodontis*) *javanicus* and were elucidated using NMR and FAB MS techniques (Satake et al., 1998) (**Figure 14**).

Several **other CTX3C congeners** were described by Yasumoto et al. (2000) and Yasumoto (2001) using Fast-Atom Bombardment Tandem Mass Spectrometry (FAB-MS/MS) and NMR techniques: 49-*Epi*CTX3C (or CTX3B), M-*Seco*CTX3C (or 3B), M-*Seco*-2,3-dihydro-2-hydroxy-49-methoxyCTX3C, 2,3-Dihydro-2-hydroxyCTX3C, 51-Hydroxy-2-oxoCTX3C,

2,3-Dihydro-2,3,51-trihydroxyCTX3C, A-*Seco*-2,3-Dihydro-51-hydroxyCTX3C, M-*Seco*-49-O-methylCTX3C (or M-*Seco*CTX3C methyl acetal), M-*Seco*-2,3-dihydro-2-hydroxy-49-methoxyCTX3C (**Table 3**). Again, few information is provided on the origin of those novel congeners: M-*Seco* congeners were found in *Gambierdiscus* spp. (Yasumoto, 2001). The Dictionary of Marine Natural Products (Blunt and Munro, 2008) also reports two other CTX3C congeners isolated from fish, i.e. 51-Hydroxy-3-oxoCTX3C ($C_{57}H_{82}O_{18}$, M = 1054.5501 Da) and 2,3-Dihydro-3-hydroxyCTX3C ($C_{57}H_{84}O_{17}$, M = 1040.5709 Da).

The isolation of 51-HydroxyCTX3C and 2,3-Dihydro-2,3-dihydroxyCTX3C (Satake et al., 1998) and the further discovery of several other oxidized derivatives of CTX3C (Yasumoto, 2001; Yasumoto et al., 2000) also supported the theory of oxidative modification of CTXs during the food chain transmission. A recent study conducted by Ikehara et al. (2017) demonstrated the enzymatic oxidation of the reference toxins CTX4A, CTX4B and CTX3C using *in vitro* experiments with human CYP3A4 and fish liver S9 and fractions and LC-LRMS/MS analysis. Still, oxidized CTX4A/B and/or CTX3C congeners have also been found in several *Gambierdiscus* strains using LC-MS techniques (Caillaud et al., 2011; Chinain et al., 2010a; Laza-Martinez et al., 2016; Roeder et al., 2010; Yogi et al., 2011).

3.2.2.3. Other Pacific CTX congeners.

Terao et al. (1991) and Terao et al. (1992) conducted histological studies on different tissues of mice exposed to CTX1B and to **CTX4C**, a CTX congener from a strain of *Gambierdiscus* sp. isolated from the Gambier Islands. Even if CTX4C presented CTX-type cytotoxicity, its molecular structure has never been characterized. A thorough search of the relevant literature yielded only one related conference (Legrand et al., 1990).

Lewis and Jones (1997) found eleven potential undescribed congeners of CTXs in trace amounts in a specimen of the moray eel *L. javanicus* collected from Republic of Kiribati (Central Pacific Ocean) and in a specimen of a coral cod (*Cephalopholis* sp.) collected from Australia. These congeners were found using LC-LRMS and were not structurally characterized.

Chinain et al. (2010a) studied the toxin profile of two strains of *G. polynesiensis* using a bioguided fractionation approach (MBA and $RBA_{(R)}$) followed by HPLC-LRMS analyses in full scan and in single ion monitoring (SIM) modes. The strains examined were TB-92, from Matauara (Tubai Island, Australes Archipelago, French Polynesia) and RG-92, from Avatoru (Rangiroa, French Polynesia). The authors detected some known congeners (CTX3C, 49-*Epi*CTX3C, M-*Seco*CTX3C, CTX4A, CTX4B) and tentatively identified new congeners of CTXs in trace amounts (2-3%) as either (i) stereo-isomers of known congeners; (ii) congeners with one or four open rings; (iii) CTX3C congeners presenting one or two extra double bonds.

3.2.3. Caribbean ciguatoxins

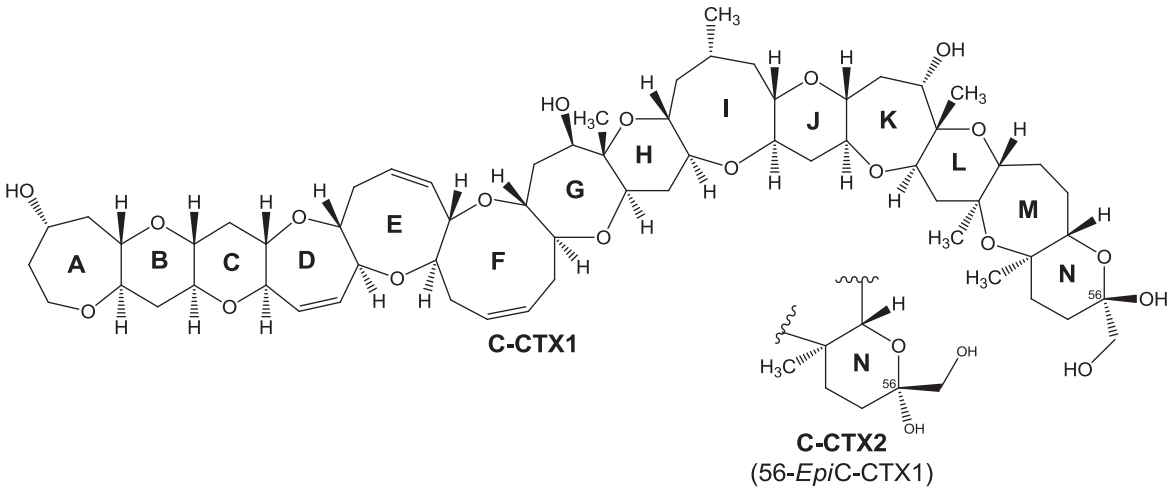

Figure 15. Structure of Caribbean C-CTX1 (Lewis et al., 1998). The inset shows the acid-catalyzed rearrangement of the hemiketal at C_{56} of C-CTX1 (C-CTX2 or 56-*Epi*C-CTX1).

Caribbean CTXs (C-CTXs) were isolated from the tissues of numerous fish species including invertebrate feeders and benthic or pelagic predators (Vernoux and Talha, 1989). Contrarily to the Pacific, the involvement of herbivorous fish has never been demonstrated in the Caribbean and no CTX precursors from *Gambierdiscus*/*Fukuyoa* have been identified to date. Lewis et al. (2016) found that five strains of *Gambierdiscus*/*Fukuyoa* from the Caribbean Sea (*F. ruetzleri* Gam1, *G. belizeanus* CCMP399, *G. carolinianus* Dive1 Gam1, *Gambierdiscus* sp. ribotype 2 St. Maarteens Gam6 and *Gambierdiscus* sp. ribotype 2 Mixed PR Gam3) produced detectable CTX-type toxicity using a SH-SY5Y cell-based FLIPR$^{TETRA®}$ assay. HPLC fractionation of those strains suggested the involvement from one to six different C-CTX congeners, depending on the strain.

The structure and configuration of **C-CTX1** ($C_{62}H_{92}O_{19}$, M = 1140.6233 Da), the major C-CTX isolated from the horse-eye jack *Caranx latus* (Vernoux and Lewis, 1997), and its C_{56}-epimer **C-CTX2**, were determined using NMR techniques in 1998 (Lewis et al., 1998) (**Figure 15**). Detection of C-CTX1 by mass spectrometry (LC-LRMS and LC-LRMS/MS) has been reported in the main families of carnivorous fish found in the Caribbean: Sphyraenidae, Carangidae, Serranidae and Lutjanidae (Pottier et al., 2002a, 2002b; Pottier et al., 2001) (and references therein).

As previously suggested in earlier studies (Poli et al., 1997; Vernoux and Lewis, 1997), multiple C-CTXs may be involved in ciguatera poisoning in the Caribbean and could explain the variability in the CFP symptomatology. Pottier et al. (2002b) found ten minor C-CTX congeners in individual toxic specimens of *C. latus* using LC-LRMS analysis (**Table 4**). These novel congeners were proposed to be isomers or derivatives of C-CTX1/2 but they have not been structurally characterized yet. A further study conducted by Abraham et al. (2012) also reported the presence of those C-CTX congener in Caribbean fish. Other five putative C-CTX congeners with MW < 900 Da were found via LC-MS techniques in the flesh of a toxic grouper specimen (Serranidae) collected in Guadeloupe (French West Indies) and presented molecular ions $[M+H]^+$ at *m/z* 851.51, 857.50, 875.49, 875.51, 895.54 (**Table 4**) (Pottier et al., 2002a). The authors suggested that the ion species at *m/z* 895.54 might correspond to brevetoxin-2 (BTX2), as *Karenia brevis* blooms have been reported in Caribbean waters. The ion species at *m/z* 875.49 and/or 875.51 may correspond to pectenotoxin-1 (PTX1) although the latter has never been described in tropical waters.

Table 4. List of the Caribbean ciguatoxin congeners (C-CTXs) known to date with relevant information and references.

Compound name	$[M+H]^+$	Additional information	Ref.
C-CTX1, C-CTX2 and three other isomers (C-CTX1141a/b/c)	1141.6306	Structure elucidation (NMR) of C-CTX1 and C-CTX2 only (elemental formula: $C_{62}H_{92}O_{19}$). C-CTX2: 56-*Epi*C-CTX1.	[1-4]
C-CTX1127	1127.57	Demethylated derivative of C-CTX1.	[2, 3]
C-CTX1143 and one other isomer (C-CTX1143a)	1143.60	Oxidation products of a demethylated derivative of C-CTX1 or reduced derivatives of C-CTX1.	[2-4]
C-CTX1157 and two other isomers (C-CTX1157a/b)	1157.57	Mono-hydroxyl derivatives of C-CTX1.	[2-4]
C-CTX1159	1159.58	Hydroxylated derivative of C-CTX1 (addition of H_2O across a double bond).	[2, 4]
putative C-CTX851	851.51	Loss of one molecule of H_2O in MS spectrum.	[3]
putative C-CTX857	857.50	Loss of two molecules of H_2O in MS spectrum.	[3]
putative C-CTX895	895.54	Loss of one molecule of H_2O in MS spectrum. Maybe BTX2? (MW_{BTX2} = 894.5 Da)	[3]
two putative C-CTX875	875.49 875.51	Loss of three molecules of H_2O in MS spectrum. Maybe PTX1? (MW_{PTX1} = 874.5 Da)	[3]

[1] R. J. Lewis, J.-P. Vernoux, I. M. Brereton, *Journal of the American Chemical Society* **1998**, *120*, 5914.
[2] I. Pottier, J. P. Vernoux, A. Jones, R. J. Lewis, *Toxicon* **2002**, *40*, 929.
[3] I. Pottier, J. P. Vernoux, A. Jones, R. J. Lewis, *Food Additives and Contaminants* **2002**, *19*, 1034.
[4] A. Abraham, E. L. E. Jester, H. R. Granade, S. M. Plakas, R. W. Dickey, *Food Chemistry* **2012**, *131*, 192.

3.2.4. Indian ciguatoxins

Six Indian CTXs (I-CTX1 to 6) have been identified in fish specimens collected from Indian Ocean (Diogène et al., 2017; Hamilton et al., 2002a; Hamilton et al., 2002b) (**Table 5**). More precisely, I-CTX1 to I-CTX4 were first described from whole fish extracts of the red emperor *Lutjanus sebae* and the red bass *Lutjanus bohar*, both collected from Republic of Mauritius (Nazareth, Saya de Malha, Soudan) (Hamilton et al., 2002a; Hamilton et al., 2002b). They were subsequently found in a stomach extract of a specimen of the bull shark *Carcharhinus leucas* from Fenerive-Est (Madagascar) (Diogène et al., 2017). I-CTX5 and I-CTX6 were described for the first time in *C. leucas* (Diogène et al., 2017). Structure elucidation via NMR of any of the six congeners has not been provided yet. The elemental formulae of I-CTXs were assigned based on high resolution mass spectrometry (HRMS) data (Diogène et al., 2017; Hamilton et al., 2002a). To date, no I-CTX precursor from microalgae has been identified.

Table 5. List of the Indian ciguatoxin congeners (I-CTXs) known to date with relevant information and references.

Compound name	Elemental formula	Exact mono-isotopic mass (M)	Additional information	Ref.
I-CTX1 I-CTX2	$C_{62}H_{92}O_{19}$	1140.6233	Same accurate mass as C-CTX1, but different retention times. No interconversion observed. Major in *L. sebae*, minor in *C. leucas*.	[1-3]
I-CTX3 I-CTX4	$C_{62}H_{92}O_{20}$	1156.6182	Mono-hydroxyl derivatives of I-CTX1/2. Less potent than I-CTX1/2. Major in *C. leucas*, minor in *L. sebae*.	[1-3]
I-CTX5	$C_{62}H_{90}O_{19}$	1138.6076	Loss of 2H (double bond) compared to I-CTX1/2. Found in *C. leucas* only.	[3]
I-CTX6	$C_{62}H_{90}O_{20}$	1154.6025	Loss of 2H (double bond) compared to I-CTX3/4. Found in *C. leucas* only.	[3]

[1] B. Hamilton, M. Hurbungs, A. Jones, R. J. Lewis, *Toxicon* **2002**, *40*, 1347.
[2] B. Hamilton, M. Hurbungs, J. P. Vernoux, A. Jones, R. J. Lewis, *Toxicon* **2002**, *40*, 685.
[3] J. Diogène, L. Reverté, M. Rambla-Alegre, V. del Río, P. de la Iglesia, M. Campàs, O. Palacios, C. Flores, J. Caixach, C. Ralijaona, I. Razanajatovo, A. Pirog, H. Magalon, N. Arnich, J. Turquet, *Scientific Reports* **2017**, *7*.

In the study conducted by Hamilton et al. (2002a), comparison of HRMS spectral signals of the Indian I-CTX1 and I-CTX2 from *L. sebae* with reference material of the Caribbean C-CTX1 indicated that the three molecules have the same accurate mass. Diogène et al. (2017) also provided HRMS spectra of I-CTX1 and I-CTX2 from *C. leucas*. Thus, the four CTX congeners I-CTX1, I-CTX2, C-CTX1 and C-CTX2 (or 56-*Epi*C-CTX1) share the same elemental formula, i.e. $C_{62}H_{92}O_{19}$ (M = 1140.6233). Hamilton et al. (2002b) separated I-CTX1, I-CTX2 and C-CTX1 (reference material) in the same analytical conditions using a reversed-phase chromatography column. In support of the chromatographic separation, MS and MS/MS spectra of I-CTX1 and I-CTX2 presented different ion ratios than those exhibited by C-CTX1 (Hamilton et al., 2002a; Hamilton et al., 2002b). I-CTX1 and I-CTX2 are believed to be epimers, but, unlike CTXs from Pacific or Caribbean areas, interconversion did not occur in acidic conditions (Hamilton et al., 2002b).

Hamilton et al. (2002a) already hypothesized that the congeners I-CTX3 and I-CTX4 from *L. sebae*, both having a nominal mass of 1156.6 Da, are mono-hydroxyl derivatives of I-CTX1/2. Later, Diogène et al. (2017) confirmed this hypothesis with HRMS analysis of *C. leucas* extracts and assigned to I-CTX3 and I-CTX4 the molecular formula of $C_{62}H_{92}O_{20}$ (M = 1156.6182). Interestingly, $RBA_{(R)}$:MS ratios suggested that I-CTX3 and I-CTX4 are ca. 2-fold less potent than I-CTX1 and I-CTX2 (Hamilton et al., 2002b). Therefore, contrarily to CTXs from the Pacific, potency of I-CTXs does not seem to increase when the degree of oxidation increases.

For the other two congeners from *C. leucas*, I-CTX5 ($C_{62}H_{90}O_{19}$, M = 1138.6076) and I-CTX6 ($C_{62}H_{90}O_{20}$, M = 1154.6025), the assignment of the elemental formulae was supported by isotopic pattern and mass accuracy of the pseudomolecular ions $[M+Na]^+$ and $[M+NH_4]^+$ (Δppm < 10) (Diogène et al., 2017).

3.2.5. Ciguatoxins from North Atlantic Ocean?

Otero et al. (2010) screened two fish specimens (*Seriola dumerili* and *Seriola fasciata*) collected from the North Atlantic Ocean (Selvagens Islands, Madeira Archipelago) for the presence of CTXs, using both a functional assay (cerebellar granule cells) and chemical analysis (UPLC-LRMS). Identification of CTXs was based on both bioactivity and MS behavior (sodium and ammonium adducts, losses of molecules of water). Interestingly, those fishes resulted positive for the presence of seven potential CTX congeners with $[M+H]^+$ presenting the following *m/z*: 1111.6, 1023.5, 1061.0 (three peaks), 1140.6 and 1141.6. The peaks with *m/z* 1111.6 and 1023.5 were assigned to the Pacific CTX1B and CTX3C, respectively, as confirmed by standard reference material. The peaks with *m/z* 1061.0 and 1040.6 may be attributable to the Pacific CTX4A (or CTX4B) and 51-HydroxyCTX3C, respectively. The peak with *m/z* 1141.6 could correspond to a Caribbean or Indian CTX, either C-CTX1/2 or I-CTX1/2. If those congeners were confirmed, the classification of CTXs in Pacific, Indian or Caribbean should be revised.

Silva et al. (2015) screened 22 species of benthic organisms collected from North Atlantic Ocean for the presence of CTX congeners using UPLC-IT-TOF-MS analysis. Only two starfish species (*Ophidiaster ophidianus* and *Marthasterias glacialis*) collected from the Portuguese islands Madeira (Madeira archipelago) and São Miguel (Azores archipelago) were positive for three potential CTX candidates. HRMS spectra of these three CTX candidates showed molecular ion species $[M+H]^+$ with measured accurate masses of *m/z* 1111.5790, 1109.5262 and 1123.5862 associated with losses of 3 up to 5 molecules of H_2O and pseudomolecular ions (Na^+ and/or K^+ and/or NH_4^+ adducts). The authors suggested that the ion species at *m/z* 1111.5760 could correspond to the Pacific CTX1B (theoretical exact mass of $[M+H]^+$ *m/z* 1111.5836, Δppm = -6.8), but they could not confirm its identity because reference CTX1B was

not available in that study. This study brought to light the potential role of echinoderms as ciguatera vectors.

3.2.6. Mechanism of action and biological effects

The voltage-gated sodium channels (VGSCs) are transmembrane proteins responsible for the rapid increase in membrane Na^+ conductance which triggers the depolarizing phase of the action potential in many excitable cells (Hodgkin and Huxley, 1990). This transient increase in Na^+ conductance lasts only milliseconds and then spontaneously inactivates, K^+ permeability increases and the membrane can be repolarized. This process is critical for the generation and propagation of electrical signals in most excitable cells (such as nerve, muscle and endocrine cells).

The VGSC consists of a large core protein, the α-subunit (220-280 kDa, ~ 2000 amino acids), that can be associated with smaller (22-40 kDa) β-subunits: β_1, β_2 and β_3 (Catterall, 1992; Isom et al., 1992; Isom et al., 1995; Morgan et al., 2000) (**Figure 16A**). The α-subunit contains the functional ion conduction pore as an aqueous cavity that is selectively permeable for sodium ions, the β-subunits have auxiliary regulatory functions. Different regions of the α-subunit of VGSCs have been identified as molecular targets or "receptor sites" for several families of neurotoxins. Competition radioligand binding experiments have shown that ciguatoxins (CTXs) compete with another family of lipid-soluble cyclic polyether toxins, brevetoxins (BTXs), for the receptor site 5 of the α-subunit on the intramembrane portion of VGSCs (Lombet et al., 1987) (**Figure 16B**). Transmembrane segments IS6 and IVS5 have both been identified to participate in the formation of receptor site 5 (Trainer et al., 1994; Trainer et al., 1991). Interestingly, the affinity of the different congeners of CTXs for the VGSC is proportional to their respective intraperitoneal LD_{50}s in mice (Lewis, 1994).

The action of CTXs on VGSCs mainly consists of (i) a shift in the voltage dependence of the VGSC activation to more negative (depolarized) potentials and (ii) a blockage of VGSC inactivation (Shmukler and Nikishin, 2017). As a consequence, CTXs remarkably alter the ordinary functioning of neuronal cells, causing increased Na^+ influx, repetitive firing of action potentials and resting membrane depolarization (Benoit et al., 1996; Bidard et al., 1984; Yamaoka et al., 2011; Yamaoka et al., 2004).

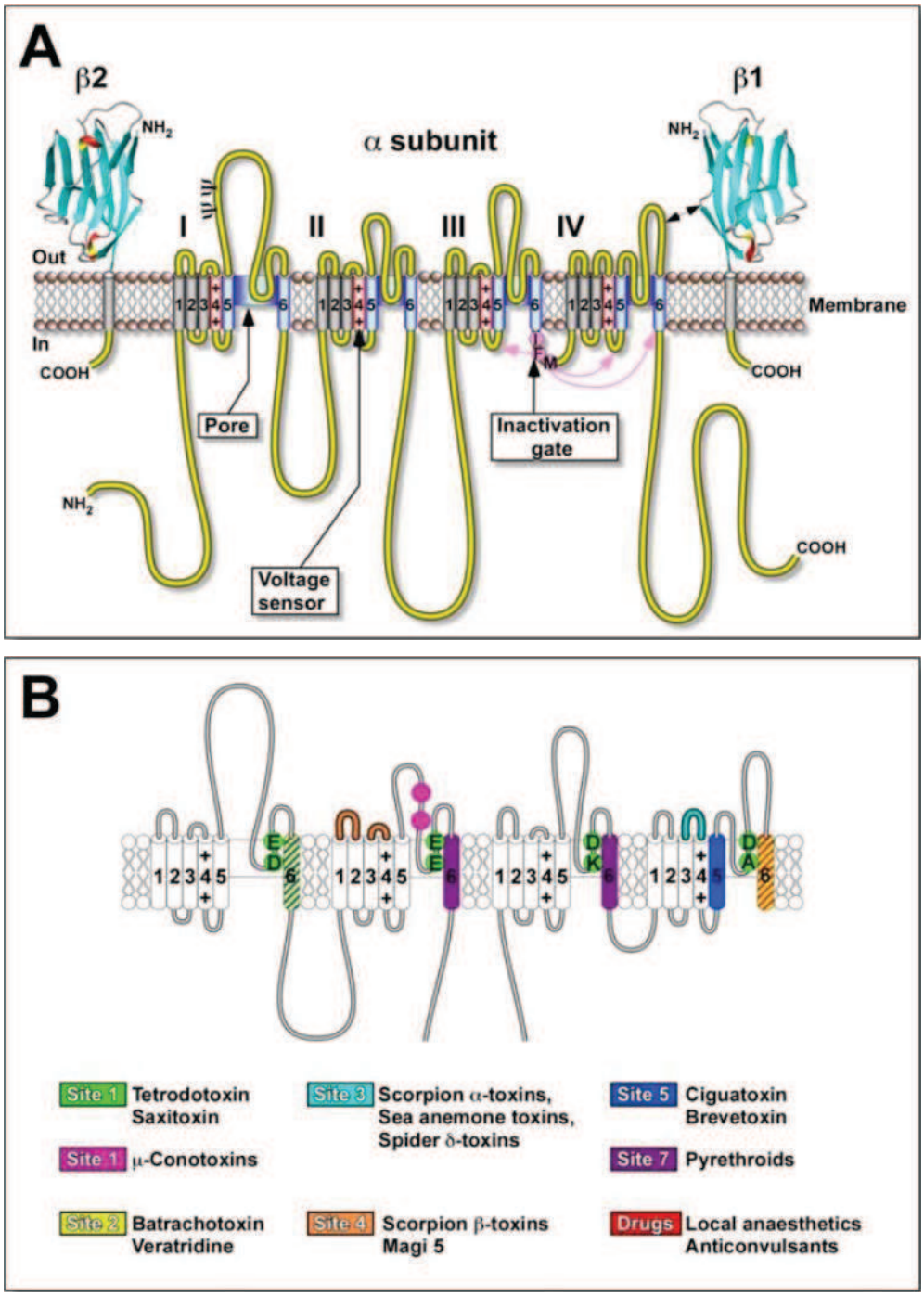

Figure 16. Molecular structure and neurotoxin receptor sites of the voltage-gated sodium channel (VGSC) (Nicholson and Lewis, 2006). **(A)** Schematic representation of the subunit structure of the VGSC showing the functional α-subunit and auxiliary β-subunits β_1 and β_2. **(B)** Location of known neurotoxin receptor sites on VGSCs.

The voltage-gated potassium channel (VGPC) may represent a secondary target of CTXs. Indeed, some studies showed that CTX1B affects K^+ currents at nanomolar concentrations (Birinyi-Strachan et al., 2005b; Hidalgo et al., 2002; Mattei et al., 2014). Ionic disturbances induced by CTXs have several impacts at the cellular and physiological levels, such

as membrane excitability (Birinyi-Strachan et al., 2005b), axonal and Schwann edema (Benoit et al., 1996; Mattei et al., 1997; Mattei et al., 1999a; Mattei et al., 2014) and perturbation of Ca^{2+} homeostasis (Hidalgo et al., 2002; Molgó et al., 1991; Molgó et al., 1993a; Molgó et al., 1993b).

The CTX-induced neuronal excitability produces activation of peripheral sensory neurons expressing TRPA1, a transient receptor potential cation channel, as demonstrated in cultured mouse dorsal root ganglion (DRG) cells (Vetter et al., 2012b; Voets, 2012). The TRPA1-dependent Ca^{2+} influx in response to mild cooling is responsible for the development of pain and cold allodynia (Vetter et al., 2014). The intense pruritus observed in the clinical picture of ciguatera may also be related to the CTX-induced TRPA1 activation, as Wilson et al. (2011) demonstrated that TRPA1-expressing fibers also mediate itch.

The CTX-induced swelling produces increased electrical capacitance of the nodal membrane and alterations to nerve conduction velocity, phenomena which are likely to be involved in the sensory disturbances characteristic of ciguatera poisoning (Cameron and Capra, 1993; Cameron et al., 1991a, 1991b, 1993). The CTX-induced perturbation of Ca^{2+} homeostasis is involved in the positive inotropic actions of CTXs in cardiac myocytes (Lewis, 1988; Lewis and Endean, 1986) and neurotransmitters' release (Meunier et al., 2009). The release of norepinephrine and ATP from adrenergic nerve terminals is a consequence of the CTX-induced asynchronous discharge of preganglionic perivascular axons (Brock et al., 1995) and has been associated to vas deferens contraction, a cardiovascular symptom of ciguatera poisoning (Nicholson and Lewis, 2006). The release of acetylcholine (ACh) is a consequence of the indirect effect of CTXs on the Na^{+}-Ca^{2+} exchanger (NCX). The CTX-induced depolarization and spontaneous repetitive firing in synaptosomes reduces the ability of NCX to extrude Ca^{2+} ions and can trigger the Na^{+}-Ca^{2+} exchanger to operate in a reverse mode. Therefore, cytoplasmic Ca^{2+} concentration increases and promotes Ca^{2+}-dependent ACh release (Molgó et al., 1993a). In support, the CTX-induced release of ACh is completely blocked by inhibitors of the Na^{+}-Ca^{2+} exchanger such as benperidol and cotidal, thus confirming the involvement of Na^{+}-Ca^{2+} exchanger in ACh release (Gaudry-Talarmain et al., 1996). The release of acetylcholine (ACh) from neuromuscular junction has been associated to: (i) perturbation of the gastro-intestinal symptoms, at the origin of the severe diarrhea observed

among the clinical signs of ciguatera poisoning (Nicholson and Lewis, 2006), (ii) disturbance in mechanical activity of cardiac tissue, at the origin of bradycardia experienced by some ciguatera patients (Sauviat et al., 2002).

Recent studies on the RAW 264.7 murine macrophage cell line demonstrated that CTXs also act as potent modulators of mRNA expression, interfering with oxidative stress and immune response mechanisms (Kumar-Roine et al., 2008; Matsui et al., 2010). Indeed, CTX1B was shown to cause a high and prolonged up-regulation of inducible nitric oxide synthetase (iNOS), an enzyme which produces nitric oxide radical (NO) from L-arginine (Kumar-Roine et al., 2008). The CTX-induced activation of NO pathway has been associated to the swelling of frog red blood cells (Sauviat et al., 2006) and may also be involved in some of the symptoms of the ciguatera poisoning, e.g. the chronic fatigue syndrome (Pall, 2001; Racciatti et al., 2001) and cardiovascular dysfunctions (Moncada and Higgs, 2006; Strunk et al., 2001; Wang et al., 1999). Matsui et al. (2010) also demonstrated that CTX1B up-regulated three pro-inflammatory (TNF-α, IL-1b and IL-6) and one anti-inflammatory (IL-10) cytokines, which could be consistent with the fever and the neurological and neuropsychiatric disorders experienced by patients affected by ciguatera poisoning (Friedman et al., 2008).

3.3. **Maitotoxins**

3.3.1. Background

Maitotoxins (MTXs) constitute a family of ladder-shaped polyether toxins produced by *Gambierdiscus*/*Fukuyoa* spp., soluble in polar solvents (e.g. water, methanol), which may be found in the viscera of fish (Yasumoto and Kanno, 1976). Three MTX congeners are known to date: maitotoxin (MTX, $C_{164}H_{256}O_{68}S_2Na_2$, M = 3423.5811 Da) (Murata et al., 1993), maitotoxin-2 (MTX2, MW = 3298 Da for the mono-sodium salt) (Holmes et al., 1990) and maitotoxin-3 (MTX3, MW = 1060.5 Da for the di-sodium salt) (Holmes and Lewis, 1994).

Maitotoxin (MTX) is among the most potent marine toxins identified to date, with an intraperitoneal (i.p.) lethal dose 50 (LD_{50}) in mice of 0.050 µg kg^{-1} (Murata et al., 1993). Its oral potency, however, is much lower (Kelley et al., 1986), probably due to low intestinal absorption caused by its high molecular weight and hydrophilicity. MTX is primarily found in the tissues associated with the digestive tract of fish and could play a role in ciguatera fish poisoning (CFP) if gut and liver tissues are consumed (Ajani et al., 2017). MTX activates voltage-independent Ca^{2+} entry mechanisms that significantly raise intracellular Ca^{2+} levels in a wide variety of cells (Gusovsky et al., 1990; Ogura et al., 1984; Ohizumi and Kobayashi, 1990). The specific molecular target of MTX, however, is still unknown (Reyes et al., 2014).

The two other MTX congeners known to date, MTX2 and MTX3, were isolated during the 1990s (Holmes and Lewis, 1994; Holmes et al., 1990). Since the molecular structures of MTX2 and MTX3 are still unknown, the only structural feature that can be considered common to the MTX class of toxins is the presence of (at least) one sulfate ester group. Desulfatation experiments conducted by Holmes and Lewis (1994) on the three MTX congeners suggested that at least one of the sulfate ester groups is critical for the bioactivity. In particular, a study conducted by Murata et al. (1991) showed that desulfatation or hydrogenation of MTX sensibly decreased its ability to induce Ca^{2+} influx or phosphoinositide breakdown in insulinoma or glioma cells.

Table 6 presents a schematic summary of the three MTXs known to date with relevant chemical information.

Davis et al. (1994) described the purification and isolation of a Caribbean MTX, i.e. a MTX congener isolated from a Caribbean strain of *Gambierdiscus* sp. (MTX-C). Lu et al. (2013) investigated the activity of MTX-C in insulin-secreting transformed HIT-T15 cells (hamster pancreatic islet) using the fluorescence Ca^{2+}-indicator Indo-1AM. Like MTX, MTX-C caused non-selective ion channels to open, and, subsequently, elicited an abnormal Ca^{2+}-influx. A recent study conducted by Lewis et al. (2016) indicated that several strains of *Gambierdiscus*/*Fukuyoa* produce multiple MTX congeners, in one case more than four (*G. belizeanus* CCMP399), suggesting broader chemical diversity than what is known so far within MTX group. These congeners are not listed in **Table 6** since no further information beyond activity was provided in those studies.

Table 6. List of the three MTXs known to date with relevant chemical information. *Gambierdiscus* sp. GII-1 was isolated from Gambier Islands (French Polynesia), *Gambierdiscus* sp. NQ1 from Queensland (Australia) and *Gambierdiscus* sp. WC1/1 from Australia. FAB^-: fast atom bombardment ionization in negative ion acquisition mode. IS^+: ionspray ionization in positive ion acquisition mode. IS^-: ionspray ionization in negative ion acquisition mode. UNKN: unknown.

Name	Abbr.	Formula	Mass (Da)	Structural studies	Toxicity (i.p. LD_{50} in mice, µg kg^{-1})	Source	Ref.
Maitotoxin	MTX	$C_{164}H_{256}O_{68}S_2Na_2$	3423.5811	IR, UV (λ_{max} = 230 nm) LRMS/MS: FAB^- NMR with complete stereochemistry	0.050	GII-1	[1-4]
Maitotoxin-2	MTX2	UNKN (mono-sodiated salt of a molecule containing one sulfate ester)	3298	UV (λ_{max} = 230 nm) LRMS: IS^+, IS^-, FAB^-	0.080	NQ1	[5]
Maitotoxin-3	MTX3	UNKN (di-sodiated salt of a molecule containing two sulfate esters)	1060.5	UV (λ_{max} = 200, 235 nm) LRMS: IS^+	UNKN	WC1/1	[6]

[1] M. Murata, H. Naoki, T. Iwashita, S. Matsunaga, M. Sasaki, A. Yokoyama, T. Yasumoto, *J. Am. Chem. Soc.* **1993**, *115*, 2060.
[2] M. Sasaki, N. Matsumori, T. Maruyama, T. Nonomura, M. Murata, K. Tachibana, T. Yasumoto, *Angew. Chem. Int. Ed. Engl.* **1996**, *35*, 1672.
[3] T. Nonomura, M. Sasaki, N. Matsumori, M. Murata, K. Tachibana, T. Yasumoto, *Angew. Chem. Int. Ed. Engl.* **1996**, *35*, 1675.
[4] W. J. Zheng, J. A. DeMattei, J. P. Wu, J. J. W. Duan, L. R. Cook, H. Oinuma, Y. Kishi, *J. Am. Chem. Soc.* **1996**, *118*, 7946.
[5] M. J. Holmes, R. J. Lewis, N. C. Gillespie, *Toxicon* **1990**, *28*, 1159.
[6] M. J. Holmes, R. J. Lewis, *Nat. Toxins* **1994**, *2*, 64.

3.3.2. Maitotoxin congeners

3.3.2.1. Maitotoxin (MTX)

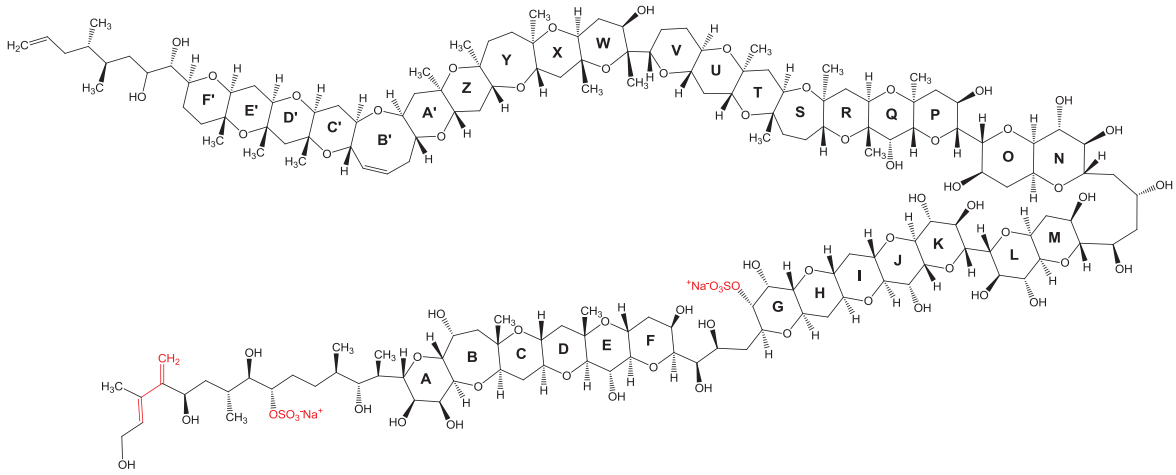

Figure 17. Structure of maitotoxin (MTX) with absolute stereochemistry according to Sasaki et al. (1996) and Nonomura et al. (1996). Note the ladder-shaped polyether structure and the following characteristic features (in red): one conjugated diene at C_2-C_3-C_4-C_{144}, responsible for UV absorbance (λ_{max} = 230 nm) (Yokoyama et al., 1988), and two sodiated sulfate ester groups at C_9 and C_{40}.

Maitotoxin (MTX, $C_{164}H_{256}O_{68}S_2Na_2$, M = 3423.5811 Da, **Figure 17**) was first detected in 1976 in the viscera of the bristletooth surgeonfish *Ctenochaetus striatus* (in Tahitian "maito", hence its name) collected in Tahiti (French Polynesia) and it was initially suspected of contributing to the diversity of ciguatera symptoms (Yasumoto et al., 1976; Yasumoto et al., 1971). Eleven years later, the toxin was isolated from the dinoflagellate *Gambierdiscus* (strain name/origin not provided) by Yasumoto et al. (1987) confirming the source of the toxin isolated from contaminated fish. Purified MTX exists as a di-sodiated white amorphous solid that is soluble in polar solvents (e.g. water, methanol and dimethylsulfoxide). MTX is relatively stable in alkaline but not in acidic conditions (Yokoyama et al., 1988). In aqueous solution, pure MTX tends to adhere to both glass and plastic surfaces (Murata et al., 1994; Murata and Yasumoto, 2000). When dissolved in methanol-water mixtures, MTX exhibits a single UV absorbance maximum at 230 nm (Yokoyama et al., 1988) due to the presence of a conjugated diene at one extremity of the molecule (C_2-C_3-C_4-C_{144}, **Figure 17**). The complete chemical structure of MTX was elucidated in 1993 following isolation from the *Gambierdiscus* strain GII-1 from the Gambier Islands (Murata et al., 1993). MTX is the largest non-polymeric marine toxin identified to date, consisting of a ladder-shaped cyclic

polyether that is composed of 32 fused ether rings, 28 hydroxyl groups, 21 methyl groups, two sulfates and 98 chiral centers (molecular formula: $C_{164}H_{256}O_{68}S_2Na_2$, mono-isotopic mass of 3423.5811 Da for the di-sodium salt). In 1996, the stereochemistry of the entire molecule was also assigned (Nonomura et al., 1996; Sasaki et al., 1996; Zheng et al., 1996) (**Figure 17**). Gallimore and Spencer (2006) contested the stereochemistry of the junction between J and K rings according to a mechanistic hypothesis for the biosynthesis of marine ladder-shaped polyethers. Nicolaou and Frederick (2007) and Nicolaou et al. (2007), however, supported the originally assigned structure based on reported NMR spectroscopic data, computational studies and providing chemical synthesis and NMR analysis of the GHIJK ring system. X-ray crystal structure of MTX is needed to solve this controversy.

3.3.2.2. Maitotoxin-2 (MTX2)

Maitotoxin-2 (MTX2) was obtained from a single Australian *Gambierdiscus* strain from Queensland (NQ1) (Holmes et al., 1990). It had an i.p. LD_{50} in mice of 0.080 µg kg^{-1} (Holmes and Lewis, 1994), i.e. 1.6-fold less toxic than MTX (Murata et al., 1993). When dissolved in acetonitrile-water mixtures, MTX2 showed a single UV absorbance maximum at 230 nm (Holmes and Lewis, 1994), identical to that reported for MTX in methanol-water (Yokoyama et al., 1988). The molecular structure of MTX2 has not been elucidated yet. Lewis et al. (1994a) conducted LC-LRMS analyses on material isolated from strain NQ1 in ionspray ionization in positive ion acquisition mode (IS^+), ionspray ionization in negative ion acquisition mode (IS^-) and fast atom bombardment ionization in negative ion acquisition mode (FAB^-) and suggested that MTX2 is mono-sulfated with a nominal mass of 3298 Da (as mono-sodium salt) (**Figure 18**, **Figure 19**).

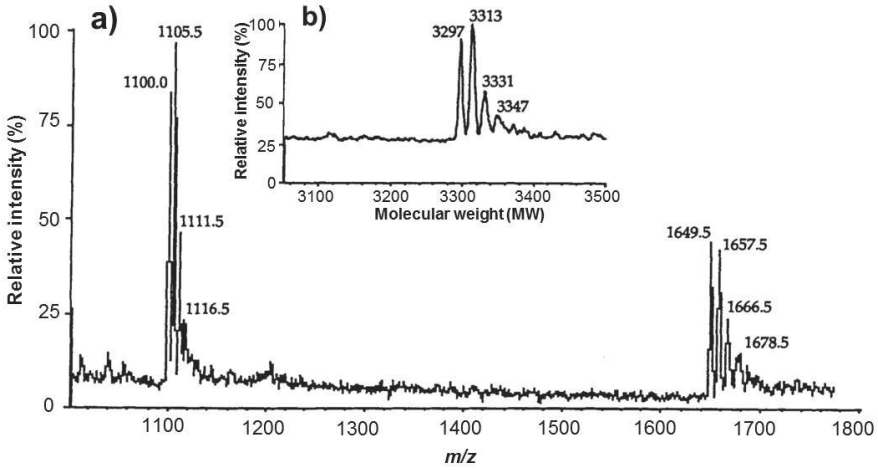

Figure 18. LRMS spectra of MTX2 obtained using positive ion acquisition mode according to Lewis et al. (1994a). **(a)** Positive ionspray (IS^+) LRMS spectrum of MTX2 (50 µg mL^{-1}) obtained using an orifice potential (OR) of 60 V. MTX2 yielded bi-charged and tri-charged pseudomolecular cations (Na^+ and K^+ adducts). **(b)** Reconstructed spectrum of MTX2 showing prominent signals at M_r = 3297 and 3313 for the sodium and potassium salts, respectively.

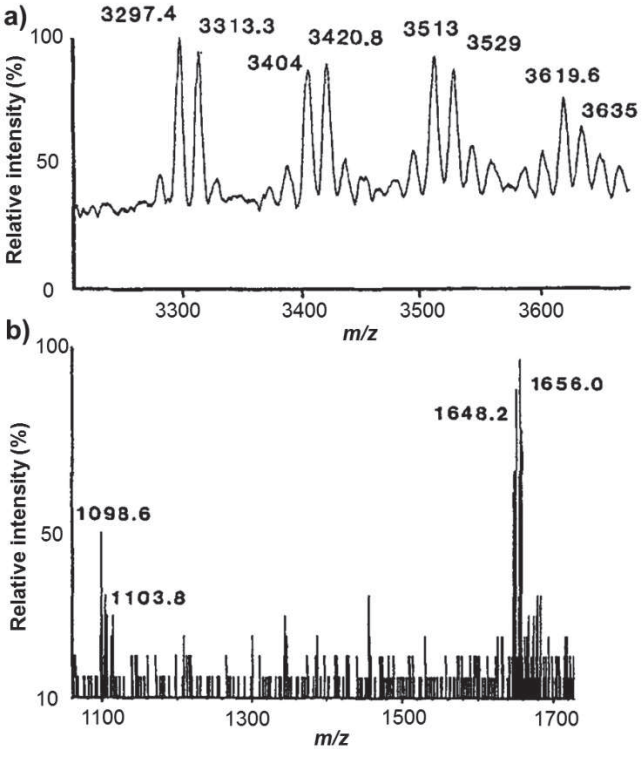

Figure 19. LRMS spectra of MTX2 obtained using negative ion acquisition mode according to Lewis et al. (1994a). **(a)** Negative fast atom bombardment (FAB^-) LRMS spectrum of MTX2 dissolved in glycerol:thioglycerol (1:1, v/v). **(b)** Negative ionspray (IS^-) LRMS spectrum of MTX2 (OR = - 120 V) dissolved in MeOH:H_2O (1:1, v/v). MTX2 yielded bi-charged and tri-charged pseudomolecular anions (Na^+ and K^+ adducts).

3.3.2.3. Maitotoxin-3 (MTX3)

Maitotoxin-3 (MTX3) was isolated from the Australian *Gambierdiscus* strain WC1/1 (Holmes and Lewis, 1994). Maitotoxin-3 was found to be toxic in mice, inducing similar symptoms than those observed for MTX and MTX2, but scarcity of the compound did not permit the determination of MTX3 potency (i.p. LD_{50} in mice). On a reversed-phase column, MTX3 elutes earlier than MTX2 and later than MTX when using a linear gradient of acetonitrile-water (Holmes and Lewis, 1994). When dissolved in acetonitrile-water mixtures, MTX3 showed a UV spectrum composed of two peaks, a minor peak at 200 nm and a major peak at 235 nm, the latter being at a slightly higher wavelength than MTX and MTX2 (230 nm) (Holmes and Lewis, 1994). Lewis et al. (1994a) conducted LC-LRMS analyses of material isolated from strain WC1/1 in IS^+ acquisition mode. Their results suggested that MTX3 is di-sulfated with a MW = 1060.5 Da (as di-sodium salt) (**Figure 20**). The actual molecular structure of MTX3 has yet to be determined.

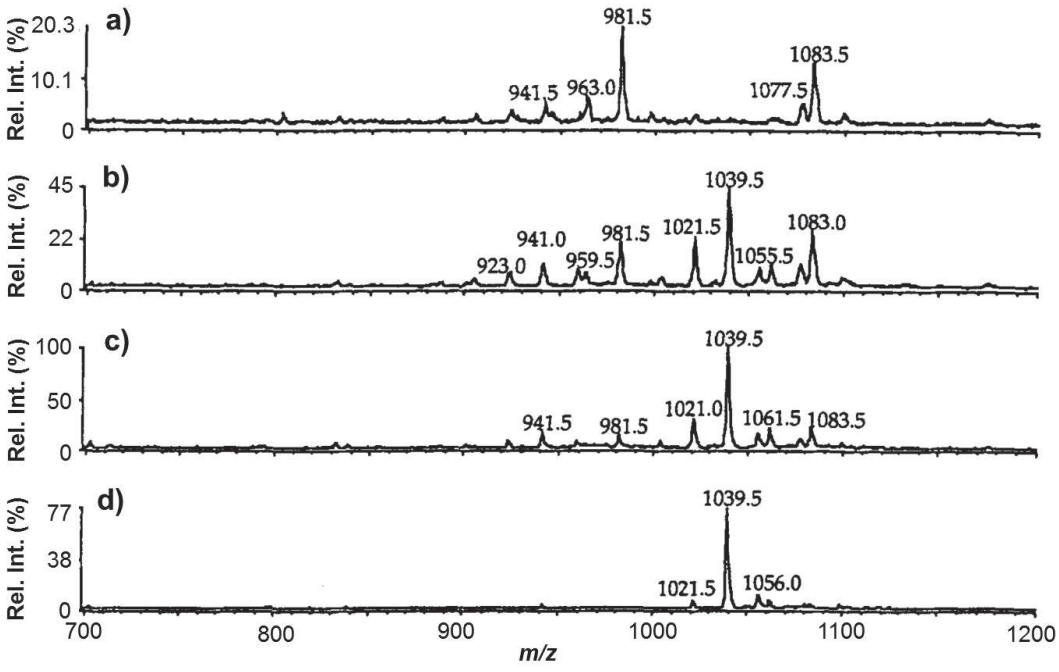

Figure 20. LRMS spectra of MTX3 obtained using ionspray ionization in positive ion acquisition mode (IS^+) according toLewis et al. (1994a). Spectra were acquired with different orifice potentials (OR): **(a)** OR = 160 V; **(b)** OR = 120 V; **(c)** OR = 100 V and **(d)** OR = 60 V. MTX3 yielded only mono-charged cations.

3.3.3. Mechanism of action and biological effects

Initially, MTX was considered a specific activator of voltage-gated calcium channels (Cataldi et al., 1999; Freedman et al., 1984; Takahashi et al., 1982). In actuality, MTX increases $_{i}Ca^{2+}$ by activating a voltage-independent Ca^{2+} entry mechanism in the plasma membrane, without directly promoting the release of Ca^{2+} from intracellular storage compartments (Brereton et al., 2001; Gutierrez et al., 1997; Morales-Tlalpan and Vaca, 2002). So far, MTX has been shown to activate non-selective ion channels (NSCCs), probably involving TRPC1 (transient receptor potential canonical 1) (Brereton et al., 2001; Flores et al., 2017; Martinez-François et al., 2002; Meunier et al., 2009; Morales-Tlalpan and Vaca, 2002). The activation of a sodium-calcium exchanger (NCX) in reverse mode has also been observed in rat aortic smooth muscle cells (Frew et al., 2008). The activation of the sodium-hydrogen exchanger (NHE) equally appears to play a role in MTX cytotoxic activity in cortical neurons (Wang et al., 2009) and it may be a consequence of MTX-induced intracellular acidification, probably involving voltage-gated sodium channels (VGSCs) (Martin et al., 2014). Maitotoxin is also likely to convert the Ca^{2+}-ATPase (PCMA) pump into a Ca^{2+}-permeable non-selective ion channel, as demonstrated in PCMA-overexpressed *Spodoptera frugiperda* (Sf9) insect cells and human embryonic kidney (HEK293) cells (Sinkins et al., 2009). To date, it is unclear whether MTX directly interacts with any of these targets. Several research groups have postulated that MTX may bind a still undescribed MTX-receptor (de la Rosa et al., 2007; Gutierrez et al., 1997; Lundy et al., 2004; Nicolaou and Frederick, 2007). Murata et al. (2008) also hypothesized that a self-assemblage of four or more molecules of MTX could occur to form a pore on cell membranes for non-selective ion influx; however, this hypothesis has not been confirmed. Since the specific molecular target of MTX is still unknown, its structure-activity relationship (SAR) can only be inferred based on its NMR structural features and similarities with other ladder-shaped polyether toxins. Konoki et al. (1999) first hypothesized that the hydrophobic side of MTX (rings R through F', **Figure 17**) penetrates the phospholipid bilayer of cell membranes and the hydrophilic portion of the molecule, presenting the polyhydroxy- groups and the two sulfate ester groups (rings A through Q, **Figure 17**), remains outside the cell. Modeling studies conducted by Reyes et al. (2014) corroborate this hypothesis.

Experiments using purified MTX showed it causes a rapid influx of external Ca^{2+} and a steep increase of intracellular Ca^{2+} ($_{i}Ca^{2+}$) concentration in a wide variety of cells (Gusovsky and Daly, 1990; Reyes et al., 2014). The Ca^{2+} influx elicited by MTX leads to numerous secondary events, including: depolarization in neuronal cells (Ogura et al., 1984), phosphoinositide breakdown (Gusovsky et al., 1990), smooth muscle contraction (Ohizumi et al., 1983a; Ohizumi and Yasumoto, 1983a, 1983b), induction of acrosome reaction in sperm (Amano et al., 1993; Nishiyama et al., 1986; Treviño et al., 2006), secretion of neurotransmitters (e.g. dopamine (Taglialatela et al., 1986), noradrenaline (Ohizumi et al., 1983b; Takahashi et al., 1983)), hormones (e.g. insulin (Leech and Habener, 1997; Soergel et al., 1990)) and inflammatory intermediates (e.g. arachidonic acid (Choi et al., 1990) and histamine (Columbo et al., 1992)), formation or activation of large cytolytic/oncotic pores (COPs) (Estacion and Schilling, 2001; Schilling et al., 1999a; Schilling et al., 1999b). The methylated fragment of MTX from ring Q through A' showed a significant growth inhibition against several cancer cells (Nicolaou et al., 2014). MTX2 and MTX3 bioactivities have not been characterized yet. (Holmes et al., 1991) reported that MTX2 and MTX3 elicited symptoms in mice (i.p. injection) similar to what previously reported for MTX, and hypothesized a similar mode of action of the three MTX congeners.

3.4. **Other cyclic polyether compounds**

3.4.1. Gambieric acids

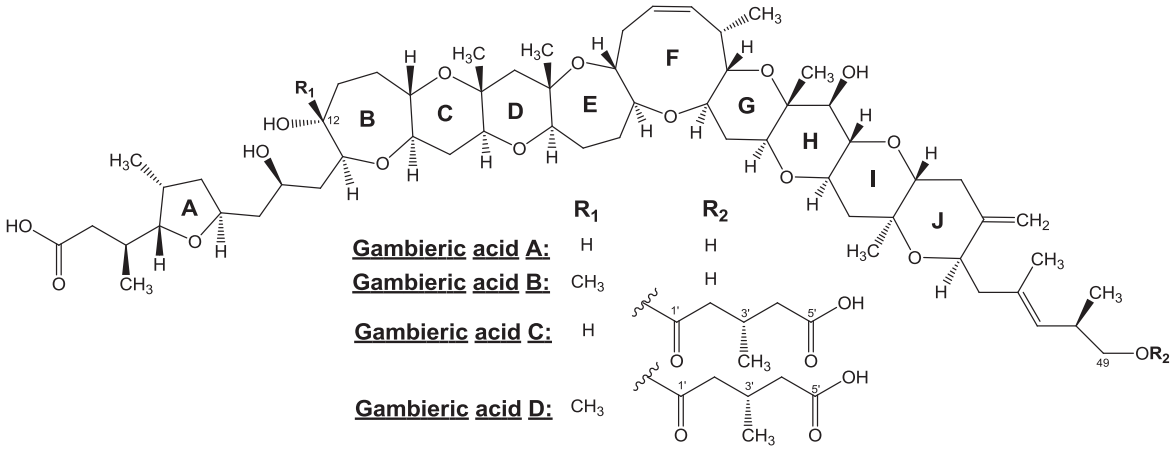

Figure 21. Molecular structure of gambieric acids according to Nagai et al. (1992a) and Nagai et al. (1992b).

Gambieric acids (GAs) were isolated from filtered medium of cultured cells of *Gambierdiscus* sp. GII-1 (Gambier Islands, French Polynesia) by Nagai et al. (1992a) and Nagai et al. (1992b). Four congeners of GAs have been described to date: gambieric acid A (GA A, $C_{59}H_{92}O_{16}$, M = 1056.6385 Da), gambieric acid B (GA B, $C_{60}H_{94}O_{16}$, M = 1070.6542 Da), gambieric acid C (GA C, $C_{65}H_{100}O_{19}$, M = 1184.6859 Da) and gambieric acid D (GA D, $C_{66}H_{102}O_{19}$, M = 1198.7015 Da).

Gambieric acid A has a ladder-shaped polyether structure consisting of nine contiguous ether rings (rings B-J), and one isolated tetrahydrofuran (ring A) (**Figure 21**). Gambieric acid B differs from GA A only for the presence of a methyl group at C_{12}. Gambieric acid C and GA D are 3-methylhemiglutarates derivatives of the primary hydroxyl group at C_{49} of GA A and GA B, respectively (**Figure 21**).

Gambieric acids have highly potent antifungal activity: potency of GA A, GA B and a mixture of GA C and D exceeded that of amphotericin B by a factor 2000, as demonstrated against *Aspergillus niger* (paper disk method) in a study conducted by Nagai et al. (1992b). Antifungal activity was observed against filamentous fungi but not against yeasts, and was potentiated in the presence of ferric compounds such as $FeCl_3$ or $Fe_2(SO_4)_3$ (Nagai et al., 1993). Gambieric acid A and GA B showed no *in vivo* toxicity in mice at a dose of 1 mg kg^{-1} (i.p. injection), while a mixture of GA C and

GA D was moderately cytotoxic (IC_{50} = 1.1 µg mL^{-1}) to mouse lymphoma cells L5178Y (Nagai et al., 1992a).

Mechanism of action of GAs has not been described yet. Inoue et al. (2003) showed that GA A inhibits the binding of radiolabeled brevetoxin-3 ([^{3}H]BTX3) to the site 5 of VGSCs in synaptosomes isolated from rat brain.

Gambieric acid A also seems to serve as an endogenous enhancer of *Gambierdiscus* growth, as showed in laboratory cultures of a strain of *Gambierdiscus* sp. isolated from Waianae Boat Harbor, Oahu (Hawaii) (Sakamoto et al., 1996). Recent evidence demonstrated production of GA A by the genus *Fukuyoa*, i.e. *F. paulensis* Dn135EHU (Formentera, Balearic Islands, Western Mediterranean Sea) (Laza-Martinez et al., 2016). The presence of GA D in the flesh of a ciguatoxic bull shark (*Carcharhinus leucas*) caught in Madagascar (Indian Ocean) was also demonstrated using high resolution mass spectrometry (HRMS) analysis (Diogène et al., 2017). This finding represented the first evidence of the trophic transfer of GAs into the marine food chain.

3.4.2. Gambierol

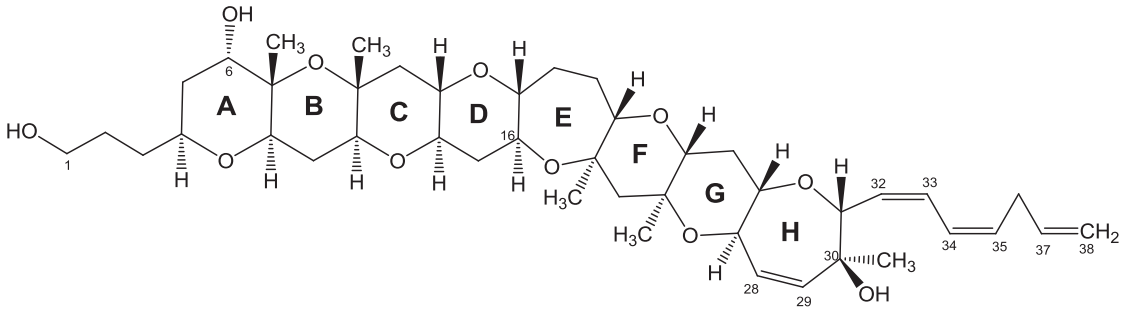

Figure 22. Molecular structure of gambierol (Satake et al., 1993a) with complete stereochemistry (Morohashi et al., 1999).

Gambierol ($C_{43}H_{64}O_{11}$, M = 756.4449 Da, **Figure 22**) was isolated from cultured cells of *Gambierdiscus* sp. RGI-1 (Rangiroa Atoll, Tuamotu Archipelago, French Polynesia) (Satake et al., 1993a). Gambierol is a ladder-shaped polyether toxin, consisting of eight transfused ether rings (rings A-H) containing 18 stereogenic centers (**Figure 22**). The toxin was structurally characterized using HR-FABMS in positive mode and one-dimensional (1D) and two-dimensional (2D) NMR techniques (Satake et al., 1993a). The complete stereochemistry was further assigned using a modified Mosher analysis (Morohashi et al., 1999) (**Figure 22**).

Gambierol is characterized by a single UV absorbance maximum in methanol at a wavelength of 237 nm, due to the conjugated diene at C_{32}-C_{35}. Total synthesis of gambierol has been described using different synthetic approaches (Furuta et al., 2010; Furuta et al., 2009; Fuwa et al., 2002b; Johnson et al., 2006; Kadota et al., 2003b). The availability of synthetic material permitted to deepen the knowledge on gambierol mechanism of action and biological activity.

Toxicity of gambierol in mice has been assessed as follows: i.p. LD_{50} of 50-80 μg kg^{-1} (Ito et al., 2003; Satake et al., 1993a), i.v. LD_{50} of 80 μg kg^{-1} and p.o. LD_{50} of 150 μg kg^{-1} (Ito et al., 2003), thus indicating efficient absorption from the intestine to the blood stream. Symptoms caused in mice were reported to be similar to those elicited by CTXs, suggesting the involvement of gambierol in ciguatera poisoning (Ito et al., 2003; Satake et al., 1993a). Histopathological studies in mouse tissues indicated that gambierol affects several organs, in particular lungs and heart, causing systemic congestion (poor blood circulation) (Ito et al., 2003).

Biological activity of gambierol is essentially caused by its inhibitory action on voltage-gated potassium channels (VGPCs). Binding site of gambierol is constituted by a lipid-exposed surface of the pore domain of VGPCs, located at T427 in the Segment 6 (Kopljar et al., 2009). Kopljar et al. (2013) demostrated that gambierol affects the gating mechanism of $K_v3.1$ channels stabilizing their resting state. Gambierol was shown to inhibit K^+ currents at nanomolar concentrations in different cell models, such as mouse taste cells (Ghiaroni et al., 2005), rat embryonic chromaffin cells, vertebrate motor nerve terminals (Schlumberger et al., 2007) and skeletal muscle cells (Schlumberger et al., 2010). Taste alterations experienced by ciguatera patients may be a consequence of the gambierol-elicited alteration of action potential discharge in taste cells (Meunier et al., 2009). A secondary effect of gambierol is the release of neurotransmitters. Indeed, gambierol indirectly stimulates acetylcholine (ACh) release from motor nerve terminals via K^+ currents inhibition, as observed in frog and mouse neuromuscular preparations (Schlumberger et al., 2007).

Some studies suggest that gambierol, at micromolar concentrations, also acts on voltage-gated sodium channels (VGSCs) (Inoue et al., 2003; LePage et al., 2007; Louzao et al., 2006). Inoue et al. (2003) and LePage et al. (2007) showed that gambierol inhibits the binding of radiolabeled

brevetoxin-3 ([^{3}H]BTX3) to the site 5 of VGSCs in rat brain synaptosomes. Also, gambierol was shown to act as a partial agonist of VGSCs in human neuroblastoma cells (Louzao et al., 2006).

Total syntheses of several gambierol congeners have also been performed and permitted to have insights on the structure-activity relationship (SAR) of gambierol-like polyethers (Alonso et al., 2012; Alonso et al., 2017; Fuwa et al., 2003; Fuwa et al., 2002a; Fuwa et al., 2004; Kadota et al., 2003a). Perez et al. (2012) identified that the biological activity of gambierol resides in its right-half moiety (rings E-H and the unsaturated triene side chain, **Figure 22**). In particular, the stereochemistry of C_{16} (Kadota et al., 2003a), the double bond at C_{28}-C_{29} within the H ring and the unsaturated triene side chain seem to be crucial for the bioactivity (Fuwa et al., 2003; Fuwa et al., 2004). The hydroxy groups at C_1 and C_6, the methyl group at C_{30} and the double bond at the extremity of the molecule (C_{37}-C_{38}, **Figure 22**) have been shown to contribute to neurotoxicity against mice (Fuwa et al., 2004). Potential therapeutical application of gambierol and its truncated tetra- and hepta-cyclic congeners into Alzheimer's disease have also been highlighted (Alonso et al., 2012; Alonso et al., 2017).

3.4.3. Gambieroxide

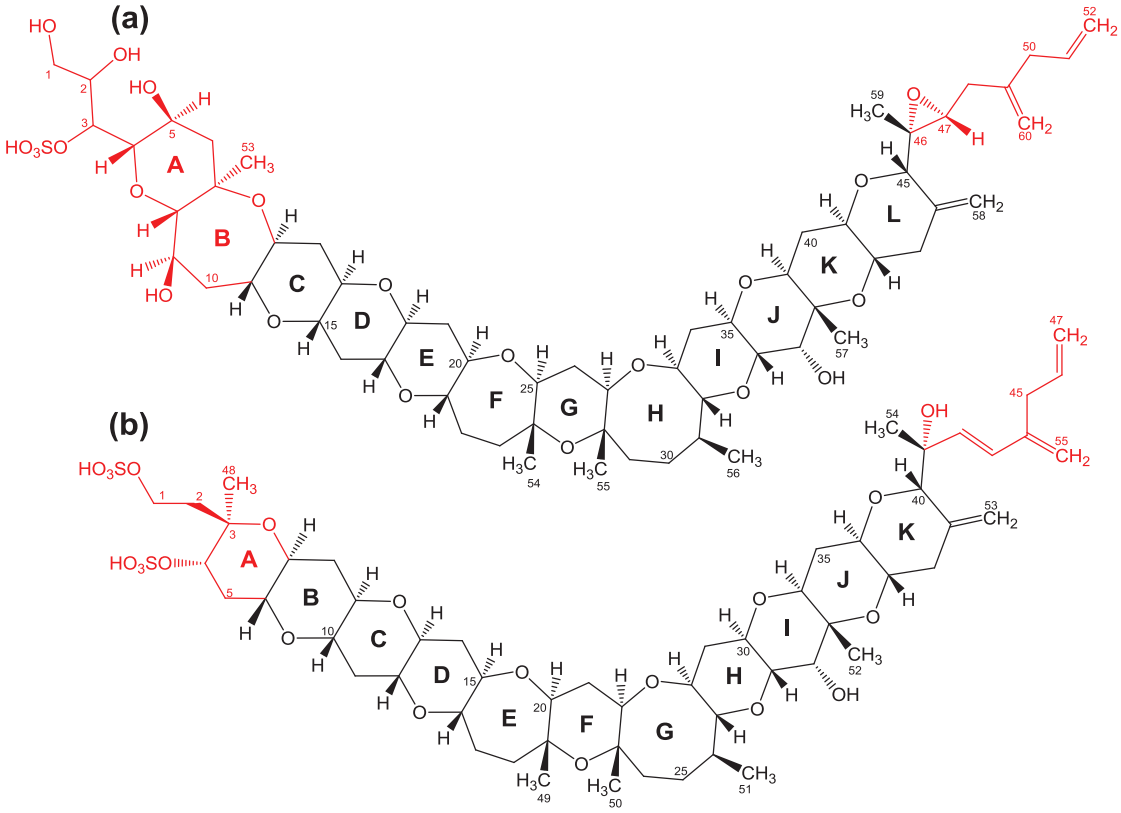

Figure 23. Molecular structures of **(a)** gambieroxide (Watanabe et al., 2013), from the dinoflagellate *Gambierdiscus* and **(b)** yessotoxin (free acid form) (Murata et al., 1987; Takahashi et al., 1996) from dinoflagellates of the genera *Protoceratium* and *Lingulodinium*. Black color indicates the structural features in common between the two molecules. Red color indicates the structural features characteristic to each of the two molecules. Nota bene: stereochemistry of (a) has not been resolved at C_2 and C_3.

Gambieroxide ($C_{60}H_{90}O_{22}S$, M = 1194.5644 Da, **Figure 23a**) was isolated from cultured cells of *Gambierdiscus* sp. GTP2 (Papeete, Tahiti, French Polynesia) (Watanabe et al., 2013). Molecular structure was elucidated and stereochemistry assigned (except for C_2 and C_3) using (i) a 800 MHz NMR spectrometer, both in one-dimensional (^{1}H NMR) and two-dimensional NMR techniques and (ii) HR-ESI-TOFMS/MS analysis, both in positive and negative ionization modes (Watanabe et al., 2013). Gambieroxide is a ladder-shaped polyether compound consisting of a skeleton composed of 12 transfused ether rings (rings A-L) and two side chains at the extremities (**Figure 23a**). Characteristic structural features are described as follows: the left side chain contains a sulfate ester group at C_3; the right side chain contains an epoxide at C_{46}-C_{47} (**Figure 23a**). The chemical structure of gambieroxide (Watanabe et al., 2013) is strikingly similar to that of yessotoxin (YTX) (Murata et al., 1987; Takahashi et al.,

1996) (**Figure 23b**), a toxin produced by the dinoflagellates *Protoceratium reticulatum* (Loader et al., 2007; Samdal et al., 2004; Satake et al., 1997b) and *Lingulodinium polyedrum* (Paz et al., 2004). Gambieroxide does not absorb into the UV-VIS wavelength range, consistently with the absence of conjugated unsaturations. Biological activity of gambieroxide has yet to be evaluated.

3.4.4. Gambierone

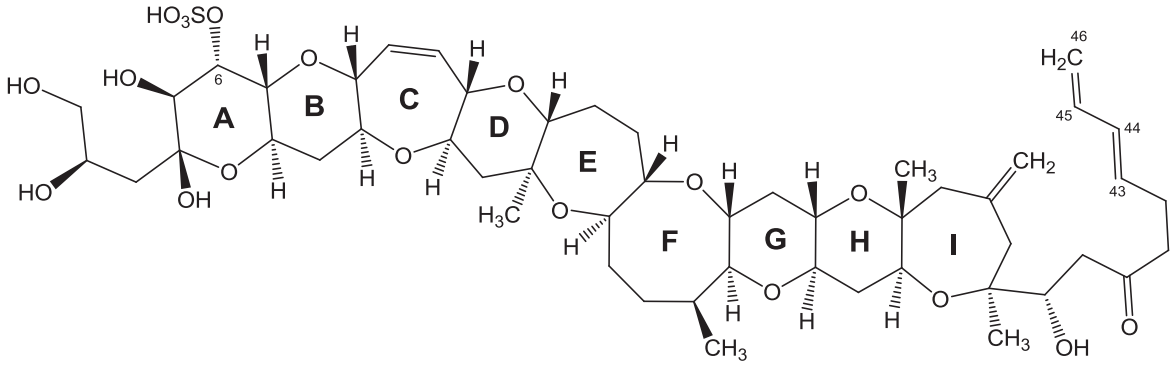

Figure 24. Molecular structure of gambierone and stereochemistry assignment according to modeling studies (Rodríguez et al., 2015).

Gambierone ($C_{51}H_{76}O_{19}S$, M = 1024.4702 Da, **Figure 24**) was isolated from cultured cells of *G. belizeanus* CCMP401 (Barthelemy Island, Caribbean Sea, North Atlantic Ocean) (Rodríguez et al., 2015). Molecular structure was elucidated using both HRMS and 750 MHz 2D NMR techniques. Gambierone is a ladder-shaped polyether toxin constituted of nine transfused rings (rings A-I) and presenting the following characteristic features: (i) a sulfate ester group on ring A at C_6 and (ii) a conjugated vinyl end (C_{43}-C_{46}) (**Figure 24**). The absolute configuration has not completely been resolved yet. **Figure 24** represents the most probable absolute configuration (91.2% confidence) based on modeling studies conducted by Rodríguez et al. (2015).

Biological activity of gambierone was evaluated using (i) electrophysiological measurements of Na^+ currents in HEK-293 cells expressing hNa_v 1.3 and 1.6 and (ii) measurement of cytosolic calcium (Ca^{2+}) levels in neuroblastoma SH-SY5Y cells via Fura-2-AM fluorescence. Results demonstrated that gambierone activates voltage-gated sodium channels in a similar way as CTXs, although with much less potency, and that, unlike MTXs, the increase in intracellular Ca^{2+} is a consequence of Na^+ influx (Rodríguez et al., 2015).

3.5. **Extraction and purification procedures**

The procedures for isolation of cyclic polyether compounds from *Gambierdiscus*/*Fukuyoa* described in literature included several clean-up steps, depending on the compound considered (**Table 7**).

Except for gambieric acids, which were isolated from filtered culture medium (Nagai et al., 1993; Nagai et al., 1992a; Nagai et al., 1992b), the first step generally consisted of repeated extractions of the cellular content using absolute methanol, aqueous methanol mixtures or acetone.

The resulting crude extract underwent several purification steps, which may include: liquid-liquid partitioning, solid phase extraction (SPE), size-exclusion, reversed-phase and normal-phase chromatographies. Except for CTX4B (Murata et al., 1990), the first clean-up step generally consisted of one (sometimes two) liquid-liquid partitioning, mostly using dichloromethane (CH_2Cl_2) *versus* aqueous (aq.) methanol mixtures. This step is assumed to separate the two main families of toxins produced by *Gambierdiscus* and *Fukuyoa*: ciguatoxins (CTXs), which are lipophilic compounds, and maitotoxins (MTXs), which are amphiphilic compounds. Liquid-liquid partitioning between CH_2Cl_2 and aq. methanol is generally used when evaluating the toxicity specific to each of the two families, even if carryover should not be neglected (Lewis et al., 2016). Subsequent clean-up steps and column characteristics (e.g. brand, length, diameter, particle size or porosity) were often poorly detailed.

Due to the lack of information on purification factors and recovery yields, it is difficult to discuss the relevance of each of the clean-up steps adopted. From a general point of view, procedures that involve the smallest number of steps with the maximum degree of orthogonality (i.e. different principles of separation) could be considered more relevant and should be preferred, e.g. the procedure used for isolation of CTX3C (Satake et al., 1993b).

Table 7. Procedures of extraction and purification of cyclic polyether compounds from the dinoflagellate *Gambierdiscus*. The fractions that contained the compound are highlighted in bold. (?) = unknown; n = number of steps.

Compound	*Gambierdiscus* species / strain	Extraction	Liquid-liquid partitioning		Chromatography			n	Ref.
			Apolar	Polar	Type	Stationary phases	Mobile phases		
CTX4B	(?) / (?)	Me_2CO	/	/	Mg silicate	Florisil (26*300 mm)	1) C_6H_{14}:Me_2CO (4:1) 2) **Me_2CO:MeOH (9:1)** 3) Me_2CO:MeOH (1:1)	7	[1]
					RP-LC	Develosil Iop ODS	1) MeOH:H_2O (7:3) 2) **MeOH:H_2O (9:1)** 3) **MeOH:H_2O (1:0)**		
					SEC	Toyopearl HW-40	MeOH		
					SEC	Toyopearl HW-40	MeOH:H_2O (17:3)		
					RP-HPLC	Develosil ODS-7	Linear gradient MeOH:H_2O (17:3 → 1:0)		
					RP-Polymer base	Asahipak ODP-50	MeCN:H_2O (17:3)		
CTX3C[(a)]	(?) / RGI-1	MeOH	**CH_2Cl_2**	MeOH:H_2O (3:2)	Mg silicate	Florisil	Me_2CO:MeOH (9:1)	5	[2]
					SEC	Toyopearl HW-40	MeOH		
					RP-LC	Asahipak ODP-50	MeOH:H_2O (75:25)		
CTX4A	(?) / RGI-1	MeOH	**CH_2Cl_2**	MeOH:H_2O (3:2)	Mg silicate	Florisil	1) C_6H_{14}:Me_2CO (4:1) 2) **Me_2CO:MeOH (9:1)**	7	[3]
					SEC	Toyopearl HW-40	MeOH:H_2O (17:3)		
					RP-LC	Asahipak ODP-50 (10*250 mm) (x2)	Linear gradient MeCN:H_2O (3:1 → 1:0)		
					RP-LC	Capcell Pak C_8 (4.6*150 mm)	MeOH:H_2O (13:3)		
					RP-LC	Asahipak ODP-50 (4.6*150 mm)	MeOH:H_2O (17:3)		

Compound	*Gambierdiscus* species / strain	Extraction	Liquid-liquid partitioning		Chromatography			n	Ref.
			Apolar	Polar	Type	Stationary phases	Mobile phases		
MTX	(?) / (?)	Boiling MeOH	1) **Et_2O**	H_2O	NP-LC	Silicic acid	$CHCl_3$:MeOH, stepwise	6	[4]
					RP-LC	Develosil ODS	MeOH:H_2O, stepwise		
			2) **1-BuOH**	H_2O	RP-LC	Develosil TMS	MeCN:H_2O (7:13)		
MTX[b]	(?) / GII-1	MeOH (2x) MeOH:H_2O (1:1) (2x) under reflux	1) CH_2Cl_2	**MeOH:H_2O (4:1)** (3x)	NP-LC	SiO_2 gel (32*120 mm)	1) $CHCl_3$:MeOH (7:3) (0.3 L) 2) **$CHCl_3$:MeOH (1:1)** (0.3 L)	8	[5, 6]
					RP-LC	C_{18} ODS Fuji Gel Q3 (30-50 µm, 20*200 mm)	1) MeOH:H_2O (1:1) (0.2 L) 2) **MeOH:H_2O (7:3)** (0.2 L) 3) MeOH:H_2O (1:0) (0.2 L)		
			2) **1-BuOH**	H_2O	RP-LC	Develosil C_8 15/30 (10*480 mm)	1) MeCN:H_2O (1:3) (0.1 L) 2) MeCN:H_2O (3:7) (80 mL) 3) **MeCN:H_2O (4:6)** (0.1 L) 4) MeCN:H_2O (1:1) (80 mL)		
					RP-LC	Develosil C_8 15/30 (10*500 mm)	MeCN:H_2O (1:2) (*RV* = 36-72 mL)		
					RP-LC	Develosil TMS-5 (10*250 mm)	MeCN:H_2O (7:13) (*RV* = 35 mL)		
Gambieric acids[c]	(?) / GII-1	Polystyrene resin Amberlite XAD-2 (80*400 mm) H_2O (10 L) **MeOH** (5 L)	1) **Et_2O**	H_2O	SEC	Toyopearl HW-40 (25*300 mm)	MeOH:H_2O (1:1)	8	[7-9]
			2) **1-BuOH**	H_2O	RP-LC	Develosil ODS 15/30 (10*40 mm)	1) MeOH:H_2O (1:1) 2) MeOH:H_2O (7:3) 3) **MeOH:H_2O (1:0)**		
					RP-LC	Develosil ODS-7 (10*250 mm)	MeCN:H_2O (9:1)		
					RP-LC	Develosil ODS-5 (8*250 mm)	MeCN:H_2O (9:1)		
					NP-LC	Develosil 60-5 (8*250 mm)	$CHCl_3$:MeOH:H_2O (200:10:1)		
Gambierol[d]	(?) / RGI-1	MeOH	**CH_2Cl_2**	MeOH:H_2O (3:2)	Bioguided fractionation using MBA, no further information provided			?	[10]
Gambieroxide[e]	(?) / GTP2	MeOH (3x)	1) CH_2Cl_2	**MeOH:H_2O (2:3)**	NP-LC	Al_2O_3 column (250*10 mm)	1) $CHCl_3$:MeOH (1:1) 2) MeOH 3) **1% NH_4OH aq.:MeOH (1:1)**	7	[11]
			2) **1-BuOH**	H_2O	RP-LC	ODS Q3 (Fuji gel) (250*10 mm)	MeOH:H_2O (4:1)		
					RP-LC	Develosil C_8 (250*10 mm)	MeCN:H_2O (2:3)		
					RP-LC	Capcell pak C_{18} (250*10 mm)	MeCN:H_2O (55:45) containing 5 mmol L^{-1} $AcONH_4$		

Compound	*Gambierdiscus* species / strain	Extraction	Liquid-liquid partitioning		Chromatography			n	Ref.
			Apolar	Polar	Type	Stationary phases	Mobile phases		
Gambierone[f]	*G. belizeanus* / CCMP401	MeOH (3 x 0.8 L)	1) **Et_2O** (0.3 L)	H_2O (0.3 L)	Mg silicate	Florisil	1) EtOAc:MeOH (9:1) 2) EtOAc:MeOH (8:2) 3) EtOAc:MeOH (1:1) 4) EtOAc:MeOH (0:1)	9	[12]
			2) C_6H_{14} (0.4 L)	**MeOH:H_2O (9:1)** (0.2 L)	RP-SPE	C_{18}	1) MeOH:H_2O (1:1) 2) **MeOH:H_2O (3:2)** 3) **MeOH:H_2O (7:3)** 4) MeOH:H_2O (4:1) 5) MeOH:H_2O (9:1) 6) MeOH:H_2O (1:0)		
					NP-SPE	SiO_2	1) $CHCl_3$:MeOH (9:1) (0.3 L) 2) $CHCl_3$:MeOH (8:2) (0.3 L) 3) **$CHCl_3$:MeOH (7:3)** (0.3 L) 4) $CHCl_3$:MeOH (1:1) (0.3 L)		
					RP-SPE	C_{18} cartridge	1) **MeOH:H_2O (1:1)** (0.2 L) 2) **MeOH:H_2O (51:49)** (0.14 L) 3) MeOH:H_2O (55:45) (0.14 L)		
					RP-SPE	C_8 cartridge	1) MeCN:H_2O (1:4) (40 mL) 2) **MeCN:H_2O (1:3)** (40 mL) 3) MeCN:H_2O (3:7) (20 mL) 4) MeCN:H_2O (4:6) (20 mL) 5) MeCN:H_2O (1:1) (20 mL)		
					Preparative RP-HPLC	X-Select Phenyl-Hexyl (10*250 mm, 5 µm)	Isocratic elution MeOH:H_2O (3:2) (*RT*: 25 min)		

SPE: solid phase extraction, SEC: size-exclusion chromatography, RP: reversed-phase, NP: normal-phase, HP: high performance, LC: liquid Chromatography, C_6H_{14}: hexane, Me_2CO: acetone, MeOH: methanol, H_2O: water, MeCN: acetonitrile, 1-BuOH: 1-butanol, Et_2O: diethyl ether, EtOAc: ethyl acetate, CH_2Cl_2: dichloromethane, $CHCl_3$: chloroform, Al_2O_3: alumina, $AcONH_4$: ammonium acetate, SiO_2: silica, NH_4OH: ammonium hydroxide, aq.: aqueous, *RV*: retention volume, *RT*: retention time.

[a] 0.7 mg from 1100 L of *Gambierdiscus* culture (cell density not provided); [b] 20 mg from 4 x 10^9 *Gambierdiscus* cells; [c] GA A (0.6 mg), GA B (0.15 mg), GA C and GA D (5.8 mg) from 5000 L of filtered culture medium; [d] 1.2 mg from 1100 L of *Gambierdiscus* culture (cell density not provided); [e] 0.4 mg from 3020 L of *Gambierdiscus* culture (cell density not provided); [f] 2.24 mg from 19 g (wet weight) of cell pellet, i.e. 9200 L of *Gambierdiscus* culture (cell density not provided).

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4. Methodologies for toxin determination

4.1. General introduction

The European Union legislation does not define regulatory limits for ciguatoxins in fish, but the regulation requires that no fish products containing CTX-group toxins are placed on the market (Commission Regulation No. 854/2004). Some studies estimated that levels above 0.01 ng CTX1B eq g^{-1} fish flesh or 0.1 ng C-CTX1 eq g^{-1} fish flesh may represent a threat for consumer health (Dickey, 2008; Lehane, 2000; Lehane and Lewis, 2000; Vernoux and Lewis, 1997) and have been proposed as safety thresholds (10x safety factor) by the U.S. Food and Drug Administration (FDA) (FDA, 2011) and the European Food Safety Authority (EFSA) (EFSA, 2010). Maitotoxins are not considered to be involved in CFP and no safety guidance exists to date. Nevertheless, MTX is an extremely potent toxin, with an i.p. LD_{50} in mice of 0.050 µg kg^{-1} (Murata et al., 1993), it can be found in the digestive tract of fish (Kohli et al., 2014b; Yasumoto et al., 1976; Yasumoto et al., 1971) and may potentially be found in the flesh as well (Kohli et al., 2014b). Still, MTXs can be considered a potential threat for consumer health, especially when guts and livers of fish are consumed.

Unlike other microalgal toxins implicated in food poisonings, no standardized method exists to date for the content determination of ciguatera toxins. The development of sensitive and specific methodologies for CTX and MTX content determination is required for routine monitoring of food. Determination of ciguatera toxins' content in fish or phytoplankton samples may help identification of ciguatera hotspots and improve geographical risk assessment. In addition, the monitoring of foodstuffs other than fish may also have importance for the identification of new vectors of ciguatera toxins in the marine food chain. The confirmation of the presence of ciguatera toxins in the blood of patients may also help the clinical diagnosis of CFP (Dechraoui Bottein et al., 2007), enabling differential diagnoses from other forms of ichtyosarchotoxism. Finally, determination of CTX and MTX profiles in monoclonal cultures of *Gambierdiscus*/*Fukuyoa* is necessary to associate toxin production with a particular *Gambierdiscus* or *Fukuyoa* species/strain and may help prediction of ciguatera outbreaks. In this section, a brief review of the

different methodologies for CTX and MTX detection and quantification is presented, including biological (*in vivo* or *in vitro*) assays and physico-chemical techniques.

4.2. **Assay technologies**

4.2.1. In vivo bioassays

Bioassays in animals have been traditionally used in ciguatera endemic regions to monitor suspected toxic fish samples, flesh or viscera. Examples of *in vivo* models involve cats (Bagnis and Fevai, 1971), mongooses (Banner and Boroughs, 1958), chickens (Kosaki and Anderson, 1968), mosquitos (Chungue et al., 1984), larvae of *Diptera* (Labrousse and Matile, 1996), crayfish and other crustaceans (Keene et al., 1968). These assays are based on different routes of exposure, which may include oral administration or parenteral injection.

Mouse bioassay (MBA). The most widely used *in vivo* bioassay for CTX and MTX determination is the mouse bioassay (MBA) consisting of an intraperitoneal (i.p.) administration of fish or algal extracts to mice (Banner et al., 1960; Hoffman et al., 1983; Lewis et al., 1993). Qualitative observations of symptoms, behavior, body temperature and survival time are used to recognize the presence of the toxins in a given sample. Quantification of the toxin content is based on the i.p. median lethal dose (LD_{50}), which is the dose necessary to kill half of a 20 g mice population, 24 h after i.p. injection, and it is generally expressed in mouse units (MU) (Endean et al., 1993). When evaluating CTX- and MTX- toxicities of a given strain of *Gambierdiscus*/*Fukuyoa*, CTXs and MTXs need to be separated first, i.e. crude extracts undergo liquid-liquid partitioning between dichloromethane (CH_2Cl_2) and an aqueous methanol mixture (MeOH:H_2O, 3:2, v/v). Relative toxicities between CTX congeners published by the European Food and Safety Agency (EFSA) are based on their respective LD_{50} in MBA, i.e. one MU is equivalent to 5 ng, 18 ng and 48 ng for the Pacific CTX1B, 52-*Epi*-54-DeoxyCTX (CTX2) and 54-DeoxyCTX (CTX3), respectively, and 72 ng for Caribbean C-CTX1 (EFSA, 2010).

The disadvantages of the MBA can be summarized as follows: (i) lack of specificity to distinguish different groups of toxins; (ii) requirement of qualified personnel and animal housing; (iii) unsuitability for individual CTX detection as market test; (iv) relatively high time and sample consumption

for analysis; (v) insufficient sensitivity for low-toxic fish or false positives if lipids are present; (vi) it is ethically questionable (Caillaud et al., 2010a; Nicholson and Lewis, 2006). Nonetheless, MBA is still a toxicological tool that is accessible to many laboratories and capable of some clinical recognition (Caillaud et al., 2010a).

4.2.2. In vitro cell-based assays (CBAs)

In vitro cell-based assays permit to study the effects of a toxin on a particular type of cell or tissue, e.g. histological damages, alteration of metabolic rates, cell growth, activation or inhibition of the transcription of genes that control basic functions. In addition, CBAs are interesting tools to explore the mechanism of action of a toxin when cells presenting specific molecular targets (e.g. receptors) are used.

Erythrocyte lysis assay (ELA). The erythrocyte lysis assay (ELA) was developed by Eschbach et al. (2001) and is based on lysis of erythrocytes due to hemolytic compounds and subsequent photometrical determination of the released hemoglobin at 414 nm. In the context of marine dinoflagellates, the ELA has broadly been used on different red blood cell lines to detect hemolytic compounds from different microalgae such as *Alexandrium* (Emura et al., 2004; Ma et al., 2011; Tatters et al., 2012), *Karenia* (Tatters et al., 2010), *Ostreopsis* (Nascimento et al., 2012) and *Gambierdiscus* (Holland et al., 2013). The ELA was initially developed to detect sodium-channel activator toxins, i.e. ciguatoxins (Shimojo and Iwaoka, 2000). Indeed, studies by Sauviat et al. (2006) showed that CTX1B and C-CTX1 are capable of deforming frog erythrocytes, inducing swelling via the NO pathway. Subsequently, Holland et al. (2013) used the ELA with human red blood cells to compare toxicity of crude extracts (in ELA buffer) of 56 isolates of different species/phylotypes of *Gambierdiscus*/*Fukuyoa*. It should be noted that hemolysis could not be univocally associated with a specific group of toxins. Indeed, lysis of erythrocytes may be due to the presence of reactive oxygen species (Chiu et al., 1982; Damude and Kinney, 2008; Marshall et al., 2003), poly-unsaturated fatty acids (de Boer et al., 2009; Yasumoto et al., 1990) or glycolipids (Emura et al., 2004; Law et al., 1980; Ma et al., 2011; Pagliara and Caroppo, 2011). Inhibition and heat treatment studies conducted by Holland et al. (2013) demonstrated that in the case of *Gambierdiscus*/*Fukuyoa* extracts, the primary cause of hemolysis is due to the presence of MTXs. Also, when crude extracts

underwent liquid-liquid partitioning, the CTX-containing dichloromethane soluble fraction accounted for less than 1% of the total hemolytic activity. Therefore, the erythrocyte lysis assay could be classified as a method for MTX determination, at least in the case of *Gambierdiscus*/*Fukuyoa* extracts, especially when sample preparation includes liquid-liquid partitioning.

Neuroblastoma cell assays. To date, the most sensitive CBA for CTX determination is the mouse neuroblastoma neuro-2a (N2a) cytotoxicity assay. The assay uses the N2a *Mus musculus* neuroblastoma cell line, it permits detection of CTXs at sub-picogram levels and can be run as high-throughput screening (HTS) assay (96-well microplates), allowing determination of several samples at a time. The N2a assay was initially developed by Manger et al. (1993) and underwent slight procedural modifications through time according to the study considered (Dickey et al., 1999; Diogène et al., 2017; Hardison et al., 2016). To date, there is no standardized procedure for maintaining N2a cells (e.g. different culture media) neither for conducting the assay (e.g. different concentrations of ouabain and veratridine), but all protocols share the same basic principles. As CTXs do not induce N2a cell death by themselves at relevant concentrations, N2a cells have to be pretreated with ouabain (O), an inhibitor of the Na^+/K^+-ATPase pump, and veratridine (V), which holds Na^+ channels in an open position. The combined action of O and V makes N2a cells highly sensitive to VGSC-activator toxins. Quantitation of CTX content is based on the quantitative colorimetric 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) assay (Mosmann, 1983). This assay assesses the mitochondrial activity of N2a cells (an indicator of N2a cell survival) after 24h exposure to toxin (Manger et al., 1993). Blue formazan is a metabolite of MTT whose absorbance can easily be detected and quantified with a plate reader set at 490, 544 or 565 nm, according to the protocol used. If MTX interference is suspected (i.e. if MTXs have not been previously separated from CTXs using liquid-liquid partitioning), an inhibitor of MTX activity such as SK&F 96365 may be used to distinguish CTX-activity from MTX-activity, allowing quantification of both groups of toxins (Caillaud et al., 2010b). The establishment of a standardized protocol for conducting the assay may lead the N2a assay to be validated as an official method for CTX detection and quantification (Darius et al., 2017).

The main advantages of the N2a cytotoxicity assay are the high sensitivity to CTXs and the suitability for routine screening purposes due to the HTS format (Caillaud et al., 2010a). Nevertheless, the N2a assay also presents some disadvantages. As is true for any toxicological test, the assay is not able to discriminate amongst different neurotoxins acting on the same biological target, e.g. different CTX (or even BTX) congeners. Thus, chemical methods are required to confirm the identity of the toxic compounds. Moreover, N2a cells have to be maintained in culture, the assay is time-consuming (about 3 days) and the lack of a universally standardized protocol prevents the comparison between different studies. A tentative attempt to make the N2a assay a ready-to-ship kit was made in 1999 for the determination of VGSC-blocking toxins (Jellett et al., 1999). The kit consisted of a modification of the N2a assay with shippable plates and reagents to be used by unspecialized laboratories. Unfortunately, the test proved to be unsuccessful, probably due to a lack of stability during shipment or unreliable laboratory conditions.

The use of “mouse neuroblastoma x rat glioma” hybrid cells (NG108-15) could represent a suitable alternative to N2a cells as demonstrated for other groups of marine toxins (Cañete et al., 2010; Cañete and Diogène, 2008).

The SH-SY5Y cell-based FLIPR$^{TETRA®}$ assay is another functional bioassay based on neuroblastoma cell model (Lewis et al., 2016). SH-SY5Y cells are human neuroblastoma cells that express several VGSC subtypes. CTXs can be detected and quantified measuring the enhancement of veratridine-induced Ca^{2+}-influx (Vetter et al., 2012a). Intracellular Ca^{2+} concentration is monitored using a fluorescent dye (Calcium-4 No-Wash). The SH-SY5Y assay showed similar sensitivity as the N2a cytotoxicity assay (~ 1 pg CTX1B eq per well) with the following advantages: (i) it requires fewer reagents for pre-treatment (ouabain is not necessary) and (ii) it provides a direct measurement of MTX activity as well. The assay was successfully used by Lewis et al. (2016) in bioguided fractionation experiments of 12 strains of *Gambierdiscus*/*Fukuyoa* (one Caribbean *F. ruetzleri*, one Pacific *G. australes*, one Caribbean *G. belizeanus*, one Pacific and one Caribbean *G. caribaeus*, one Atlantic, one Caribbean and one Pacific *G. carolinianus*, one Caribbean and one Pacific *G. carpenteri*, two Caribbean *Gambierdiscus* sp. ribotype 2). The authors found that only five strains out of the 12, all from the Caribbean Sea

(*F. ruetzleri* Gam1, *G. belizeanus* CCMP399, *G. carolinianus* Dive1 Gam1, *Gambierdiscus* sp. ribotype 2 St. Maarteens Gam6 and Mixed PR Gam3) produced detectable levels of CTXs, probably involving from one to six different congeners, depending on the strain.

4.2.3. In vitro receptor binding assays (RBAs)

Receptor binding assays (RBAs) are based on the binding competition between CTXs and BTXs on the receptor site 5 of the alpha-subunit of VGSCs. Rat brain synaptosomes containing Na^+-channels are incubated with a fixed amount of a labeled BTX congener (BTX3 or BTX2). Subsequently, the sample is added and if CTXs are present, the labeled BTX is released from the receptor and quantitation of CTXs is possible by the measurement of radioactivity ($RBA_{(R)}$) or fluorescence ($RBA_{(F)}$). As for the N2a assay, RBAs can be run in HTS format, and have the additional advantages of being stable over long periods, cost-effective and less time-consuming. Nevertheless, they are 10-fold less sensitive compared to the N2a assay and they do not measure toxicity, but rather binding affinity. To date, two different RBAs have been developed, with the only difference being the way of labeling the BTX congener: the radioactive ($RBA_{(R)}$) and the fluorescent RBA ($RBA_{(F)}$). The **$RBA_{(R)}$** was initially developed by (Hollenberg and Nexø, 1981) and mostly uses tritiated BTX3 ([^{3}H]-BTX3) as standard calibrant. $RBA_{(R)}$ was successfully used in a monitoring program for ciguatera risk assessment in French Polynesia (Darius et al., 2007). The **$RBA_{(F)}$** was developed by Hardison et al. (2016) and uses a fluorescently labeled BTX2 (BODIPY®-BTX2) as standard calibrant. The main advantage of the $RBA_{(F)}$ is that fluorescence can be used in any laboratory, while the use of radioactivity for $RBA_{(R)}$ is limited to facilities that are certified to use radioisotopes. The $RBA_{(F)}$ was successfully used to detect and quantify CTXs in fish extracts and gave consistent results with N2a and $RBA_{(R)}$ assays and LC-MS/MS analyses (Hardison et al., 2016).

4.2.4. Immunoassays

Immunoassays have been considered as a very promising approach for designing a rapid, reliable and cost-effective method for mass screening of fish, prior to consumption, mainly because of their specificity. From an immunological point of view, CTXs can be considered to be haptens, i.e. low molecular weight compounds, with one or more antigenic determinants

(epitopes), able to trigger an immunological response if they are covalently conjugated to a large molecule acting as an immunogenic carrier (Caillaud et al., 2010a).

Early studies on sheep polyclonal antibodies (PAbs) and mouse monoclonal antibodies (MAbs) against CTXs permitted the development of a radioimmunoassay (Kimura et al., 1982a; Kimura et al., 1982b) and an enzyme-immunoassay (Hokama et al., 1983). MAb-based assays were more sensitive and specific than PAb-based assays (Hokama et al., 1990; Hokama et al., 1992). Further development of solid-phase immunobed assays led to the commercialization of two ready-to-use kits, Ciguatect™ (Dickey et al., 1994) and Cigua-Check® (Bienfang et al., 2011), however, these kits were not successfully validated.

The main limitation for the development of such assays is the extreme scarcity of CTX standards. Since the beginning of the 2000s, more promising studies in the field are being conducted in Japan and they are based on (i) synthetic ring fragments of CTXs (to overcome the lack of the natural toxins) and (ii) the use of modern molecular biology techniques (to provide a set of immunological reagents with improved specificity and affinity) (Inoue et al., 2009; Nagumo et al., 2001; Nagumo et al., 2004; Oguri et al., 2003; Tsumoto et al., 2008; Tsumuraya et al., 2010; Tsumuraya et al., 2006; Tsumuraya et al., 2012; Ui et al., 2008).

4.3. **Chemical analyses**

4.3.1. HPLC-UV

High-performance liquid chromatography coupled with UV detection (HPLC-UV) methods have extensively been applied as the endpoint of purification procedures for the isolation of several CTX congeners from fish tissues and dinoflagellate extracts (Hamilton et al., 2002a; Hamilton et al., 2002b; Legrand et al., 1998; Legrand et al., 1989; Lewis and Sellin, 1992; Lewis et al., 1991; Lewis et al., 1998; Satake et al., 1998; Satake et al., 1997a; Satake et al., 1993b; Vernoux and Lewis, 1997). Reversed-phase HPLC-UV with C_{18}, C_8 or polymeric columns was generally performed using isocratic or linear gradients from 50:50 to 100:0 (MeCN:H_2O with or without buffers) after a series of clean-up steps involving liquid-liquid partitioning (e.g. between CH_2Cl_2 and MeOH:H_2O 3:2, v/v), solid-phase extraction (SPE) and/or size-exclusion chromatography (SEC) (**Table 7**). It should be

noted that since most CTXs do not possess distinctive chromophore groups (i.e. two or more conjugated unsaturations) in their molecular structure, they do not strongly absorb ultraviolet/visible (UV/VIS) wavelengths. Therefore, detection of CTXs has to be made at 210-215 nm, a poorly sensitive and non-selective range of detection.

HPLC-UV methods have also been applied for MTX detection during purification procedures. Indeed, MTX absorbs maximally at a wavelength of 230 nm, due to the presence of a conjugated diene at one extremity of the molecule. MTX2 also absorbs maximally at λ = 230 nm, whereas MTX3 absorbs maximally at λ = 235 nm.

4.3.2. HPLC-FLD

The presence of a primary hydroxyl group at the side chain of some CTX congeners (e.g. CTX1B) allows them to be derivatized into fluorescent esters being suitable for high-performance liquid chromatography with fluorescence detection (HPLC-FLD). The HPLC-FLD methods for CTX determination described in literature used different derivatization procedures. The most used fluorescent reagents are (i) 1-anthroylnitrile (i.e. anthrylcarbocyanide) (Dickey et al., 1992; Goto et al., 1983) or (ii) carbonyl azides (or carbonyl nitriles) of coumarin derivatives (e.g. 7-diethylaminocoumarin-3-carbonyl azide) (Dickey et al., 1992). An alternative derivatization procedure consists of using a post-column alkaline oxidation with periodic acid and ammonium hydroxide (Sick et al., 1986).

Disadvantages of HPLC-FLD include: (i) low sensitivity (limit of detection 0.5-1 ng on-column); (ii) non-applicability to detection of CTX congeners which do not present a primary hydroxyl group (e.g. CTX3C) and (iii) low specificity: a primary hydroxyl group may often be present in other compounds from such biological matrices (i.e. requirement of a laborious sample preparation with several clean-up steps) (Caillaud et al., 2010a).

4.3.3. HPLC-MS for CTX determination

High-performance liquid chromatography coupled with mass spectrometry detection (HPLC-MS) includes: (i) the chromatographic separation of the different CTX congeners and (ii) the acquisition of MS spectra in positive ion acquisition mode, in full scan (MS) or targeted (MS/MS) modes. HPLC-MS represents a very useful tool for (i) the

structure elucidation of novel CTX congeners, from fish or dinoflagellate, and (ii) the establishment of the toxin profile of a fish specimen or dinoflagellate strain, with the identification and quantification of known congeners.

Chromatographic separation generally involves the use of a reversed-phase stationary phase, and binary mobile phases of MeCN:H_2O or MeOH:H_2O with the addition of modifiers (trifluoroacetic acid, formic acid or ammonium acetate), in isocratic or gradient modes.

Low resolution mass spectrometry (LRMS). In most of the studies described in literature, MS detection is provided by low resolution mass spectrometry (LRMS) equipment. Source of ionization is either fast atom bombardment (FAB) (Lewis et al., 1991; Yasumoto, 2001; Yasumoto et al., 2000) or electrospray (ESI) (Ikehara et al., 2017; Lewis et al., 1994a; Lewis et al., 1997; Lewis and Sellin, 1992; Pottier et al., 2002b; Vernoux and Lewis, 1997; Yogi et al., 2011; Yogi et al., 2014). Structure elucidation of most of the Pacific CTX congeners known to date was aided by the analysis of charge-remote fragments from both termini of sodium adducts ($[M+Na]^+$) using FAB LRMS/MS^+ techniques (Yasumoto, 2001; Yasumoto et al., 2000). Atmospheric pressure chemical ionization (APCI) may be more suitable than ESI for positive MS ionization of cyclic polyether compounds, as recently demonstrated for brevetoxin-2 (BTX2) (Mead et al., 2014). Few studies also used high resolution mass spectrometry (HRMS) equipments (Diogène et al., 2017; Hamilton et al., 2002a; Hamilton et al., 2002b; Laza-Martinez et al., 2016; Silva et al., 2015).

Positive MS spectra of CTXs (**Figure 25**) are characterized by singly charged molecular ions with (i) sodium, ammonium and/or potassium adducts and (ii) losses of up to five molecules of water, due to the presence of numerous hydroxyl groups in the molecule.

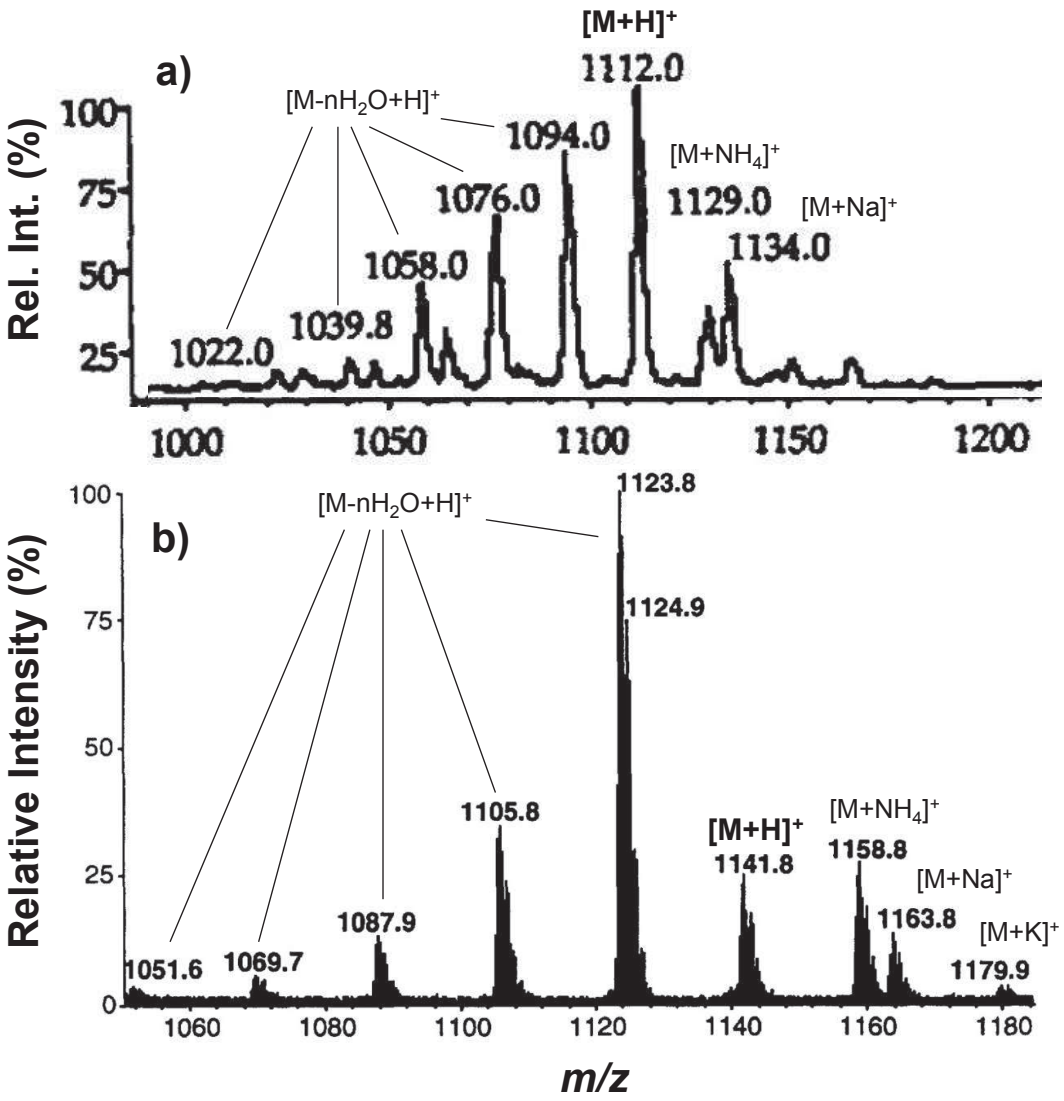


Figure 25. Positive low resolution mass spectrometry (LRMS) spectra of **(a)** Pacific CTX1B (Lewis et al., 1994a) and **(b)** Caribbean C-CTX1 (Vernoux and Lewis, 1997). Both LRMS spectra were obtained using an API-III triple quadrupole mass spectrometer equipped with an ionspray (IS) source. The orifice potential was set at 100 V for (a), at 120 V for (b). The assigned ion species are indicated in the figure. Note the presence of singly charged ion species: the molecular and pseudomolecular ions and losses of up to 5 molecules of water. C-CTX2 (Vernoux and Lewis, 1997), I-CTX1 and I-CTX2 (Hamilton et al., 2002a; Hamilton et al., 2002b) present the same MS spectrum as (b) with different ion ratios. NB: in the original study (Lewis et al., 1994a), $[M+NH_4]^+$ of CTX1B was indicated as $[M+H_2O+H]^+$. Assignment of *m/z* 1129.0 to $[M+NH_4]^+$ was provided by Lewis et al. (1999).

Based on the MS behavior of known CTXs, i.e. the loss of up to five molecules of water (**Figure 25**), other congeners were tentatively identified but they have not been structurally characterized yet. This is the case of (i) minor congeners of Pacific CTXs, found in dinoflagellate (*G. polynesiensis*) (Chinain et al., 2010a) and in fish (moray eel *Lycodontis javanicus*) (Lewis et al., 1997); (ii) minor congeners of Caribbean C-CTXs, found in fish (horse-eye jack *Caranx latus*) (Pottier et al., 2002b) and (iii) four Indian CTXs, found in fish (red bass *Lutjanus bohar* and red emperor *Lutjanus sebae*) (Hamilton et al., 2002a; Hamilton et al., 2002b).

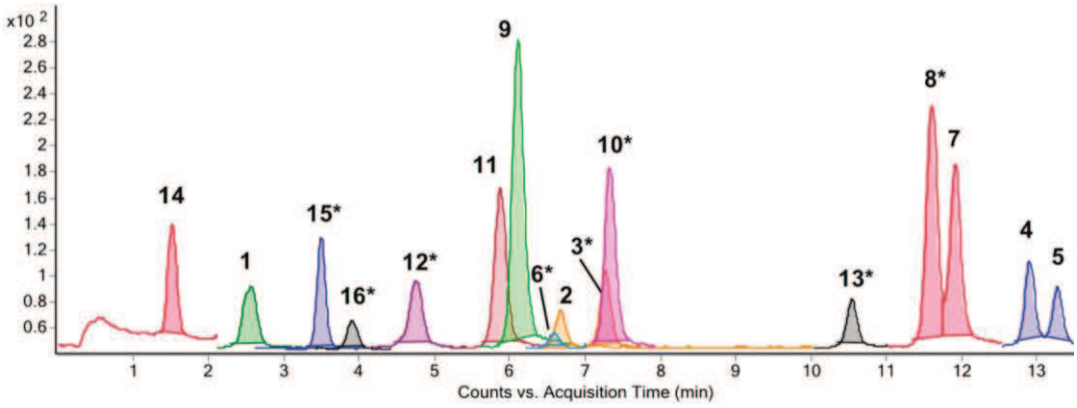
Losses of up to five molecules of water served as the MS feature used to develop HPLC-MS/MS methods for CTX determination in dinoflagellate and fish samples. Lewis et al. (1999) first described an HPLC-MS/MS method for identification and quantitation of Pacific CTX1B and Caribbean C-CTX1 in crude extracts of fish. The MRM transitions for CTX identification involved the pseudomolecular ion species $[M+NH_4]^+$ as the parent ion (*m/z* 1128.7 for CTX1B; *m/z* 1158.6 for C-CTX1) and the ion species $[M-nH_2O+H]^+$ as fragment ions, with n=1 up to 3 (*m/z* 1094.0, 1076.0 and 1058.0 for CTX1B; *m/z* 1123.6, 1105.6 for C-CTX1). The method successfully identified C-CTX1 in toxic fish specimens collected from the Caribbean Sea, with a sensitivity at sub-part per billion levels (limit of detection, LOD = 0.04 ppb for CTX1B and 0.10 ppb for C-CTX1). Improvements in sample preparation and MS instrumentation allowed for rapid extraction procedures and increased sensitivity, with LODs for CTX1B reaching 0.1 ppb (Lewis et al., 2009) and 0.03 ppb (Stewart et al., 2010). A further study conducted by Wu et al. (2011) permitted detection of CTX1B from a 5 g fish sample even at 0.01 ppb, meeting the food safety requirements. Nevertheless, in that case, two clean-up steps (two successive SPEs) were required before running the analysis. Mak et al. (2013) also proposed to search for CTXs into a different matrix, the whole fish blood. The method was successfully applied to four species of edible coral reef fish and CTX content in blood was found to be coherent with toxicity data (N2a assay). The main advantages of this method are detailed as follows: (i) it constituted a non-lethal method for screening of ciguateric fish and (ii) matrix effects were found to be significantly lower than those observed in fish muscles. Nevertheless, a clean-up step (SPE) is required before running the analysis.

HPLC-MS/MS studies based on MRM transitions with multiple losses of water successfully identified several known CTX congeners in fish flesh and/or viscera (Abraham et al., 2012; Boada et al., 2010; Hamilton et al., 2010; Hardison et al., 2016; Hossen et al., 2015; Otero et al., 2010; Robertson et al., 2014; Wong et al., 2014) and in dinoflagellate strains (Caillaud et al., 2011; Chinain et al., 2010a; Roeder et al., 2010). Nevertheless, it should be noted that such subsequent losses of H_2O do not represent a fragmentation pattern characteristic to CTXs: any polyhydroxylated compound would behave in the same way and CTXs may be misidentified.

Alternative ways to confirm MS identification of CTXs are presented as follows. Dechraoui Bottein et al. (2011) analyzed liver extracts of the monk seals *Monachus schauinslandi*, from Hawaii, using an API4000 QTrap hybrid triple-quadrupole/linear ion trap mass spectrometer, in both multiple ion monitoring (MI) and multiple reaction monitoring (MRM) modes. The authors reported four characteristic fragment ions of CTX3C at the following *m/z*: 907.6, 447.5, 155.1 and 125.1. This finding gave interesting insights to understand the behavior of CTXs in positive ionization and MS/MS fragmentation. Indeed, the identification of fragmentation patterns characteristic to the CTX group would help the development of more specific HPLC-MS/MS methods. The authors suggested that the MS/MS ions at *m/z* 155.1 and 125.1 derived from fragmentation of the right side of CTX3C (L and M rings, **Figure 14**, **section 3.2.1.2**) and may serve to specifically identify all CTXs which share the same terminal structure as that of CTX3C.

A completely different approach was adopted by Yogi et al. (2011). The authors developed an HPLC-MS/MS method for detection and quantification of 16 cyclic polyether compounds, i.e. 13 Pacific CTXs and three related polyethers (gambierol, gambieric acids A and B). The method was successfully applied (i) to determine CTX profiles in *Gambierdiscus* sp. RGI-1 (Yogi et al., 2011) and in eight species of Pacific fish (Yogi et al., 2014) (ii) to demonstrate the oxidation of CTX4A, CTX4B and CTX3C due to fish and human enzymes (Ikehara et al., 2017). CTX identification was achieved, with very high sensitivity (LOD = 0.25 pg on-column), using the MRM transition $[M+Na]^+/[M+Na]^+$ (40 eV). Thus, the method did not involve any fragmentation pathway. Toxin identity was confirmed by comparison of retention time and mass with reference material. The asset of this study was to provide a chromatogram of as many as 16 reference compounds (**Figure 26**), obtained from either chemical synthesis or natural sources. Almost all reference compounds were well separated on a reversed-phase column (Zorbax C_{18}) using a slow linear gradient of acidified MeOH/H_2O mobile phases (1% MeOH increase per minute). The chromatogram shown in **Figure 26** gave interesting information on the relative retention times between CTXs and may help their identification in future studies using similar chromatographic conditions. The weak point of this study is that MRM transitions from parent ion to parent ion cannot unambiguously identify any compound, in particular in the absence of reference material.

To date, only one CTX congener, Pacific CTX3C, is commercially available (Wako Chemicals Ltd.) and it is not certified.



Rt	Compounds	*Rt*	Compounds	*Rt*	Compounds	*Rt*	Compounds
1.5	gambierol (**14**)	4.7	M-*seco*-CTX3C (**12**)	6.7	52-*epi*-54-deoxyCTX1B (**2**)	11.5	49-*epi*CTX3C (**8**)
2.6	CTX1B (**1**)	5.9	2,3-dihydroxyCTX3C (**11**)	7.2	54-deoxyCTX1B (**3**)	11.8	CTX3C (**7**)
3.5	gambieric acid A (**15**)	6.1	51-hydroxyCTX3C (**9**)	7.3	2-hydroxyCTX3C (**10**)	12.9	CTX4A (**4**)
3.9	gambieric acid B (**16**)	6.6	M-*seco*-CTX4A/B (**6**)	10.5	M-*seco*-CTX3C methyl acetal (**13**)	13.3	CTX4B (**5**)

Figure 26. Chromatogram with retention time (R_t, min) data for 16 reference compounds, i.e. 13 Pacific ciguatoxins, gambierol and gambieric acids A and B (Yogi et al., 2011).

High resolution mass spectrometry (HRMS). The use of high resolution mass spectrometry (HRMS) for CTX determination is recent (Diogène et al., 2017; Hamilton et al., 2002a; Hamilton et al., 2002b; Laza-Martinez et al., 2016; Silva et al., 2015).

Low mass accuracy of LRMS equipments might lead to misidentification, especially when reference material is not available. This was demonstrated by a study conducted in our laboratory (unpublished data, article in preparation): as shown in **Figure 27**, the peak at 5.363 min observed in a strain of *Gambierdiscus* (*Gambierdiscus* sp. from Southwater, USA) could be incorrectly attributed to CTX3C using LRMS. The misidentification was confirmed in full scan HRMS analysis by differences observed between the two MS spectra (peak at 5.363 min, **Figure 27a**, *versus* peak of CTX3C, reference material, at 5.391 min, **Figure 27b**).

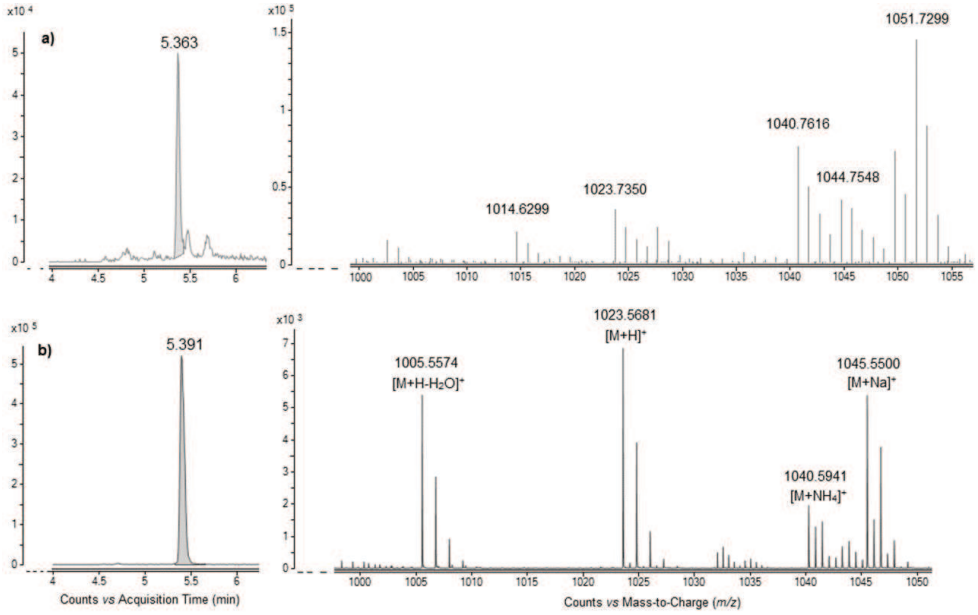


Figure 27. High resolution LC-MS spectra (ESI$^+$, *m/z* 1000-1060) corresponding to major peak eluting at 5.363 min in **(a)** *Gambierdiscus* sp. from Southwater (USA) and at 5.391 min in **(b)** CTX3C standard (Wako Chemicals, Ltd.).

Hamilton et al. (2002a) and Hamilton et al. (2002b) analyzed toxic specimens of *Lutjanus* spp. from Indian Ocean, using a Sciex API QSTAR Pulsar (Quadrupole/TOF) equipped with electrospray (ESI) ionization source. The authors discovered four Indian CTX congeners and provided accurate mass data for I-CTX1 and I-CTX2.

Silva et al. (2015) provided a positive HRMS spectrum (IT-TOF-MS system with ESI interface) of reference CTX3C obtained by chemical synthesis (Hirama et al., 2001; Inoue and Hirama, 2004) (**Figure 28**).

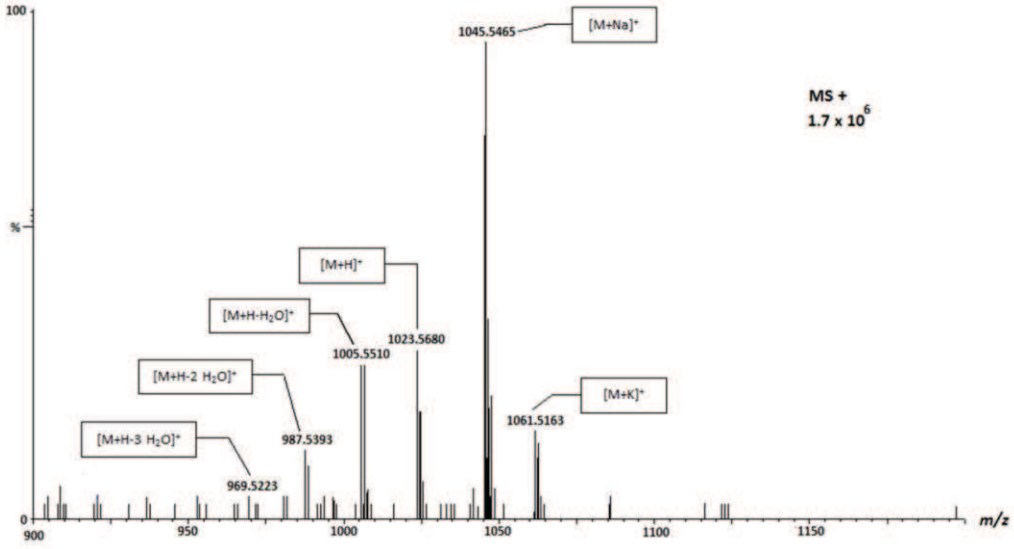


Figure 28. HRMS spectrum of synthetic CTX3C obtained by UPLC-MS-IT-TOF analysis (Silva et al., 2015).

Laza-Martinez et al. (2016) analyzed the toxin profile of *F. paulensis* Dn135EHU, a strain isolated from the temperate island of Formentera (Balearic Islands, Western Mediterranean Sea), using an Exactive® mass spectrometer equipped with an Orbitrap® mass analyzer. The authors disposed of a standard mix solution of CTX4A, CTX4B, CTX3C, 52-*Epi*-54-deoxyCTX (CTX2) and 54-DeoxyCTX (CTX3) as reference material. The strain was positive for the presence of trace amounts of a compound having mass characteristics of 54-DeoxyCTX ($[M+Na]^+$, Δppm = 10.56). 54-DeoxyCTX is a CTX congener generally found in fish caught in the Pacific. If the findings of this study were confirmed, the classification of CTXs according to their geographical origin would be obsolete, as previously suggested by Caillaud et al. (2011) and Silva et al. (2015). Also, the theory of the oxidative modification of CTXs along the marine food chain should be revised. Nevertheless, the mass error (Δppm = 10.56) appears large compared to the instrument capability and thus repeat analyses should be undertaken.

Diogène et al. (2017) analyzed the toxin profile of a shark (*Carcharhinus leucas*) involved in a fatal food poisoning in the Indian Ocean, using an Orbitrap®-Exactive® HCD mass spectrometer equipped with heated electrospray source (H-ESI II). Six Indian CTX congeners were identified in the stomach content of *C. leucas* after bioguided fractionation. Gambieric acid D was also identified in the flesh. Accurate mass data permitted to assign elemental formulae to the four I-CTXs previously described from *L. sebae* (Hamilton et al., 2002a; Hamilton et al., 2002b) and to two novel congeners, I-CTX5 and I-CTX6.

Table 8 and **Table 9** summarize all the *Gambierdiscus* and *Fukuyoa* strains which have been screened for the presence of CTXs and CTX-related compounds using LC-MS techniques (Caillaud et al., 2011; Chinain et al., 2010a; Kohli et al., 2014a; Kretzschmar et al., 2017; Laza-Martinez et al., 2016; Munday et al., 2017; Rhodes et al., 2016; Rhodes et al., 2017b; Rhodes et al., 2010; Rhodes et al., 2017c; Roeder et al., 2010; Roué et al., 2016; Smith et al., 2016; Yogi et al., 2011).

Table 8. *Fukuyoa* and *Gambierdiscus* strains that do not produce any of the following Pacific CTX congeners: CTX3C, 49-*Epi*CTX3C (CTX3B), CTX4A and CTX4B. Strain underwent LC-LRMS/MS analysis in presence of reference material for the four congeners mentioned above.

Species	Strain names	Ref.
F. cf. *yasumotoi*	CAWD210, CAWD211	[1]
F. paulensis	CAWD238	[2]
G. australes	CAWD216, CAWD149, S8xii, S4G, S5(6)GC2a, CAWD244, S9a, CAWD246 CAWD255, CAWD256, Mac2-a, -b,-c, Mac3-a, -b, -e, -i, -m, -n, Mac4-e, -f, -fuk, -g, Mac5-a, -b, -c, -e, -f, -g, -j, -l, Mac6-a	[1-4]
G. carpenteri	Merimbula, CAWD237	[2, 5]
G. cheloniae	CAWD232, CAWD236	[2, 6]
G. honu	CAWD233, CAWD242	[2, 7]
G. lapillus	HG1, HG4, HG5, HG6, HG7, HG26	[8]
G. pacificus	CAWD213, CAWD227, C14CI, NLD-10, NLD-11, NLD-14	[1, 2]
G. polynesiensis	CAWD254	[4]
G. toxicus	HIT0	[9]

[1] L. Rhodes, T. Harwood, K. Smith, P. Argyle, R. Munday, *Harmful Algae* **2016**, *55*, 295.
[2] R. Munday, S. Murray, L. Rhodes, M. Larsson, D. Harwood, *Marine Drugs* **2017**, *15*, 208.
[3] L. L. Rhodes, K. F. Smith, R. Munday, A. I. Selwood, P. S. McNabb, P. T. Holland, M. Y. Bottein, *Toxicon* **2010**, *56*, 751.
[4] L. L. Rhodes, K. F. Smith, S. Murray, D. T. Harwood, T. Trnski, R. Munday, *Mar Drugs* **2017**, *15*.
[5] G. S. Kohli, S. A. Murray, B. A. Neilan, L. L. Rhodes, D. T. Harwood, K. F. Smith, L. Meyer, A. Capper, S. Brett, G. M. Hallegraeff, *Harmful Algae* **2014**, *39*, 134.
[6] K. F. Smith, L. Rhodes, A. Verma, B. G. Curley, D. T. Harwood, G. S. Kohli, D. Solomona, T. Rongo, R. Munday, S. A. Murray, *Harmful Algae* **2016**, *60*, 45.
[7] L. Rhodes, K. F. Smith, A. Verma, B. G. Curley, D. T. Harwood, S. Murray, G. S. Kohli, D. Solomona, T. Rongo, R. Munday, S. A. Murray, *Harmful Algae* **2017**, *65*, 61.
[8] A. L. Kretzschmar, A. Verma, T. Harwood, M. Hoppenrath, S. Murray, *J. Phycol.* **2017**, *53*, 283.
[9] M. Roué, H. T. Darius, S. Picot, A. Ung, J. Viallon, N. Gaertner-Mazouni, M. Sibat, Z. Amzil, M. Chinain, *Harmful Algae* **2016**, *57*, 78.

Table 9. *Fukuyoa* and *Gambierdiscus* strains that were found to be positive for the presence of CTXs and CTX-related compounds using low-resolution (LR) LC-MS analyses (except for [1], HRMS).

Species	Strain names	CTXs and CTX-related compounds	Confirmation	Ref.
F. paulensis	Dn135EHU	54-DeoxyCTX (CTX3)[(e)]	RM[(a)], HRMS[(b)]	[1]
F. ruetzleri	NOAA3	2,3-Dihydro-2,3-dihydroxyCTX3C	-	[2]
G. australes	CCMP1653	2,3-Dihydro-2,3-dihydroxyCTX3C	-	[2]
G. caribaeus	CCMP401, CCMP1651, CCMP1652, CCMP1733	2,3-Dihydro-2,3-dihydroxyCTX3C	-	[2]
G. carpenteri	CCMP1654, NOAA12	2,3-Dihydro-2,3-dihydroxyCTX3C	-	[2]
G. pacificus	CCMP1650	2,3-Dihydro-2,3-dihydroxyCTX3C	-	[2]
	G10-DC	CTX3C or 49-*Epi*CTX3C (CTX3B), CTX4A or CTX4B, 2,3-Dihydro-2,3-dihydroxyCTX3C, 51-HydroxyCTX3C, M/L-*Seco*CTX3C, C-CTX1/2 or I-CTX1/2	-	[3]
G. polynesiensis	CAWD212	49-*Epi*CTX3C (CTX3B)[(c)], CTX3C, CTX4A, CTX4B ([CTXs] = 0.44-18.2 pg cell^{-1})	RM	[4, 5]
	TB-92, RG-92	CTX3C (41-48%), 49-*Epi*CTX3C (CTX3B) (26-34%), CTX4A (13-16%), CTX4B (1%), M-*Seco*CTX3C (2-13%), Others (2-3%)	RM	[6, 7]
Gambierdiscus sp.	RGI-1	CTX3C, 49-*Epi*CTX3C (CTX3B), CTX4A, CTX4B, M-*Seco*CTX3C, M-*Seco*CTX4A/B, M-*Seco*-49-O-methylCTX3C, M-*Seco*CTX4A/B methyl acetal[(d)], 51-HydroxyCTX3C[(e)], gambierol[(e)], 52-*Epi*-54-DeoxyCTX (CTX2)[(e)], 54-DeoxyCTX (CTX3)[(e)]	RM	[8]
Gambierdiscus sp.	Viet Nam	2,3-Dihydro-2,3-dihydroxyCTX3C, CTX3C, CTX4A[(e)] or CTX4B[(e)]	-	[2]
G. toxicus	NOAA5	2,3-Dihydro-2,3-dihydroxyCTX3C	-	[2]

(a) RM = reference material, **(b)** HRMS = high resolution mass spectrometry, **(c)** major compound, **(d)** tentatively assigned based on $[M+Na]^+$ (*m/z* 1115) and retention time (no RM available), **(e)** trace amounts.

[1] A. Laza-Martinez, H. David, P. Riobo, I. Miguel, E. Orive, *Journal of Eukaryotic Microbiology* **2016**, *63*, 481.
[2] K. Roeder, K. Erler, S. Kibler, P. Tester, H. Van The, L. Nguyen-Ngoc, G. Gerdts, B. Luckas, *Toxicon* **2010**, *56*, 731.
[3] A. Caillaud, P. de la Iglesia, E. Barber, H. Eixarch, N. Mohammad-Noor, T. Yasumoto, J. Diogène, *Harmful Algae* **2011**, *10*, 433.
[4] L. Rhodes, T. Harwood, K. Smith, P. Argyle, R. Munday, *Harmful Algae* **2016**, *55*, 295.
[5] R. Munday, S. Murray, L. Rhodes, M. Larsson, D. Harwood, *Marine Drugs* **2017**, *15*, 208.
[6] M. Roué, H. T. Darius, S. Picot, A. Ung, J. Viallon, N. Gaertner-Mazouni, M. Sibat, Z. Amzil, M. Chinain, *Harmful Algae* **2016**, *57*, 78.
[7] M. Chinain, H. T. Darius, A. Ung, P. Cruchet, Z. Wang, D. Ponton, D. Laurent, S. Pauillac, *Toxicon* **2010**, *56*, 739.
[8] K. Yogi, N. Oshiro, Y. Inafuku, M. Hirama, T. Yasumoto, *Anal Chem* **2011**, *83*, 8886.

4.3.4. HPLC-MS for MTX determination

Two LC-MS methods for detection and quantification of MTXs were developed by a research group of the Cawthron Institute (Nelson, New Zealand) using a low resolution Xevo® TQ-S triple quadrupole mass spectrometer equipped with an electrospray source operating in negative ion acquisition mode (ESI^-) (Kohli, 2013). These methods rely on quantitation of either a specific oxidative cleavage product (MTX) or the intact molecule (MTX and putative MTX3). They permitted to detect and quantify MTX and p-MTX3 in *Gambierdiscus* and *Fukuyoa* strains (Kohli, 2013; Kretzschmar et al., 2017; Munday et al., 2017; Rhodes et al., 2016; Rhodes et al., 2017b; Rhodes et al., 2017d; Smith et al., 2016), as well as to prove the uptake of MTX from the MTX-producing *G. australes* CAWD149 to carnivorous snapper (*Pagrus auratus*) via juvenile mullet (*Aldrichetta forsteri*) in a laboratory-controlled feeding study (Kohli et al., 2014b). Since the LRMS description of MTX2 in Lewis et al. (1994a) from *Gambierdiscus* sp. NQ1, MTX2 has never been reported.

The **intact molecule method** identifies MTX using the MRM transition *m/z* 1689.6 → 1689.6 (CE = 50 eV, "parent ion → parent ion") and a putative MTX3 (p-MTX3) using the MRM transition *m/z* 1037.6 → 96.8 (CE not specified, "parent ion → fragment ion"). The ion at *m/z* 1689.6 corresponds to the bicharged molecular anion $[M-2H]^{2-}$ of MTX. The fragment ion at 96.8 *m/z* corresponds to the hydrogenated sulfate anion ($[HOSO_3]^-$) and the parent ion at 1037.6 *m/z* had been incorrectly assigned to the molecular monocharged anion $[M-H]^-$ of MTX3 (Kohli, 2013). Indeed, according to the original study (Lewis et al., 1994a), the ion 1037.6 *m/z* should actually correspond to $[M+Na-2H]^-$ of MTX3. A total of 65 strains have been examined using this LC-LRMS/MS method: one strain of *F. paulensis*, two strains of *F.* cf. *yasumotoi*, 41 strains of *G. australes*, one strain of *G. belizeanus*, two strains of *G. carpenteri*, two strains of *G. cheloniae*, two strains of *G. honu*, six strains of *G. lapillus*, six strains of *G. pacificus* and two strains of *G. polynesiensis* (**Table 10**).

Table 10. *Fukuyoa* and *Gambierdiscus* strains which have been screened for the presence of MTX congeners using LC-LRMS/MS, i.e. intact molecule method, Kohli (2013). ND = not detected; D = detected.

Species	n	Strain names	MTX (pg cell^{-1})	Putative MTX3 (peak area cell^{-1})	Ref.
F. paulensis	1	CAWD238	ND	1	[1]
F. cf. *yasumotoi*	2	CAWD210, CAWD211	ND	D	[2-3]
G. australes	2	CAWD216, CBo	ND	D	[1, 3-5]
	15	CAWD149, CAWD245, #2V, #3-5, #5a, #5h, CAWD246, CAWD244, CBc, CAWD248, CBaii, S8xii, S4G, S5(6)GC2a, S9a	0.3-36.6	16-33	
	24	CAWD255, CAWD256, Mac2-a, -b,-c, Mac3-a, -b, -e, -i, -m, -n, Mac4-e, -f, -fuk, -g, Mac5-a, -b, -c, -e, -f, -g, -j, -l, Mac6-a	3-36	D	
G. belizeanus	1	CCMP401	ND	D	[6]
G. carpenteri	2	Merimbula, CAWD237	ND	ND	[1, 7]
G. cheloniae	2	CAWD232, CAWD236	ND	0.4-1	[1, 8]
G. honu	2	CAWD233, CAWD242	ND	5	[1, 4, 9]
G. lapillus	6	HG1, HG4, HG5, HG6, HG7, HG26	ND	D	[10]
G. pacificus	6	CAWD213, NLD-10, NLD-11, NLD-14, CAWD227, C14CI	ND	7-18	[1, 3]
G. polynesiensis	2	CAWD212, CAWD254	ND	0.3	[1, 3, 5]

[1] R. Munday, S. Murray, L. Rhodes, M. Larsson, D. Harwood, *Marine Drugs* **2017**, *15*, 208.
[2] L. Rhodes, G. G. Papiol, K. Smith, T. Harwood, *N. Z. J. Mar. Freshw. Res.* **2014**, *48*, 303.
[3] L. Rhodes, T. Harwood, K. Smith, P. Argyle, R. Munday, *Harmful Algae* **2016**, *55*, 295.
[4] L. L. Rhodes, K. F. Smith, A. Verma, S. Murray, D. T. Harwood, T. Trnski, *New Zealand Journal of Marine and Freshwater Research* **2017**, 1.
[5] L. L. Rhodes, K. F. Smith, S. Murray, D. T. Harwood, T. Trnski, R. Munday, *Mar Drugs* **2017**, *15*.
[6] G. S. Kohli, PhD thesis, University of New South Wales, Sidney, Australia **2013**.
[7] G. S. Kohli, S. A. Murray, B. A. Neilan, L. L. Rhodes, D. T. Harwood, K. F. Smith, L. Meyer, A. Capper, S. Brett, G. M. Hallegraeff, *Harmful Algae* **2014**, *39*, 134.
[8] K. F. Smith, L. Rhodes, A. Verma, B. G. Curley, D. T. Harwood, G. S. Kohli, D. Solomona, T. Rongo, R. Munday, S. A. Murray, *Harmful Algae* **2016**, *60*, 45.
[9] L. Rhodes, K. F. Smith, A. Verma, B. G. Curley, D. T. Harwood, S. Murray, G. S. Kohli, D. Solomona, T. Rongo, R. Munday, S. A. Murray, *Harmful Algae* **2017**, *65*, 61.
[10] A. L. Kretzschmar, A. Verma, T. Harwood, M. Hoppenrath, S. Murray, *J. Phycol.* **2017**, *53*, 283.

These authors reported that MTX was produced by strains of *G. australes* isolated from either Cook Islands or Kermadec Islands (39 strains out of 41), with concentrations ranging from 0.3 to 36.6 pg MTX cell^{-1}. All but two (*G. carpenteri* Merimbula and *G. carpenteri* CAWD237) out of the 65 strains were positive for the presence of p-MTX3 (Kohli, 2013; Kretzschmar et al., 2017; Munday et al., 2017; Rhodes et al., 2016; Rhodes et al., 2017b; Rhodes et al., 2017d; Smith et al., 2016). In reason of the lack of standard material for MTX3, the concentration of p-MTX3 in *Gambierdiscus* and *Fukuyoa* strains was expressed in peak area cell^{-1}, and it was found to be comprised between 0.4 - 33 (Munday et al., 2017). Interestingly, Munday et al. (2017) found no correlation between p-MTX3 concentration and MBA toxicity, casting doubts about the relevance of the ubiquitous sulfated compound at 1037.6 *m/z* (MS^-).

The **oxidative cleavage method** (**Figure 29**) for MTX quantification was described by Kohli (2013) and used in the laboratory-controlled feeding study conducted by Kohli et al. (2014b). The principle of this method is similar to that recently described for palytoxin (Selwood et al., 2012). The method consists of loading MTX on a StrataX SPE cartridge and exposing it to periodic acid (HIO_4, 50 mM) for two minutes. The oxidative cleavage product is then eluted with acetonitrile-water (4:1, v/v) containing 20 mM ammonium acetate. Subsequently, the oxidative cleavage product is detected and quantified via LC-LRMS/MS using the MRM transition 971.4 *m/z* → 96.8 *m/z* (CE = 80 eV, "parent ion → fragment ion").

The parent ion at 971.4 *m/z* is evidently the *m/z* of the product of the oxidative cleavage of MTX, but it cannot be attributed to the elemental formula $C_{43}H_{68}O_{20}S$ (Kohli, 2013) (**Figure 29c**). Indeed, the molecular anion $[C_{43}H_{67}O_{20}S]^-$ would have a nominal mass of 935.4 *m/z*. Therefore, subsequent oxidation reactions are likely to occur, most probably involving the conjugated diene function at C_1-C_2-C_3-C_{144} of MTX. In addition to uncertainties about the attribution of the ion at 971.4 *m/z*, the LOD of MTX estimated for the oxidative cleavage method (10-20 µg kg^{-1}) was at least 10 times higher than the LOD estimated for the intact molecule method (1 µg kg^{-1}) (Kohli et al., 2014b). In conclusion, the oxidative cleavage method seems less promising than the intact molecule method for MTX detection and quantification. Nevertheless, the comprehension of the oxidative cleavage pathways of MTX may be interesting in the context of structure elucidation studies for hitherto undescribed MTX congeners.

Oxidative cleavage method for MTX detection in LC-LRMS/MS (Kohli, 2013)

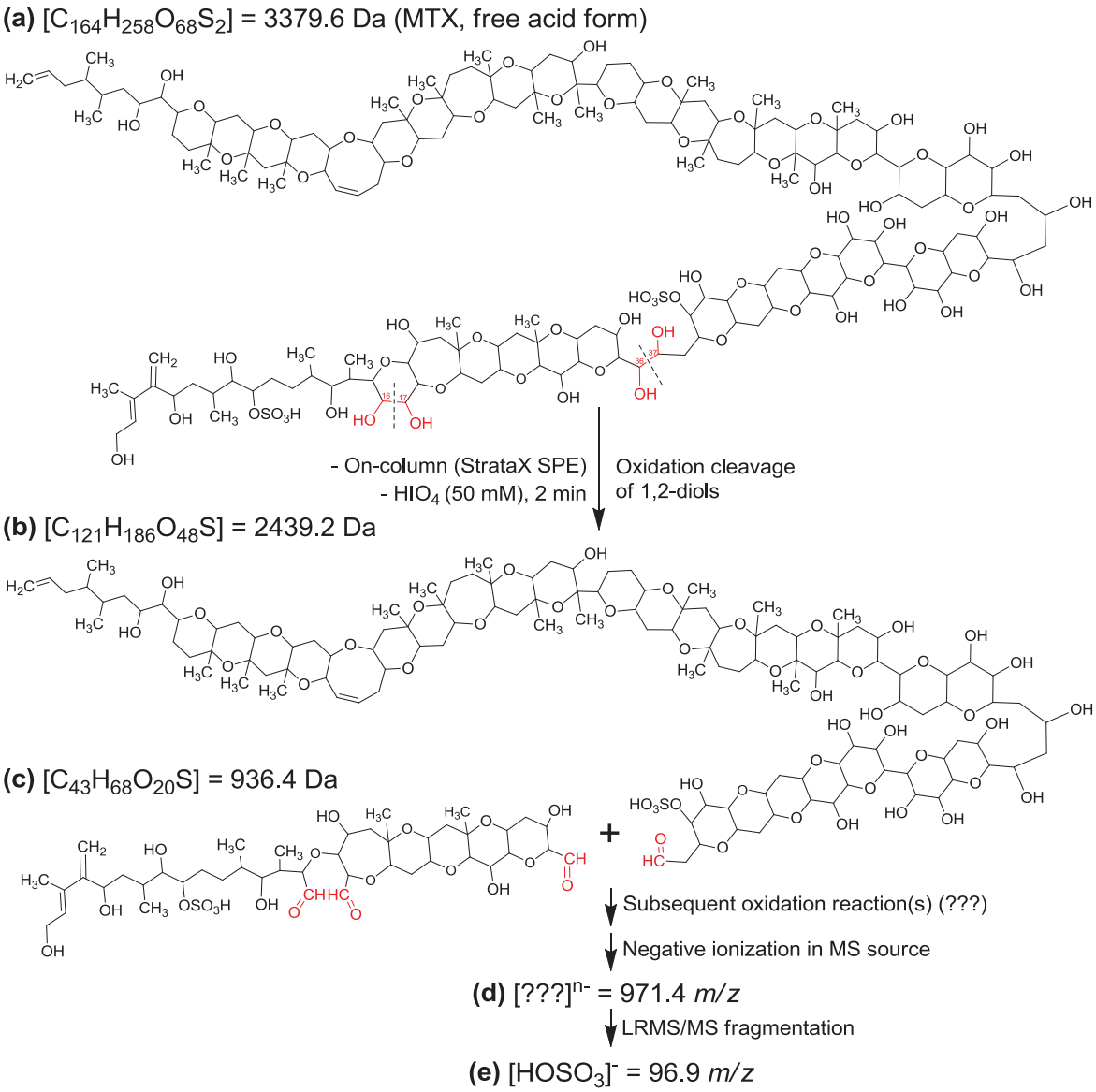


Figure 29. Schematic representation of the oxidative cleavage method for MTX detection in LC-LRMS/MS (Kohli, 2013). **(a)**: intact MTX (free acid form), **(b)** and **(c)**: products of (a) after HIO_4-induced oxidation cleavage. The breakage of carbon-carbon bonds of vicinal diols at C_{16}-C_{17} and C_{36}-C_{37} and the subsequent formation of aldehyde groups are highlighted in red; **(d)**: parent ion detected in LRMS, unknown elemental formula. It may derive from (b) or (c) after subsequent oxidation reaction(s); **(e)**: fragment anion $[HOSO_3]^-$ detected in LRMS/MS analysis.

Chapter 2: Relative toxicity of different species of *Gambierdiscus* and *Fukuyoa*

1. Toxicity screening of 13 *Gambierdiscus* strains using neuro-2a and erythrocyte lysis bioassays

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1.1. Abstract

Species in the epi-benthic dinoflagellate genus *Gambierdiscus* produce ciguatoxins (CTXs) and maitotoxins (MTXs), which are among the most potent marine toxins known. Consumption of fish contaminated with sufficient quantities of CTXs causes Ciguatera Fish Poisoning (CFP), the largest cause of non-bacterial food poisoning worldwide. Maitotoxins, which can be found in the digestive system of fish, could also contribute to CFP if such tissues are consumed. Recently, an increasing number of *Gambierdiscus* species have been identified; yet, little is known about the variation in toxicity among *Gambierdiscus* strains or species.

This study is the first assessment of relative CTX- and MTX-toxicity of *Gambierdiscus* species from areas as widespread as the North-Eastern Atlantic Ocean, Pacific Ocean and the Mediterranean Sea. A total of 13 strains were screened: (i) seven Pacific strains of *G. australes*, *G. balechii*, *G. caribaeus*, *G. carpenteri*, *G. pacificus*, *G. scabrosus* and one strain of an undetermined species (*Gambierdiscus* sp. Viet Nam), (ii) five strains from the North-Eastern Atlantic Ocean (two *G. australes*, a single *G. excentricus* and two *G. silvae* strains), and (iii) one *G. carolinianus* strain from the Mediterranean Sea. Cell pellets of *Gambierdiscus* were extracted with methanol and the crude extracts partitioned into a CTX-containing dichloromethane fraction and a MTX-containing aqueous methanol fraction. CTX-toxicity was estimated using the neuro-2a cytoxicity assay, and MTX-toxicity via a human erythrocyte lysis assay.

Different species were grouped into different ratios of CTX- and MTX-toxicity, however, the ratio was not related to the geographical origin of

species (Atlantic, Mediterranean, Pacific). All strains showed MTX-toxicity, ranging from 1.5 to 86 pg MTX equivalents (eq) cell^{-1}. All but one of the strains showed relative low CTX-toxicity ranging from 0.6 to 50 fg CTX3C eq cell^{-1}. The exception was the highly toxic *G. excentricus* strain from the Canary Islands, which produced 1,426 fg CTX3C eq cell^{-1}. As was true for CTX, the highest MTX-toxicity was also found in *G. excentricus*. Thus, the present study confirmed that at least one species from the Atlantic Ocean demonstrates similar toxicity as the most toxic strains from the Pacific, even if the metabolites in fish have so far been shown to be more toxic in the Pacific Ocean.

Keywords: Ciguatera Fish Poisoning, *Gambierdiscus*, ciguatoxins, maitotoxins, neuro-2a assay, erythrocyte lysis assay.

1.2. Introduction

Dinoflagellates in the genera *Gambierdiscus* and *Fukuyoa* produce ciguatoxins (CTXs) and maitotoxins (MTXs), cyclic polyether neurotoxins that rank in the top five most potent natural toxins isolated to date (Fusetani and Kem, 2009).

Ciguatoxins, like brevetoxins, bind voltage-gated sodium channels (VGSCs) at site 5 on the alpha-subunit causing an influx of Na^+ into affected cells that disrupts cellular function, especially in nerve cells (Benoit et al., 1986; Legrand et al., 1982; Lombet et al., 1987). Ciguatoxins are lipophilic and they could readily accumulate in the marine food chain reaching their highest concentration in fish, as hypothesized by Randall (1958), albeit with considerable lag-time between the bloom of *Gambierdiscus* sp. and CTX-related CFP outbreaks (Chateau-Degat et al., 2005). The genera *Gambierdiscus* and *Fukuyoa* are epi-benthic and are found on many substrates including macro-algae, algal turfs, sea grasses and coral rubble (Parsons and Preskitt, 2007; Rains and Parsons, 2015) but they can also be found in near bottom plankton as shown using moored screens (Tester et al., 2014). Algal turfs appear to be very suitable substrates as support for *Gambierdiscus*, even when compared to macrophytes (Leaw et al., 2016). It is commonly assumed that the primary flux occurs from herbivorous grazers of such macro-algae to carnivorous fish (Ledreux et al., 2014), though other vectors such as crustaceans, echinoderms, and bivalves have been implicated (Kelly et al., 1992;

Laurent et al., 2008; Roué et al., 2016; Silva et al., 2015). During this accumulation process, CTXs are biotransformed, frequently resulting in metabolites of greater toxicities than the algal parent compounds (Lehane and Lewis, 2000). Certain *Gambierdiscus* species also produce other bioactive polyether compounds, such as gambierol (Cuypers et al., 2008; Satake et al., 1993a), gambieric acids (Nagai et al., 1993; Nagai et al., 1992) and gambierone (Rodríguez et al., 2015). The biological activity of gambierone is known to mimic that of CTX3C, although much lower in intensity, whereas the overall toxicity of gambierol and gambieric acids has yet to be characterized. The role, if any, of these three classes of compounds in causing CFP is unknown.

Maitotoxins are amphiphilic molecules that bind non-selective ion channels, causing an influx of Ca^{2+} that significantly raises intracellular Ca^{2+} levels. This is important since Ca^{2+} is one of the major signaling ions in the cell. The increased influx of the ion abnormally activates numerous biochemical pathways, including apoptosis, which disrupt the function of neuronal, muscular and red blood cells (Gusovsky and Daly, 1990; Ogura et al., 1984; Ohizumi and Kobayashi, 1990). Even though MTXs are more toxic than CTXs when injected intraperitoneally into mice, MTXs are less likely to be involved in causing Ciguatera Fish Poisoning (CFP) because of their low capacity to accumulate in fish flesh and their low oral potency as assessed in mice (Yasumoto et al., 1976). Still, a recent study by Kohli et al. (2014) suggests that MTX could accumulate in carnivorous fish (fed in controlled conditions with *Gambierdiscus*-inoculated herbivorous fish), particularly in their digestive tract and liver, and thus MTXs may potentially contribute to CFP. Also, the large diversity of symptoms of CFP observed in different oceans has been suggested to be related to different CTX profiles (Lewis, 2001) but may also potentially be related to differences in consumer habits, e.g. the consumption of the intestinal parts of fish (Gatti et al., 2008; Hamilton et al., 2010). Consequently, the role of MTXs in contributing to CFP still remains to be clarified, in particular whether such contribution may derive from contamination of fish fillets during dissection of ciguateric fish or only from the consumption of visceral tissues of ciguateric fish.

In addition to uncertainties regarding different toxin profiles and the routes of accumulation little is known about the degree to which toxicity varies among species. One reason this has proven challenging is that the taxonomy has only recently been sufficiently resolved to examine species-

specific toxicity (Fraga and Rodríguez, 2014; Fraga et al., 2011; Fraga et al., 2016; Kretzschmar et al., 2016; Litaker et al., 2009; Nishimura et al., 2014; Smith et al., 2016). This taxonomic work includes the separation of the globular *Gambierdiscus* species into the genus *Fukuyoa* (Gómez et al., 2015).

The goal of this study was to characterize the relative toxicity of *Gambierdiscus* strains from the Pacific Ocean, the North-Eastern Atlantic Ocean and the Mediterranean Sea. A total of 13 strains were examined, representing ten known species and one strain for which species annotation is not yet complete (**Table 11, section 1.3.2**). Except two strains (CCMP1653 and the strain from Viet Nam), none of the strains studied had previously been shown to produce any known CTXs or MTXs. Hence, this study examined the strains with a targeted cellular bioassay approach to detect activity of hitherto undescribed congeners of CTXs and MTXs.

1.3. **Materials and Methods**

1.3.1. Reference toxins and chemicals

CTX3C was kindly provided by Mireille Chinain (Institut Louis Malardé, Tahiti) and used as the reference standard for the neuroblastoma neuro-2a (N2a) cytotoxicity assay. MTX was purchased from Wako Chemicals USA, Inc. (Richmond, Virginia, USA) and used as the reference standard for the erythrocyte lysis assay (ELA). CTX3C was dissolved and stored in pure MeOH prior to utilization in the N2a assay. MTX was stored in MeOH:H_2O (1:1, v/v), dried and re-dissolved in ELA buffer prior to utilization in the ELA. HPLC grade methanol and dichloromethane were purchased from Sigma Aldrich (St. Louis, Missouri, USA).

Eagle's Minimum Essential Medium (EMEM, ATCC® 30-2003) for culture of N2a cells was purchased from the American Type Culture Collection. The following additives to the N2a medium were purchased from Sigma Aldrich (St. Louis, Missouri, USA): sodium pyruvate, streptomycin, penicillin and fetal bovine serum. N2a assay reagents were also purchased from Sigma Aldrich: trypsin-(ethylenediaminetetraacetic acid) (trypsin-EDTA) and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT). Saponin for the ELA was purchased from Sigma Aldrich (St. Louis, Missouri, USA).

1.3.2. Laboratory cultures of *Gambierdiscus* spp.

The strains of *Gambierdiscus* which were examined in this study and their location of origin are listed in **Table 11**.

Table 11. Denomination and origin of *Gambierdiscus* strains examined in this study.

Location	Species / Strain	Origin	Culture Collection	Reference
Atlantic Ocean	*G. australes* / VGO1178[b]	Punta Hidalgo, Tenerife, Canary Islands	CCVIEO[(a)]	(Fraga and Rodríguez, 2014)
	G. australes / VGO1181[b]	Punta Hidalgo, Tenerife, Canary Islands	CCVIEO	Sequencing of LSU rDNA (D1-D3 region) (GENBANK KY549925)
	G. excentricus / VGO791[b]	Punta Hidalgo, Tenerife, Canary Islands	CCVIEO	(Fraga et al., 2011)
	G. silvae / VGO1167[b] (species formerly known as *G.* ribotype 1)	Punta Hidalgo, Tenerife, Canary Islands	CCVIEO	(Fraga and Rodríguez, 2014)
	G. silvae / VGO1180[b] (species formerly known as *G.* ribotype 1)	Punta Hidalgo, Tenerife, Canary Islands	CCVIEO	(Fraga and Rodríguez, 2014)
Mediterranean Sea	*G. carolinianus* / Greece Gam2[a]	Crete, Greece	CCFHR[(b)]	Species-specific qPCR assays (Vandersea et al., 2012)
Pacific Ocean	*G. australes* / CCMP1653 (NOAA 24)[b] (strain previously reported as T39 strain)	Tern Island, Hawaii	NCMA[(c)]	(Babinchak et al., 1986; Litaker et al., 2009)
	G. balechii / VGO917[b]	Manado, Celebes Sea, Indonesia	CCVIEO	(Bravo et al., 2014; Fraga et al., 2016)
	G. caribaeus / Bill Hi Gam8[a]	Waikiki Beach, Honolulu, Hawaii	CCFHR	Species-specific qPCR assays (Vandersea et al., 2012)
	G. carpenteri / Pat Hi Jar7 Gam11[a]	Waikiki Beach, Honolulu, Hawaii	CCFHR	Species-specific qPCR assays (Vandersea et al., 2012)
	G. pacificus / CCMP1650 (NOAA 9)[a,b]	Moorea, Society Islands, French Polynesia	NCMA	(Litaker et al., 2009)
	G. scabrosus / KW070922_1[b] (species formerly known as *Gambierdiscus* sp. type 1)	Kashiwa-jima Island, Otsuki, Kochi, Japan	KU[(d)]	(Nishimura et al., 2013; Nishimura et al., 2014)
	Gambierdiscus sp. / Viet Nam[b] (strain reported as *G. toxicus* Vietnam)	Cau Island, Binh Thuan, South China Sea, Viet Nam	VNIO[(e)]	(Roeder et al., 2010)

[a] strains cultured at the CCFHR laboratory (Beaufort, NC, USA) (**section 1.3.2.1**).
[b] strains cultured at the IFREMER laboratory (Nantes, France) (**section 1.3.2.2**).
(a) Culture Collection of Harmful Microalgae of IEO (CCVIEO), Centro de Vigo, Vigo, Spain.
(b) National Oceanographic and Atmospheric Administration (NOAA), Center for Coastal Fisheries Habit Research (CCFHR), Beaufort, NC, USA.
(c) Provasoli – Guillard National Center for Marine Algae and Microbiota (NCMA), Bigelow Laboratory for Ocean Sciences, East Boothbay, Maine, USA.
(d) Kochi University (KU), Kochi, Japan.
(e) Viet Nam National Institute of Oceanography (VNIO, VAST), Vinh Nguyen, Nha Trang, Viet Nam.

Strains of *G. scabrosus* (Nishimura et al., 2014), *G. excentricus* (Fraga et al., 2011), *G. silvae* (Fraga and Rodríguez, 2014) and *G. balechii* (Fraga et al., 2016) all belong to recently described species. Molecular analysis of *Gambierdiscus* sp. Viet Nam, previously reported as *G. toxicus* Vietnam by Roeder et al. (2010), still needs to be completed, and hence it was not assigned to this species.

Culture experiments were conducted using a semi-continuous batch method at both the Phycotoxins laboratory at the French Research Institute for Exploitation of the Sea (IFREMER), Nantes, France and at the National

Oceanic and Atmospheric Administration, Center for Coastal Fisheries and Habitat Research (CCFHR), Beaufort, NC, USA. Cell densities were maintained at levels to ensure the absence of nutrient or CO_2 limitation. At pH > 8.4 cells become progressively more CO_2 limited. Cells of *Gambierdiscus* were harvested in log phase growth. Slight differences in the experimental protocols necessitated by differences in the equipment available at each location are noted below (**sections 1.3.2.1** and **1.3.2.2**). As a control, *G. pacificus* CCMP1650 was grown in both laboratories to determine if growth rate and toxin values obtained in each laboratory were comparable.

1.3.2.1. NOAA CCFHR Laboratory (Beaufort, NC, USA)

Four strains of *Gambierdiscus* (*G. caribaeus* Bill Hi Gam8, *G. carolinianus* Greece Gam2, *G. carpenteri* Pat Hi Jar7 Gam11 and *G. pacificus* CCMP1650) (**Table 11**) were grown in 75 cm^2 tissue culture flasks with vented caps (Falcon®, BD Biosciences, Bedford, MA, USA). Media consisted of 0.2 µm filtered Gulf Stream seawater (at a salinity of 33), vitamins and nutrients were added according to a modified K-medium protocol (Keller and Guillard, 1985; Keller et al., 1987). Phosphate was added in the form of Na_2 *β*-Glycerophosphoric Acid, 5-Hydrate at twice the concentration used for K-medium preparation. An EDTA-trace metal buffer system was used with the omission of copper as described by Hardison et al. (2012). The media was sterilized via microwave treatment as described in Keller et al. (1988). The culture pH was monitored (Thermo Orion 3 star pH meter, Ross ultra-combination pH electrode) to ensure pH range throughout experiments were between 8.1 and 8.4. This ensured the cells were not CO_2-limited. Cultures were maintained in a Percival Scientific incubator (Boone, IA, USA) at 27 °C, under full spectrum lights (Blue Max F20-T12, Full Spectrum Solutions, Mississippi, USA) with an incident photon flux density at 100 µmol photons m^{-2} s^{-1} and a daily light-dark cycle of 12h:12h light:dark (LD). Full spectrum light source was placed in illumination cassettes above the culture flasks. Flasks were randomly placed and the position was changed once a day in order to ensure a homogeneous exposure to light. When the culture reached ~1000-2000 cells mL^{-1}, cells were first retained on a 20 µm sieve, washed with sterile seawater and collected by centrifugation (10 min, 1800 *g*, 20 °C) in 50 mL

Falcon tubes. Cell pellets were stored at -20 °C until further extraction for toxicity screening.

1.3.2.2. IFREMER Laboratory (Nantes, France)

Ten strains of *Gambierdiscus* were cultured in 75 cm^2 culture flasks (Corning® CellBIND®, Grosseron SAS, Coueron, France): *G. australes* CCMP1653, VGO1178 and VGO1181, *G. excentricus* VGO791, *G. pacificus* CCMP1650, *G. scabrosus* KW070922_1, *G. silvae* VGO1167 and VGO1180, *G. balechii* VGO917 and *Gambierdiscus* sp. Viet Nam (**Table 11**). Media consisted of filtered (0.2 µm) natural Mediterranean seawater (at a salinity of 33) enriched with L1 nutrients with the exception of silica (Guillard and Hargraves, 1993). Cultures were maintained in a growth chamber incubator (Binder KBW240, Binder GmbH, Tuttlingen, Germany) at 25 °C, under the same light conditions described above. After three weeks of semi-continuous culture, cells were harvested by centrifugation (20 min, 3000 *g*, 4 °C) in 50 mL Falcon tubes and cell pellets were stored at -20 °C until further extraction for toxicity screening.

1.3.3. Maximum growth rate determination

In order to determine the maximum growth rates during the exponential growth phase, *Gambierdiscus* cells were grown in semi-continuous batch cultures as previously described by Hardison et al. (2012). Briefly, all the cultures were acclimated to the culture conditions specific to each of the two laboratories (**sections 1.3.2.1** and **1.3.2.2**) for several months prior to experimentation. Cultures of *Gambierdiscus* cells were inoculated in 200 mL of culture medium at an initial concentration of ~100-200 cells mL^{-1} and incubated at randomly determined sites in the incubator which were rotated daily. Cells were kept in the exponential growth phase as follows: cultures were transferred to new medium (1 to 10 dilution) when cell concentration reached ~1000-2000 cells mL^{-1} and, thus, they never experienced nutrient or CO_2 limitation. An aliquot of culture was taken every 3-4 days during a period of 53-78 days (n = 13-15 samplings, at least three generations) and analyzed for cell concentration (cells mL^{-1}) and mean cellular biovolume (Estimated Spherical Volumes, ESV, μm^3 $cell^{-1}$) using a Multisizer™ 3 Coulter Counter® (Beckman Coulter, Georgia, USA) particle counter equipped with a 280 µm aperture tube and a 1 mL sample volume. The total volume of cells per liter of culture media

(biovolume) was then calculated. Maximum growth rate (μ_{max}, d^{-1}) was the slope calculated by the linear regression of the natural logarithm of the biovolume versus time, after correcting for serial culture dilutions (Sunda and Hardison, 2007). SigmaPlot software (version 12.5) was used to calculate regression slopes and associated relative standard error and R^2 values. Maximum growth rate (μ_{max}, divisions day^{-1}) was then calculated as follows: μ_{max} (divisions day^{-1}) = μ_{max} (d^{-1}) $ln(2)^{-1}$.

1.3.4. Toxin extraction and liquid-liquid partitioning

Cultures of each strain have been grown in three separate flasks. After the cells had been harvested in log phase growth, they were suspended in MeOH (30 mL per 1 million cells) and disrupted using sonication (CCFHR laboratory) or bead beating (IFREMER laboratory). Sonication was conducted twice for 1 min at 50% of total power (500 W) using a 3 mm diameter probe sonicator (Q-Sonica, Q700, Newtown, Connecticut USA). Grinding with the bead-mill was conducted twice for 30 min at a vibration frequency of 30 Hz using a mixer mill (Retsch MM400, Germany) with glass beads (0.25 g, diameter 250-500 µm) (Serive et al., 2012). Completeness of cell disruption was verified using light microscopy. Crude extracts (CEs) were blown dry under N_2 gas at 40 °C. The residue was suspended in MeOH:H_2O (3:2, v/v) (25 mL per 1 million cells) and partitioned twice with dichloromethane (DCM) (50 mL per 1 million cells) as previously described by Satake et al. (1993b). The lipophilic CTXs were partitioned into the DCM soluble fraction (DSF) while the amphiphilic MTXs were partitioned into the aqueous methanol (aq. MeOH) soluble fraction (MSF). Once the DSF and MSF fractions were isolated, they were blown dry under N_2 gas at 40 °C and stored at -20 °C. Just prior to the bioassays, the dried DCM and aq. MeOH residues were re-dissolved in MeOH or MeOH:H_2O (1:1, v/v), respectively. An aliquot of the hydrophilic fraction was then evaporated (N_2 gas at 40 °C) and stored at -20 °C until use in the human erythrocyte lysis assay (ELA). Just prior to running the ELA the dried residue from the MSF fraction was dissolved in ELA buffer.

1.3.5. Neuroblastoma neuro-2a assay

The neuroblastoma neuro-2a (N2a) cell line is frequently used to estimate levels of CTXs in fish, shellfish or phytoplankton extracts (Pawlowiez et al., 2013). The N2a cytotoxicity assay developed by Manger

et al. (1993) and modified by Dickey et al. (1999) was performed at CCFHR laboratory (Beaufort, NC, USA), with some modification (Hardison et al., 2016). Ciguatoxins do not induce N2a cell death, however, when N2a cells are pre-incubated with ouabain (O) and veratridine (V) they become highly sensitive to sodium channel activator toxins. Assays were set up so that the N2a cells are exposed to partially purified cell extracts with and without O and V. If cell death occurs in the samples without O and V it indicates the presence of a non-specific toxic compound other than a sodium channel activator. The details of the assay were as follows.

The N2a cell line was obtained from the American Type Culture Collection (ATCC, CCL 131). Neuro-2a cells were grown and maintained as described by Hardison et al. (2016). The assay was carried out in 96-well flat-bottom CELLCOAT® tissue culture plates with Poly-D-Lysine coating (Greiner Bio-One, Kremsmünster, Austria). Plates were seeded with 30,000 N2a cells per well and were incubated for 24 h until they were >90% confluent at the bottom of each well. The CTX3C standard, controls and *Gambierdiscus* samples were added next and incubated for 24 h. The standard curve was added in presence of O/V (250 µM and 25 µM, respectively) at 50% cell viability to increase sensitivity and specificity to CTXs. The CTX3C standard curve for this assay ranged from 0.001 to 2,000 pg mL^{-1}. A sigmoidal dose-response curve was plotted and an EC_{50} of 1.66 ± 0.16 (SD, n=12) pg CTX3C mL^{-1} was calculated using GraphPad Prism 6.0 (**Figure 34**) (Hardison et al., 2016). Controls included buffer wells to provide maximum survival estimates and wells with the addition of 1% MeOH (final concentration in well) to identify any cell mortality caused by the presence of MeOH used to dissolve the dried extracts. Half of the sample aliquots (1 µL additions) from each assay were processed in the presence of O/V (O/V^+) so they were directly comparable to the CTX3C standard curve. The other half was incubated without O/V (O/V^-) to identify non-specific mortality caused by other compounds in the sample. Total well volume was 100 µL. No more than 500 *Gambierdiscus* cell equivalents were added to each well to avoid matrix effects or non-specific N2a cell death. Each of the three replicate samples was run in duplicate in the N2a assay. Cell viability was assessed after 24 h incubation using the quantitative colorimetric 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) assay (Mosmann, 1983) using a FLUOstar® Omega microplate reader (BMG Labtech, Germany) at 544 nm. As the cells

treated with O/V showed 50% of cell viability relative to control cells in solvent vehicle (1% MeOH in N2a medium), the viability of cells treated with CTX3C standard or algal extracts was estimated relative to O/V^+ wells. Quantitation of CTX3C eq in the samples using the N2a assay was operated within a range from 20% to 80% N2a cell viability with respect to the O/V^+ wells. The limit of quantification (80% N2a cell viability) was 0.197 ± 0.005 (SD, n=12) ng CTX3C mL^{-1}. When N2a cell viability was lower than 20%, a ten-fold dilution of the sample (in MeOH) was made. Extracts or strains showing activity with this assay will be referred to as "containing CTXs" or showing "CTX-toxicity", even though this is a simplification as other compounds may also show sodium specific activity using this model.

1.3.6. Erythrocyte lysis assay

The erythrocyte lysis assay (ELA), developed by Eschbach et al. (2001) was performed at the CCFHR laboratory (Beaufort, NC, USA), with some modifications (Holland et al., 2013) to estimate the hemolytic activity of *Gambierdiscus* strains. This assay is based on lysis of erythrocytes due to hemolytic compounds and subsequent photometrical determination of the released hemoglobin. In the context of marine dinoflagellates, the ELA has broadly been used on different red blood cell lines to detect hemolytic compounds from different microalgae such as *Alexandrium* (Tatters et al., 2012), *Karenia* (Tatters et al., 2010), *Ostreopsis* cf. *ovata* (Nascimento et al., 2012a) and *Gambierdiscus* (Holland et al., 2013).

Human red blood cells (hRBCs) were obtained from the Red Cross (Durham, North Carolina, USA). The hRBCs treated with saponin provided the maximal hemolysis (100% lysis) used to normalize the hemolytic activity of MTX standard or the diluted algal extracts. The hRBCs incubated solely in ELA buffer served as negative control (0% lysis). Details of how the assay was performed can be found in Holland et al. (2013). The 8-point MTX standard curve for this assay ranged from 0.0002 to 2,000 ng mL^{-1} using purified MTX from Wako Chemicals USA, Inc. (Richmond Virginia, USA). Four replicate wells were used for each of the eight concentrations. A sigmoidal dose-response curve was plotted and an EC_{50} of 14.2 ± 3.3 (SD, n=4) ng MTX mL^{-1} was calculated using GraphPad Prism 6.0 (**Figure 35**). The minimum hemolytic activity observed was 1.51 ± 0.39 (SD, n=4) % hemolysis when hRCBs were exposed to 1.0 ng MTX mL^{-1}. For each *Gambierdiscus* MSF sample, six dilutions were tested and three

replicate wells for each dilution were run. Sigmoidal dose-response curves were plotted and EC_{50} values (cell eq mL^{-1}) were calculated for each strain using GraphPad Prism 6.0. Quantitation of MTX eq in the samples using the ELA was operated converting EC_{50} values (cell eq mL^{-1}) for each strain into toxin equivalent per cell (pg MTX eq $cell^{-1}$) taking into account the EC_{50} value obtained from the MTX standard curve. Erythrocyte lysis was assessed after 24h incubation at 4 °C using a FLUOstar® Omega microplate reader (BMG Labtech, Germany) at 415 nm. Extracts or strains showing activity with this assay will be referred to as "containing MTXs" or showing "MTX-toxicity", even though this is a simplification as other compounds may also show hemolytic activity using this model.

1.3.7. Statistical analysis

Statistical analysis was performed using RStudio (Version 0.99.903) (http://www.rstudio.com) utilizing the R statistical language version 3.3.1 (https://www.R-project.org).

Multiple and linear regression models were obtained using basic functions in R on a data matrix including all qualitative information and quantitative values measured for each replicate on all strains. This corresponded to 42 observations of 7 variables [origin, laboratory, species, μ_{max} (divisions day^{-1}), biovolume (ESV, μm^3 $cell^{-1}$), fg CTX3C eq $cell^{-1}$ (DSF) and pg MTX eq $cell^{-1}$ (MSF)]. For comparison purpose, the same analyses were performed on the same data matrix with the outlier strain (*G. excentricus* VGO791) excluded, which allowed for showing consistent correlations.

Unsupervised clustering of the strains based on the mean centered and normalized values of biovolume (ESV, μm^3 $cell^{-1}$), fg CTX3C eq $cell^{-1}$ (DSF) and pg MTX eq $cell^{-1}$ (MSF) was performed by 1) calculating the distance matrix between each observation (strain) using the Euclidean distance 2) executing a hierarchical cluster analysis using the Ward's minimum variance method. The result was displayed as a cluster dendrogram, one replicate of *Gambierdiscus* sp. Viet Nam being excluded for this analysis as it presented very dissimilar results to other two replicates.

1.4. **Results**

1.4.1. Maximum growth rates and cellular biovolumes

Maximum growth rates (μ_{max}) of *Gambierdiscus* in culture ranged from 0.099 to 0.244 divisions day^{-1}, depending on the strain (**Table 12**). The slowest growing strains were *G. excentricus* VGO791 and *G. balechii* VGO917 (IFREMER laboratory conditions), while the fastest growth was observed for *G. pacificus* CCMP1650 (CCFHR laboratory conditions).

Table 12. Maximum specific growth rates (μ_{max}, divisions day^{-1}) and per-cell CTX- and MTX-toxicity of the *Gambierdiscus* strains cultivated in this study.

Location	Species / Strain	μ_{max} (divisions day^{-1}) ± RSD	R^2	n	$days_{tot}$	ESV ± RSD (n=3) (µm^3 cell^{-1} x 10^5)	DSF: fg CTX3C eq cell^{-1} ± SD (n=3)	MSF: pg MTX eq cell^{-1} ± SD (n=3)
Atlantic Ocean	*G. australes* / VGO1178	ND				1.57 ± 0.3%	1.4 ± 0.4	4.3 ± 1.2
	G. australes / VGO1181	ND				1.24 ± 1.2%	0.6 ± 0.3	4.7 ± 2.8
	G. excentricus / VGO791	0.099 ± 2.59%	0.993	13	67	2.69 ± 1.3%	1,426 ± 55	85.7 ± 41.5
	G. silvae / VGO1167	ND				0.93 ± 7.6%	10.3 ± 2.7	2.2 ± 0.3
	G. silvae / VGO1180	ND				1.02 ± 0.7%	12.4 ± 3.6	3.3 ± 0.9
Mediterranean Sea	*G. carolinianus* / Greece Gam2[a]	0.129 ± 4.77%	0.971	15	78	1.55 ± 0.2%	3.3 ± 0.6	10.6 ± 0.4
Pacific Ocean	*G. australes* / CCMP1653	0.149 ± 4.29%	0.980	13	53	1.37 ± 0.5%	2.7 ± 0.9	5.0 ± 0.5
	G. balechii / VGO917	0.100 ± 4.46%	0.979	13	53	0.82 ± 0.5%	3.4 ± 1.5	19.9 ± 2.9
	G. caribaeus / Bill Hi Gam8[a]	0.175 ± 1.81%	0.996	15	78	1.64 ± 2.4%	1.6 ± 1.0	5.3 ± 1.0
	G. carpenteri / Pat Hi Jar7 Gam11[a]	0.141 ± 5.59%	0.964	14	68	2.24 ± 0.8%	1.4 ± 0.6	6.3 ± 1.9
	G. pacificus / CCMP1650	0.226 ± 5.07%	0.950	13	53	0.74 ± 0.7%	12.1 ± 0.0	20.1 ± 5.9
	G. pacificus / CCMP1650[a]	0.244 ± 2.59%	0.991	15	73	0.70 ± 2.2%	13.5 ± 0.4	20.3 ± 1.6
	G. scabrosus / KW070922_1	0.140 ± 1.84%	0.996	15	77	1.04 ± 0.4%	27.9 ± 3.8	1.5 ± 0.2
	Gambierdiscus sp. / Viet Nam	0.124 ± 2.42%	0.992	15	74	1.21 ± 4.0%	40.8 ± 19.6	70.0 ± 44.8

[a] cultured at the CCFHR laboratory (Beaufort, NC, USA) (**section 1.3.2.1**). The other strains were cultured at the IFREMER laboratory (Nantes, France) (**section 1.3.2.2**).
$days_{tot}$ = the total duration of the culture throughout the study expressed in days; DSF = dichloromethane soluble fraction; MSF = aqueous methanol soluble fraction; ESV = Estimated Spherical Volume (Multisizer™ 3 Coulter Counter®); ND = not determined; RSD = Relative Standard Deviation; SD = Standard Deviation.

Interestingly, *G. excentricus* VGO791, which was the slowest growing strain, had the largest cellular biovolume (**Table 12**). *G. pacificus* (CCMP1650), the species with the smallest biovolume (3.8-fold < *G. excentricus*) was the fastest growing species (**Table 12**). Notwithstanding, overall the correlation was poor between growth rate and cellular volume, e.g. *G. balechii* had a similar growth rate as *G. excentricus* but a substantially smaller cellular biovolume. The overall low growth rates (< 0.5

divisions day^{-1}) observed in this study are consistent with those previously reported in the literature (Kibler et al., 2012; Litaker et al., *submitted to PLoS One on Feb 2017*; Xu et al., 2016; Yoshimatsu et al., 2014). If a comparison could be made, it can be concluded that, in the present study, *Gambierdiscus* strains behaved as slow-growers when cultured under CCHFR and IFREMER laboratory conditions, with μ_{max} < 0.25 divisions day^{-1}, which appeared similarly low or somewhat lower than those reported in other studies.

1.4.2. Screening of DSF toxicity using the neuro-2a assay

Figure 34 (**section 1.8**) shows the sigmoidal dose-response curve of CTX3C standard on the neuro-2a assay (Hardison et al., 2016).

For each strain, both dichloromethane soluble fraction (DSF, a fraction expected to contain CTXs) and the corresponding crude extract (CE) were tested on the neuro-2a assay. All DSFs tested were found to enhance the ouabain/veratridine (O/V) mediated cell mortalities consistent with CTX activation of voltage-gated sodium channels (VGSCs). There was no enhanced mortality without addition of O/V, indicating absence of non-specific toxicity after the initial purification step. In contrast, CEs showed substantial non-specific mortality (cell death in the absence of O/V), indicating the presence of bioactive compounds other than VGSC activators such as MTXs or other toxic algal compounds. Thus, the quantitative estimation of CTXs was only possible in DSFs. Results were expressed in CTX3C equivalents (eq) per cell (**Figure 30**).

Only the one *G. excentricus* (VGO791) strain examined from the Canary Islands exhibited a high level of CTX-type toxicity, i.e. 1,426 fg CTX3C eq cell^{-1}. The CTX content for all the other strains examined fell into the range of 0.6-40.8 fg CTX3C cell^{-1} (**Figure 30**).

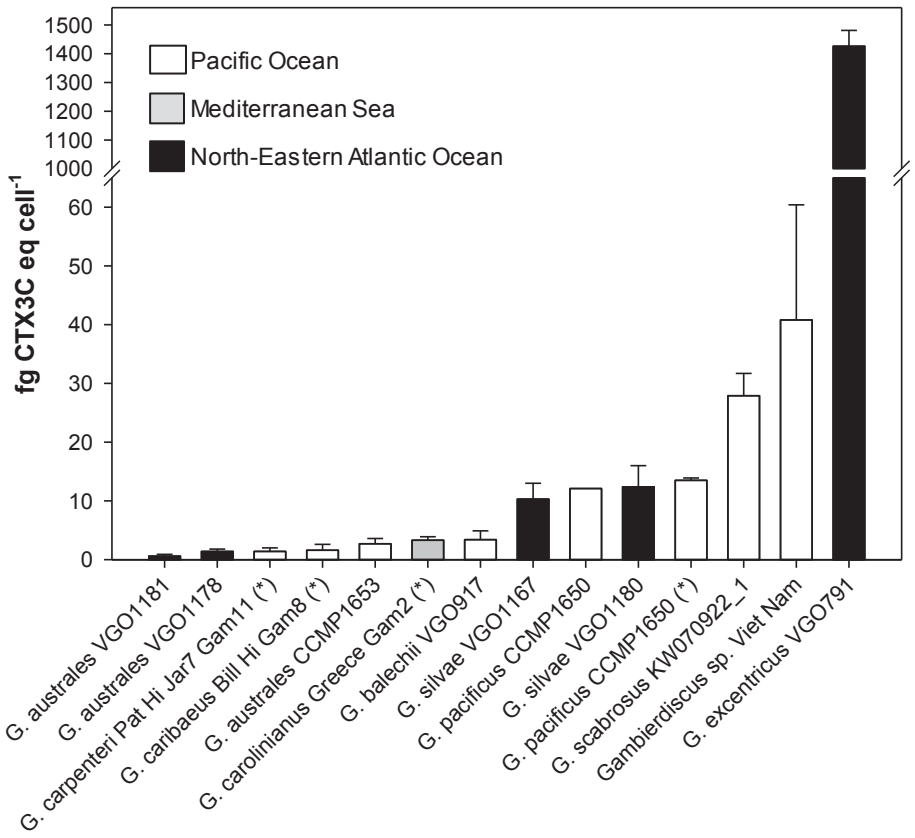

Figure 30. Neuro-2a (N2a) cytotoxicity of dichloromethane soluble fractions (DSFs, n=3) of 13 *Gambierdiscus* strains. Results are expressed in fg CTX3C eq cell^{-1}. (*) cultured at the CCFHR laboratory (**section 1.3.2.1**). The other strains were cultured at the IFREMER laboratory (Nantes, France) (**section 1.3.2.2**).

Among the Pacific strains examined in this study, the Vietnamese strain, representing an as of yet undescribed species, showed the highest N2a cytotoxicity followed by the Japanese *G. scabrosus* strain. The two *G. silvae* strains showed low CTX-type toxicity equivalent to that of *G. pacificus*, i.e. around 10 fg CTX3C eq cell^{-1}. The Mediterranean strain of *G. carolinianus* showed background levels of CTX-type activity (< 4 fg CTX3C eq cell^{-1}) similar to the Pacific *G. caribaeus*, *G. carpenteri* and *G. australes* strains. Interestingly, *G. australes* strains originating either from the Pacific or the North-Eastern Atlantic Oceans, showed similar CTX-type activity (**Figure 30**).

The strain of *G. pacificus* (CCMP1650) from French Polynesia showed similar levels of CTX-toxicity in cultures from both IFREMER and CCFHR, despite the differences in culture conditions and extraction procedure (**Table 12**). Thus, the differences in culture and extraction techniques between the two laboratories appear to not have affected the results.

1.4.3. Screening of MSF toxicity using the erythrocyte lysis assay

Figure 35 (**section 1.8**) shows the sigmoidal dose-response curve of MTX standard on the human erythrocyte lysis assay (ELA).

Figure 31 shows the MTX-type activity of the 13 strains of *Gambierdiscus* evaluated using the ELA.

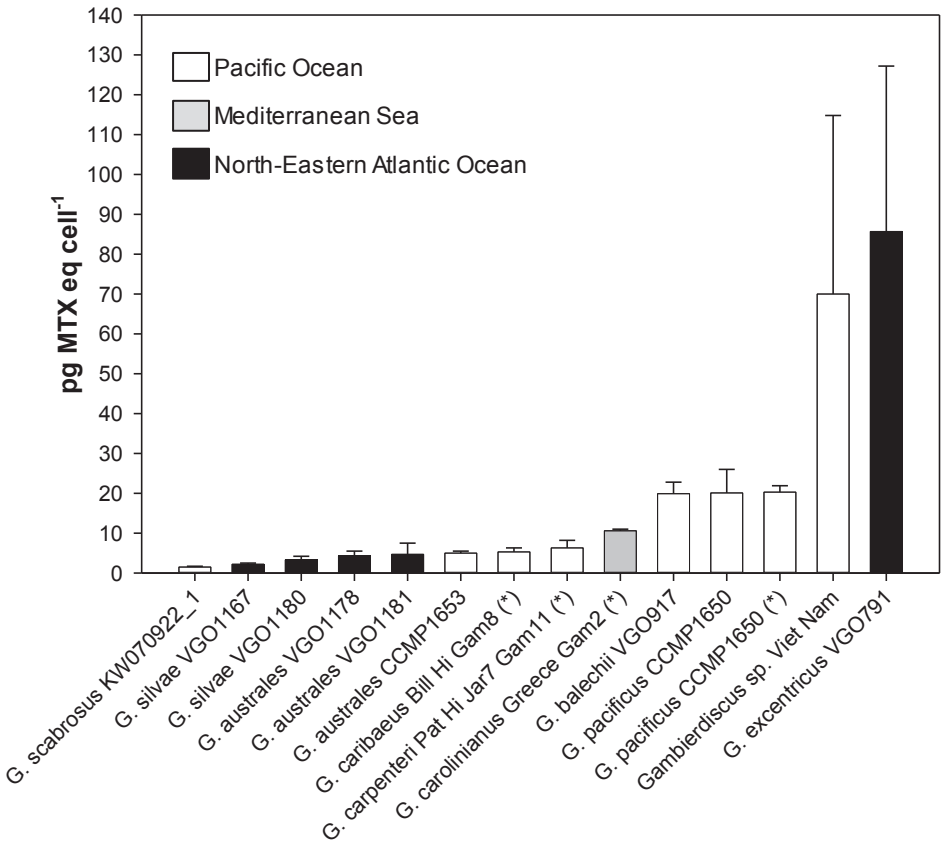

Figure 31. Hemolytic activity of aq. MeOH soluble fractions (MSFs, n=3) of 13 *Gambierdiscus* strains evaluated by means of a human erythrocyte lysis assay (ELA). Results are expressed in pg MTX eq cell^{-1}. (*) cultured at the CCFHR laboratory (**section 1.3.2.1**). The other strains were cultured at the IFREMER laboratory (Nantes, France) (**section 1.3.2.2**).

The strain of *G. excentricus* exhibited the highest hemolytic activity, followed by the Vietnamese strain. The Mediterranean strain of *G. carolinianus* was intermediate and about 5-fold more toxic than the two Atlantic strains of *G. silvae.* The Japanese strain of *G. scabrosus* showed the lowest hemolytic activity among all the strains tested in this study. Also, *G. australes*, *G. caribaeus*, and *G. carpenteri* species showed low MTX-type activity. Interestingly, among the latter ones, *G. australes* strains, originating either from Pacific or the North-Eastern Atlantic Oceans,

showed the same MTX-type activity. Overall, though variable, all strains tested showed measurable MTX-toxicity.

As in the evaluation of CTX3C equivalent toxicity, *G. pacificus* (CCMP1650) showed similar levels of MTX-toxicity in cultures from both IFREMER and CCFHR, despite the differences in culture conditions and extraction procedure (**Table 12**).

1.4.4. Relationship between CTX and MTX toxicity

The CTX toxin content per unit biovolume varied over four orders of magnitude compared to a 10-fold variation in MTX toxicity per unit biovolume (**Figure 32**). A similar pattern was observed when the data were normalized on a per cell basis. Based on a hierarchical cluster analysis (**section 1.3.7**), strains could be classified into three different groups: group I (*G. excentricus* VGO791), group II (*G. australes* CCMP1653, VGO1178, VGO1181, *G. caribaeus* Bill Hi Gam8, *G. carolinianus* Greece Gam2 and *G. carpenteri* Pat Hi Jar7Gam11) and group III (*G. balechii* VGO917, *G. pacificus* CCMP1650, *G. silvae* VGO1167, VGO1180, *G. scabrosus* KW070922_1 and *Gambierdiscus* sp. Viet Nam) (**Figure 33**). Strains of a given species classified into the same group.

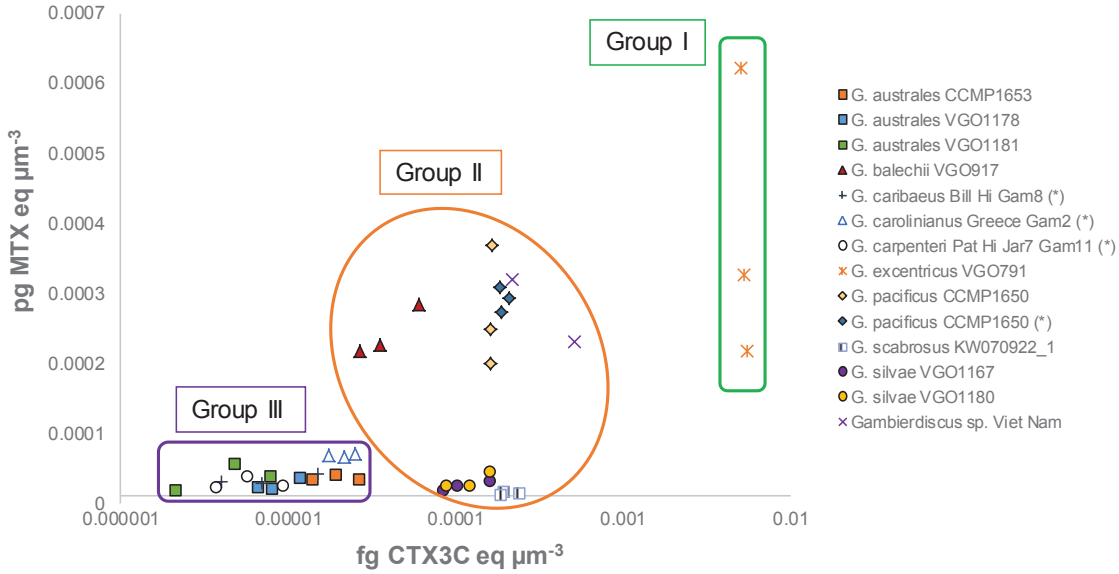

Figure 32. Plot of log CTX toxicity (fg CTX3C eq µm^{-3}) versus MTX toxicity (pg MTX eq µm^{-3}). (*) cultured at the CCFHR laboratory (**section 1.3.2.1**). The other strains were cultured at the IFREMER laboratory (Nantes, France) (**section 1.3.2.2**).

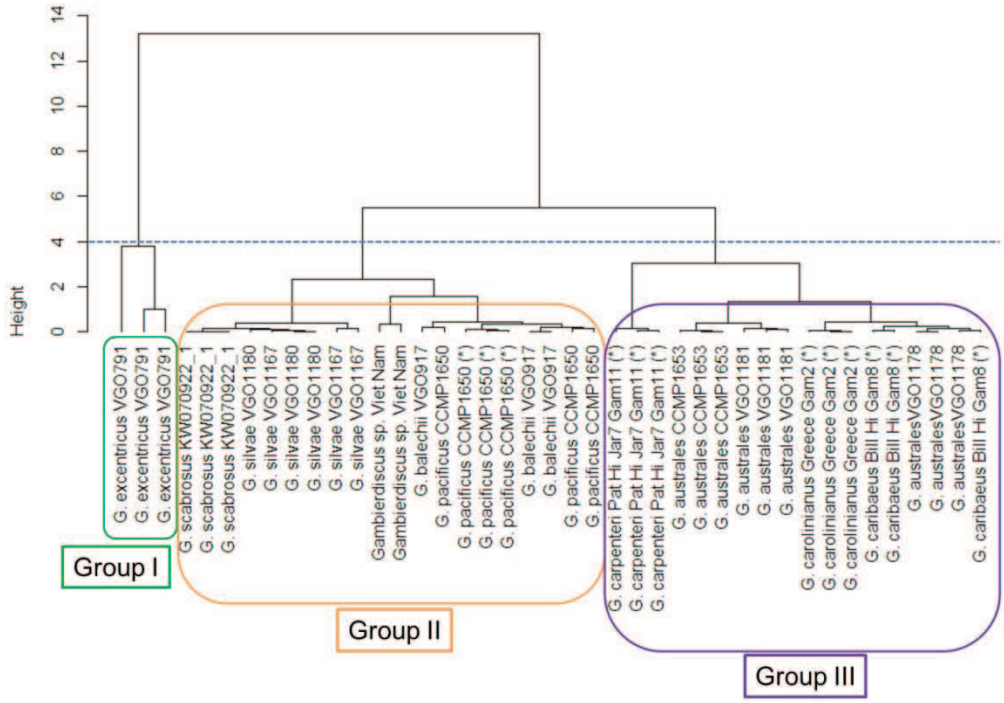

Figure 33. Dendrogram of a hierarchical cluster analysis of 13 *Gambierdiscus* strains based on the following three variables: CTX-toxicity in the DSF (fg CTX3C eq cell^{-1}), MTX-toxicity in the MSF (pg MTX eq cell^{-1}) and cell biovolume (µm^3 cell^{-1}). (*) cultured at the CCFHR laboratory (**section 1.3.2.1**). The other strains were cultured at the IFREMER laboratory (Nantes, France) (**section 1.3.2.2**).

1.5. Discussion

1.5.1. CTX-toxicity

The present study showed that *Gambierdiscus* species examined from the Pacific Ocean, North-Eastern Atlantic Ocean and Mediterranean Sea exhibited marked differences in toxicity ranging from 0.6 fg to > 1400 fg CTX3C equivalents (eq) cell^{-1} (**Figure 30**). The greatest toxicity was exhibited by *G. excentricus* (VGO791) from the Canary Islands. This result is consistent with a previous study reporting *G. excentricus* strains exhibiting between 0.37 - 1.10 pg CTX1B eq cell^{-1} (Fraga et al., 2011), which would be equivalent to 1.17 - 3.49 pg CTX3C eq cell^{-1} according to Bottein Dechraoui et al. (2007). The Canary Islands, where these strains were obtained, are a temperate region (North-Eastern Atlantic Ocean) from which Ciguatera Fish Poisoning (CFP) has recently been reported (Boada et al., 2010; Pérez-Arellano et al., 2005). More recently, *G. excentricus* has been found in Brazil, its contribution to CFP in the region has yet to be evaluated (Nascimento et al., 2012b; Nascimento et al., 2015). The observation of CFP in the Canary Islands is important because CFP is

typically considered a tropical disease (Lewis, 2001). Thus, *G. polynesiensis* and *G. excentricus* could be considered as primary toxin producing species in the South Pacific and the Eastern Atlantic Oceans, respectively (Chinain et al., 2010; Rhodes et al., 2014; Rhodes et al., 2016).

In contrast, *G. australes*, *G. balechii*, *G. carolinianus*, *G. carpenteri*, *G. pacificus* and *G. silvae* all had toxicities below 50 fg CTX3C eq cell^{-1} (**Figure 30**). In a separate study on Caribbean strains, *G. belizeanus*, *G. caribaeus*, *G. carolinianus*, *G. carpenteri*, *Gambierdiscus* ribotype II, *G. silvae* and *F. ruetzleri* have similarly low toxicities (< 20 fg CTX3C eq cell^{-1}) (Litaker et al., *submitted to PLoS One on Feb 2017*). Further, *G. carolinianus* and *G. carpenteri* strains from the Caribbean, Mediterranean and Pacific, as well as *G. silvae* and *G. australes* strains originating either from the Caribbean, Pacific or the North-Eastern Atlantic Oceans showed similar CTX-type activity, suggesting that each species produce comparable levels of toxin worldwide. The data presented here also indicate that most *Gambierdiscus* species produce relatively low levels of CTXs. This low level of toxin production raises an important question concerning the degree to which these low toxicity species contribute to the overall toxin flux into the food chain relative to the high toxicity species *G. excentricus* and *G. polynesiensis* (Litaker et al., *submitted to PLoS One on Feb 2017*). As these low toxicity species can only be of public health importance if they are able to bloom, it will be important to map abundances of the different *Gambierdiscus* species in the field.

The CTX-toxicity results from this study were consistent with other studies on individual species. The strain of *Gambierdiscus* sp. Viet Nam (previously reported as *G. toxicus* based on morphology), for example, showed the highest toxicity among all the Pacific strains examined (**Figure 30**) and has been shown to produce several CTX congeners (Roeder et al., 2010). Nishimura et al. (2013) similarly detected the CTX-like toxicity of the DSF fraction from the Japanese strain of *G. scabrosus* (species previously reported as *Gambierdiscus* sp. type 1), KW070922_1, using the mouse bioassay (MBA), i.e. 20 x 10^{-4} MU/1,000 cells. These toxicities are relatively high compared to most other species, but still low compared to *G. excentricus* or *G. polynesiensis,* consistent with the findings in this study.

The three *G. australes* strains in this study were in the same low range of toxicity, independently of their origin (Canary Islands and Hawaii). Of these strains, comparable toxicity data are only available for the Pacific CCMP1653 strain, previously reported as T39. For this particular strain, Babinchak et al. (1986) reported high toxicity of the crude extract using the MBA. Later on, a more specific assay described by Van Dolah et al. (1994) (radio-labelled brevetoxin ([^{3}H]BTX-3) displacement assay) was conducted on CCMP1653, showing from no detectable to low Na^+ ion channel activity indicating low toxicity (Sperr and Doucette, 1996). More recently, LC-MS studies conducted by Roeder et al. (2010) on CCMP1653 showed the presence of one CTX congener. Chinain et al. (2010) reported comparatively low toxicity for six *G. australes* strains originating from French Polynesia, ranging from < 0.016 (LOD) to 0.030 pg CTX3C eq cell^{-1}. Rhodes et al. (2010) found intermediate N2a cytotoxicity at sub-pg range (0.13 pg CTX3C eq cell^{-1}) for the *G. australes* CAWD149 strain (Cook Islands). Nishimura et al. (2013) reported DSF-toxicity (MBA) for a Japanese *G. australes* strain (S080911_1) of 670 x 10^{-4} MU/1,000 cells, comparable to the highly toxic *G. polynesiensis* species, i.e. 800-1500 x 10^{-4} MU/1,000 cells (Chinain et al., 1999). Such difference in toxicity between strains suggests that a larger number of strains are needed to assess intraspecific variations in CTX toxicity.

Chinain et al. (2010) observed that the slow growing species examined in their study, *G. polynesiensis* exhibited the highest level of toxicity and hypothesized that slower growing species were more toxic. In this study, *G. excentricus* was the slowest growing species and it also showed by far the highest CTX and MTX toxicity (1.4 pg CTX3C eq cell^{-1} and 86 pg MTX eq cell^{-1}) (**Figure 30**, **Figure 31**; **Table 12**). A study of *Gambierdiscus* species found in the Caribbean showed an inverse exponential relationship between CTX toxicity on a per-cell and per-biovolume basis consistent with the Chinain et al. (2010) hypothesis (Litaker et al., *submitted to PLoS One on Feb 2017*). The species *G. balechii* however appears an exception to this rule as it had a comparable growth rate to *G. excentricus* but was substantially less toxic (**Figure 30**, **Figure 32**).

1.5.2. MTX-toxicity

The MTX-toxicity of *Gambierdiscus* strains varied more than 50-fold (1.5 - 86 pg MTX eq cell^{-1}). The most maitotoxic species was *G. excentricus* (VGO791; ~80 pg MTX eq cell^{-1}) followed by the Vietnamese strain (*Gambierdiscus* sp. Viet Nam; ~70 pg MTX eq cell^{-1}). The variability was highest for the Vietnamese strain (64% RSD) with one replicate giving a much higher result that the other two, followed by variability for *G. excentricus* (48% RSD). Additional trials with more replication are needed before concluding the toxicity of these species is comparable. The measured MTX-toxicity for *G. excentricus* was lower than the ~600 pg MTX eq cell^{-1} estimated for the strains VGO790, VGO791 and VGO792 (Fraga et al. 2011) using a modified neuro-2a assay (Caillaud et al., 2010). The MTX content however was estimated using a crude extract whereas MTX-toxicity in the present study was estimated in the aqueous methanol extracts, i.e. after liquid-liquid partitioning. Possible explanations of this discrepancy include differences in the assay applied (N2a vs. ELA) and sources of standard (MTX from different sources). Similarly, the G10DC strain of *G. pacificus*, isolated from Malaysia, was estimated to have a toxicity of 50.2 pg MTX eq cell^{-1} (Caillaud et al., 2011), 2.5 times more toxic than results from the present study for CCMP1650 strain. The three *G. australes* strains assayed in this study showed low MTX-toxicity (< 5 pg MTX eq cell^{-1}) consistent with the low toxicity of *G. australes* RAV-92 strain of from Raivavae Island (Australes Archipelago) measured by MBA (Chinain et al., 1999). *G. scabrosus* strain (KW070922_1) showed a similarly low hemolytic activity (1.5 pg MTX eq cell^{-1}) consistent with the observed MSF fraction toxicity of 67 x 10^{-4} MU/1,000 cells (Nishimura et al. 2013).

1.5.3. Ratio CTX/MTX-toxicity

It should be noted that all the strains examined in this study produced measureable quantities of both CTXs and MTXs (**Figure 30**, **Figure 31**). The variation among species, however, was not consistent for the two groups of toxins. CTX-toxicity per unit biovolume varied over three orders of magnitude among species whereas the MTX-toxicity varied over one order of magnitude (**Figure 32**).

Based on a hierarchical cluster analysis (**section 1.3.7**), strains could be classified into three different groups (**Figure 33**). As visually

suggested in **Figure 32**, there is a correlation between CTX and MTX contents (on a per cell basis, in DSF and MSF fractions, respectively): the Pearson correlation coefficient for linear regression was $R^2 = 0.45$ ($P < 0.001$) when including *G. excentricus*, and $R^2 = 0.23$ ($P < 0.01$) when excluding *G. excentricus*. Though the data were limited, different strains of the same species fell into the same grouping indicating that the relationship between CTX and MTX toxicity appears constant for a given species. More data are needed to fully test this hypothesis. It should also be noted that toxicity in this study was assessed using functional assays. Those assays cannot distinguish between production of large amounts of low toxicity CTX or MTX congeners relative to smaller production of high toxicity congeners. *Gambierdiscus* cell extracts that were fractionated and assayed for toxicity using a calcium flux assay indicated that despite strains of a species possessing similar CTX and MTX toxicity, the actual congeners being produced in a given species probably vary (Lewis et al., 2016). How these relative profiles might affect grazing pressure or deter bacterial or fungal infections remains unknown.

Also unidentified is whether the different patterns of CTX and MTX can offer insights into biosynthetic pathways for these studies. Ongoing studies are focusing on growing large-scale cultures of the most toxic strains of *Gambierdiscus* for the purification of the toxic compounds through fractionation and screening using the cellular bioassays.

In summary, it should be noted that only one of the thirteen strains examined has been shown to contain pg amounts of CTX-type toxicity per cell, and this strain of *G. excentricus* needs to be examined in detail for its toxin contents to identify the algal precursor(s) of toxins involved in CFP in the Atlantic Ocean. As this strain of *G. excentricus* exhibits CTX-type toxicity in the same order of magnitude as *G. polynesiensis* from the South Pacific, *Gambierdiscus* species from both oceans should be considered to be a similar potential threat to fish consumers. All strains displayed MTX-toxicity in the pg range (1.5 - 86 pg $cell^{-1}$). Also, the variability of CTX and MTX-type toxicities between species and strains appeared to be similar to those previously reported in literature and was equivalent between Atlantic and Pacific strains of *Gambierdiscus*. Several of the findings, including correlation between growth rate and toxicity or variability within and between species, are only indicative of possible trends and more strains should be examined to corroborate the findings.

1.6. **Conflict of interest**

The authors declare that there is no conflict of interest.

1.7. **Acknowledgements**

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1.8. **Appendix A. Supplementary data**

Supplementary data associated with this article can be found, in the online version, at **http://dx.doi.org/10.1016/j.hal.2017.02.0 05**.

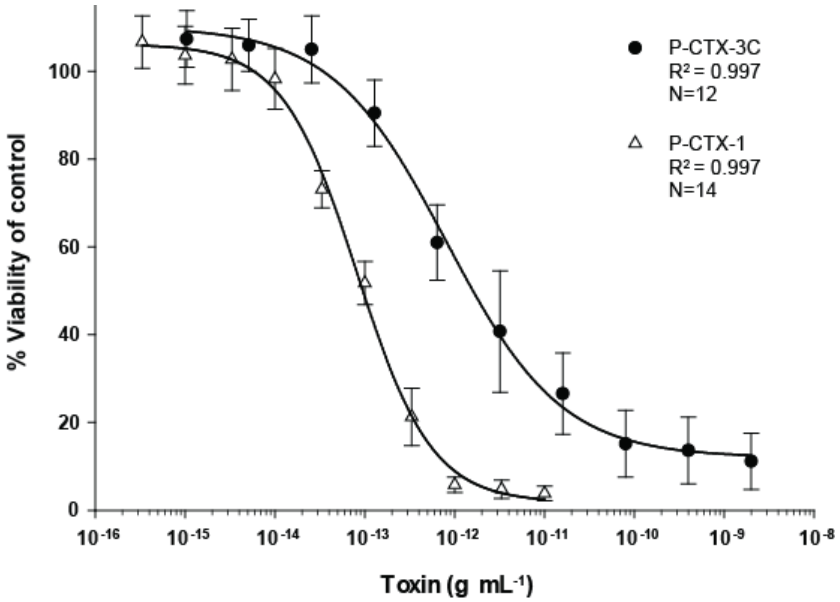

Figure 34. Sigmoidal dose-response curve of CTX3C and CTX1B standards on the neuro-2a (N2a) assay plotted using GraphPad Prism 6.0 (Hardison et al., 2016). Error bars represent the standard deviation (SD, n=12 for CTX3C, n=14 for CTX1B). The CTX standard used as reference in this study was CTX3C only.

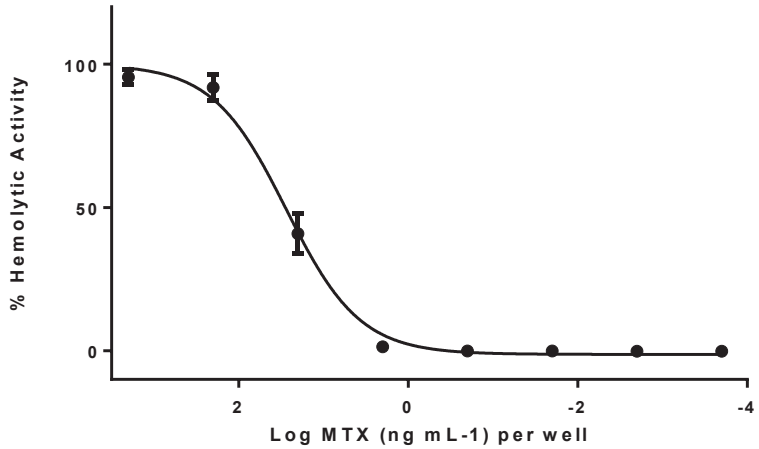

Figure 35. Sigmoidal dose-response curve of MTX standard on the human erythrocyte lysis assay (ELA) plotted using GraphPad Prism 6.0. Error bars represent the standard deviation (SD) of four replicates.

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2. Ciguatoxicity of *Gambierdiscus* and *Fukuyoa* species from the Caribbean and Gulf of Mexico

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2.1. Abstract

Dinoflagellate species belonging to the genera *Gambierdiscus* and *Fukuyoa* produce ciguatoxins (CTXs), potent neurotoxins that concentrate in fish causing ciguatera fish poisoning (CFP) in humans. While the structures and toxicities of ciguatoxins isolated from fish in the Pacific and Caribbean are known, there are few data on the variation in toxicity between and among species of *Gambierdiscus* and *Fukuyoa.* Quantifying the differences in species-specific toxicity is especially important to developing an effective cell-based risk assessment strategy for CFP. This study analyzed the ciguatoxicity of 33 strains representing seven *Gambierdiscus* and one *Fukuyoa* species using a cell based Neuro-2a cytotoxicity assay. All strains were isolated from either the Caribbean or Gulf of Mexico. The average toxicity of each species was inversely proportional to growth rate, suggesting an evolutionary trade-off between an investment in growth versus the production of defensive compounds. While there is 2- to 27-fold variation in toxicity within species, there was a 1740-fold difference between the least and most toxic species. Consequently, production of CTX or CTX-like compounds is more dependent on the species present than on the random occurrence of high or low toxicity strains. Seven of the eight species tested (*G. belizeanus*, *G. caribaeus*, *G. carolinianus*, *G. carpenteri*, *Gambierdiscus* ribotype 2, *G. silvae* and *F. ruetzleri*) exhibited low toxicities, ranging from 0 to 24.5 fg CTX3C equivalents cell^{-1}, relative to *G. excentricus*, which had a toxicity of 469 fg CTX3C eq. cell^{-1}. Isolates of *G. excentricus* from other regions have shown similarly high toxicities. If the hypothesis that *G. excentricus* is the primary source of ciguatoxins in the Atlantic is confirmed, it should be

possible to identify areas where CFP risk is greatest by monitoring only *G. excentricus* abundance using species-specific molecular assays.

2.2. Introduction

Species in the dinoflagellate genera *Gambierdiscus* and *Fukuyoa* produce cyclic polyether toxins known as ciguatoxins (CTXs) and maitotoxins (MTXs). These compounds are among the most potent naturally occurring toxins known [1]. CTXs activate voltage-gated sodium channels and disrupt normal cellular function, with nerve cells being particularly susceptible [2-6]. These toxins are lipophylic and accumulate in the food webs of many tropical, shallow water marine ecosystems reaching their highest concentrations in fish [7-9]. The consumption of fish containing sufficient CTX results in an illness known as ciguatera fish poisoning (CFP) in humans. It is the most common non-bacterial seafood-related illness and characterized by a variety of gastrointestinal and neurological symptoms, and on rare occasions, death [10, 11]. This illness is not only a concern for local populations in the tropics dependent on fish as a protein source, but also for consumers of reef fish worldwide [12, 13]. There is concern that increasing ocean temperatures in coming decades may promote range extensions of CTX-producing dinoflagellates into higher latitudes not currently impacted by CFP [14, 15]. This range expansion is supported by recent studies documenting the occurrence of *Gambierdiscus* species in more temperate waters surrounding the main islands of Japan, the Mediterranean Sea, the Canary Islands and along the eastern coasts of North and South America [14-23].

While only some *Gambierdiscus* and *Fukuyoa* isolates produce CTX or CTX-like compounds as measured by mouse, cytotoxicity or LC-MS assays, most produce varying amounts of water soluble MTXs (**Table 15**). Though MTXs are slightly more toxic than CTXs when measured by mouse bioassay using intra-peritoneal injections, they are only found in the digestive tract and liver of fish, and are unlikely to contribute to CFP unless these tisses are consumed [24-26]. Consequently, this study focused on characterizing CTX toxicity among *Gambierdiscus* and *Fukuyoa* species as these toxins pose the predominant threat to human health.

Currently there is no systematic screening protocol for testing fish for ciguatoxins. This is due largely to the expense of running the analytical assays and the limited availability of certified standards [27]. Given this

situation, estimating CFP risk is problematic. CFP frequently occurs in tropical island areas well away from metropolitan centers, so the ability to test for the toxins is limited. One approach for estimating CFP risk is to develop a cell abundance-based monitoring effort to guide the need for toxin measurements. For this approach to be effective, flux of toxins into the food web would have to be proportional to the overall abundance of *Gambierdiscus* and *Fukuyoa* species [8]. The data from a five-year survey in the Pacific by Chinain et al. [28] indicate this is not necessarily the case there. While the two years with the highest *Gambierdiscus* abundances exhibited higher than normal toxicity, across all years the relationship between *Gambierdiscus* abundance and measured toxicity was poor. Chinain et al. [28] hypothesized the variation was due to the presence of more toxic isolates or species whose relative abundances varied from year to year. Subsequent studies in the Pacific demonstrated that *G. polynesiensis* was considerably more toxic than the other species tested, suggesting changes in the relative abundance of just one species may significantly increase the CFP risk [29, 30]. The extent to which similarly toxic species or strains occur in the Caribbean and Gulf of Mexico (GOM) is the topic of this study. Thirty-three strains representing eight species of *Gambierdiscus* or *Fukuyoa* from the Caribbean were assessed using the cell based neuro-2a assay (CBA-N2a). The results showed *G. excentricus* had much higher toxicity than other co-occurring *Gambierdiscus* or *Fukuyoa* species, indicating it may be the dominant producer of CTX or CTX-like compounds in the Caribbean and GOM.

2.3. **Materials and Methods**

2.3.1. Ethics statement

The material in this manuscript has not been published in whole or in part elsewhere nor is currently being considered for publication in another journal. All the authors have been personally and actively involved in substantive work leading to the manuscript, and will hold themselves jointly and individually responsible for its content. This research used only isolates of microalgal species belonging to the genera *Fukuyoa* and *Gambierdiscus*. No human or animal subjects were involved and no collection permits were required.

2.3.2. Strain and culture conditions

Strains of seven *Gambierdiscus* (*G. belizeanus* n = 6, *G. caribaeus* n = 7, *G. carolinianus* n = 5, *G. carpenteri* n = 5, *G. excentricus* n = 1, *Gambierdiscus* ribotype 2 n = 5, *G. silvae* n = 1) and one *Fukuyoa* (*F. ruetzleri* n = 3) species obtained from the Caribbean and GOM were used to determine specific growth rates and toxicity. Four of the strains (CCMP1655, CCMP399, CCMP1733, and CCMP1651) were obtained from the National Centre for Marine Algae and Microbiota (East Boothbay, Maine, USA). All other strains were established as single cell isolates from field material as described previously [31] (**Table 13**). Where possible, isolates of the species tested were selected from geographically disparate locations.

Cells were cultured in a Percival Scientific incubator (Perry, IA, USA) maintained at 27 °C with a 12:12 h light:dark cycle. Photosynthetically active radiation (PAR) was maintained at 90-100 µmol photons m^{-2} s^{-1} by horizontally mounted fluorescent lamps (Full Spectrum Solutions, Jackson, MI, USA). Light intensity was measured using a model QSL-100 4π wand meter (Biospherical Instruments Inc., San Diego, CA, USA).

Growth medium consisted of 0.2 µm filtered Gulf Stream seawater (salinity 33) in 250 mL tissue culture flasks with vented caps (BD Biosciences, Bedford, MA, USA). Vitamins and nutrients were added according to a modified K-medium protocol [38]. Phosphate was added in the form of Na_2 *β*-glycerophosphoric acid, 5-hydrate at twice the concentration called for by K-medium protocol. An EDTA-trace metal buffer system was used with the omission of copper [39, 40]. Microwave treatment was used to sterilize the medium [41]. Culture pH was monitored using a Thermo Orion 3-Star pH meter with a Ross ultra-combination pH electrode (Thermo Fisher Scientific, Waltham, MA, USA) to ensure pH throughout experiments remained between 8.1 and 8.4. Cell densities were maintained at relatively low levels (100 to 1000 cells ml^{-1}) to avoid nutrient or CO_2 limitation.

Table 13. The species, strain designations, isolate locations, replicate growth rates and toxicities of the *Gambierdiscus* and *Fukuyoa* strains examined in this study. The citations in the species column indicate where the species was described. The reference(s) under the strain designation indicate other publications where the strain has been studied. Many of the strains analyzed for CTX-like activity in this study were also assayed for maitotoxicity in separate investigations [32, 33]. The strain growth rates (± standard deviation) were determined from triplicate, independent cultures started for each isolate. Mean species growth rates and average toxicities were determined by averaging all replicate culture data for a given species. Toxicity was normalized both as femtograms (fg) CTX3C equivalents (eq.) cell^{-1} and per biovolume attograms (ag) CTX3C eq. µm^{-3}. Correlation coefficients (R^2) for the time versus cell number relationships used to the calculate growth rates for each of the cultures exceeded 0.98.

Species	Strain	Origin	Growth rate (d^{-1})	Toxicity (fg CTX3C eq. cell^{-1})	Biovolume (µm^{-3})	Toxicity (ag CTX3C eq. µm^{-3})	Average Within Species Growth rate (d^{-1})	Average Within Species Toxicity (fg CTX3C eq. cell^{-1})	Average Within Species Toxicity (ag CTX3C eq. µm^{-3})
F. ruetzleri [31, 34]	Gam 1 [32, 35]	Carrie Bow Cay, Belize	0.17 ±0.003	24.50±0.12	66,635	0.37±0.002	0.18±0.003 (2%)	10.6±12.4 (116%)	0.16±0.019 (119%)
	NC YASU 7-21 [this study]	Continental shelf off North Carolina, USA	0.18 ±0.001	0.88±0.40	71,877	0.012±0.006			
	WH55-Gam 4 [33]	Flower Garden Banks National Marine Sanctuary (West Bank), Gulf of Mexico USA	0.17 ±0.001	6.50±1.47	75,757	0.086±0.020			
G. belizeanus [7]	CCMP399 [15, 31, 33, 35]	St. Barthelemy, Collectivity of France, Caribbean	0.20 ±0.001	0.65±0.10	100,663	0.0065±0.001	0.18±0.026 (14%)	0.85±0.81 (105%)	0.0072±0.006 (83%)
	Keys Gam 1 [32, 33]	Florida Keys, USA	0.18 ±0.001	0.54±0.03	107,489	0.0050±0.00028			
	St. Maarten Gam 12 [this study]	St. Maarten, Collectivity of France, Caribbean	0.17 ±0.001	0.49±0.26	109,449	0.0045±0.0024			
	ST1 Gam F4 [32]	St. Thomas, US Virgin Islands	0.13 ±0.001	0.43±0.38	135,352	0.0032±0.0028			
	SW Gam 2 [32]	Southwater Cay, Belize	0.15 ±0.001	2.49±1.28	128,576	0.019±0.01			
	Turks Gam 2 [this study]	The Turks and Caicos	0.18 ±0.001	0.51±0.16	107,206	0.0045±0.0015			

G. caribaeus [31]	CBC Gam 1 [32]	Carrie Bow Cay, Belize	0.16 ±0.004	0.62±0.12	243,981	0.0025±0.00049	0.17±0.024 (14%)	0.66±0.34 (52%)	0.003±0.0016 (53%)
	CCMP1651 [15, 31, 32, 36]	Grand Cayman Island, Caribbean	0.17 ±0.003	0.48±0.04	188,470	0.0026±0.0002			
	CCMP1733 [31-33, 37]	Carrie Bow Cay, Belize	0.16 ±0.002	0.80±0.43	214,306	0.0037±0.0020			
	Dive 1 FA [32, 33]	Carrie Bow Cay, Belize	0.17 ±0.001	0.69±0.19	237,758	0.0029±0.0008			
	Keys Jar 7 [32]	Florida Keys, USA	0.22 ±0.002	0.19±0.03	248,385	0.00077±0.000121			
	Mexico Algae 1 [33]	Cancun, Mexico	0.17 ±0.001	1.29±0.40	212,313	0.0061±0.0019			
	SW Gam5 [32]	Southwater Cay, Belize	0.15 ±0.003	0.52±0.26	240,568	0.0022±0.0011			
G. carolinianus [31]	Bill Aruba Gam 5 [this study]	Aruba, Caribbean	0.15 ±0.003	1.03±0.94	155,657	0.0066±0.0060	0.17±0.017 (10%)	0.27±0.43 (162%)	0.0018±0.0028 (156%)
	Jamaica algae 2 Gam 22 [this study]	Ocho Rios, Jamaica, Caribbean	0.18 ±0.005	0.10±0.03	147,450	0.00068±0.00020			
	Lobster Rock N7 [32]	Continental shelf off North Carolina, USA	0.19 ±0.003	ND	179,310	0			
	RROV5 [32, 33]	Puerto Rico, USA	0.19 ±0.004	0.02±0.01	160,763	0.00012±.000062			
	St. Maarten Gam 5 [this study]	St. Maarten, Caribbean	0.16 ±0.004	0.18±0.06	108,013	0.0017±0.00056			

G. carpenteri [31]	Bill Aruba Gam 15 [this study]	Aruba, Caribbean	0.13 ±0.002	0.71±0.21	130,630	0.0054±0.0016	0.163±0.026 (16%)	0.89±0.41 (47%)	0.0045±0.0018 (45%)
	GT4 [32, 33, 35]	Carrie Bow Cay, Belize	0.20 ±0.003	0.29±0.16	179,522	0.0016±0.00089			
	Jamaica Algae 2 Gam 1 [32, 33]	Ocho Rios, Jamaica, Caribbean	0.17 ±0.002	0.93±0.17	204,140	0.0046±0.00083			
	Mexico Algae 2 Gam 1 [this study]	Cancun, Mexico	0.15 ±0.003	1.14±0.18	206,306	0.0055±0.00087			
	WBHR21 [32, 33]	Flower Garden Banks National Marine Sanctuary (West Bank), Gulf of Mexico, USA	0.18 ±0.004	1.37±0.30	254,476	0.0054±0.0012			
G. excentricus	Pulley Ridge Gam 2 [33]	Pulley Ridge, Florida, USA	0.057 ±0.002	469±10	179,522	2.61±0.00557	0.057	469	2.61
Gambierdiscus ribotype 2 [31]	CCMP1655 [32, 36] [15, 33]	Martinique, Caribbean	0.15 ±0.001	10.9±0.36	147,700	0.074±.0024	0.128±0.010 (8%)	6.62±2.52 (38%)	0.046±0.016 (35%)
	Mixed PR Gam 4 [32, 33]	Puerto Rico, USA	0.12 ±0.001	6.63±0.54	139,322	0.048±0.0039			
	SJ3 Gam F2 [32]	St. John, US Virgin Islands	0.12 ±0.001	4.99±0.38	143,511	0.035±0.0027			
	St. Maarten Gam 10 [33]	St. Maarten, Collectivity of France, Caribbean	0.141 ±0.002	4.66±1.01	141,570	0.033±0.0071			
	SW Algae Gam 1 [33]	Southwater Cay, Belize	0.13 ±0.001	5.90±0.80	143,209	0.041±0.0056			
G. silvae [20]	Curacao Gam 11 [this study]	Curacao, Caribbean	0.098 ±0.024	19.6±4.21	70,028	0.28±0.060	0.98	19.6	0.28

2.3.3. Growth rate analysis

For each isolate examined, three independent subcultures were established and the growth rate was determined for each. These batch subcultures were grown semi-continuously by removing calculated volumes based on cell density and adding fresh media to prevent cells from entering late log phase growth. Maximal steady state growth rates were maintained for the duration of each experiment, which ranged from a minimum of 18 days to a maximum of 200 days following a period of a month or more where cells were acclimated to exponential growth conditions. Cells were counted and their biovolume was measured every three to four days using a Beckman Coulter Multisizer^{TM} 3 particle counter (Beckman Coulter Inc., Brea, CA) equipped with a 280 µm aperture and using 1.0 mL sample volumes. Samples were mixed thoroughly to ensure the cells were evenly distributed prior to counting. Specific growth rates (d^{-1}) were calculated after accounting for dilutions using a linear regression of the ln cells mL^{-1} vs. time curve [40]. This specific growth rate method provides a better estimate of average growth rate than the common practice of choosing the three steepest growth points for a growth rate determination.

When cell densities were high enough, cells were harvested for toxicity by collecting a known number of cells on a 20 µm sieve and washing them with filtered seawater (Salinity = 33) into a 50 mL centrifuge tube. The cells were pelleted using centrifugation at 3200*g* for 10 min, the supernatant carefully decanted, and the pellet was processed immediately or stored at –20 ºC prior to extraction. Because ciguatoxicity varies with growth phase, the decision was made to ensure all the cultures were maintained in steady log phase growth prior to collection of cells for toxin analysis [29, 36, 42]. This assured that the intra-strain and inter-specific toxicity measurements were not biased due to harvesting cells in different growth phases.

2.3.4. Reagents

All reagents used in this study were ACS grade or higher. Solvents were HPLC grade or higher purity. Pacific ciguatoxin-3C (CTX3C) was purchased from Wako Chemicals, USA, Inc. (Richmond, Virginia, USA) and provided by Institut Louis Malardé, Tahiti, French Polynesia (ILM). In this manuscript, we use the CTX nomenclature used by Yogi et al. [43] for the

Pacific ciguatoxins (e.g. CTX3C rather than P-CTX-3C). References to Caribbean ciguatoxins are preceded by a C (e.g. C-CTX1). CTX3C standards were stored at –20 °C and dissolved in 100% methanol prior to utilization in the CBA-N2a. All water used was Milli-Q Ultra-pure grade with 18.2 MΩ resistivity.

2.3.5. Toxin extraction

Cell pellets were sonicated for 1 min in 100% methanol at 3 mL per 100,000 cells using a Qsonica, Q700 unit (Thermo Fisher Scientific Inc., Waltham, Massachusetts) with the tip amplitude setting at 50. Once cells were disrupted, the sample was centrifuged at 3,200g for 10 min and the supernatant was transferred to a 20 mL glass scintillation vial. This was repeated two more times and the methanol was collected and dried under N_2 gas at 40 °C. The dried extract was resuspended in dichloromethane (DCM) (5mL per 100,000 cells) and washed twice in a separatory funnel with 60:40 methanol:water (2.5 mL per 100,000 cells). The dichloromethane phases (bottom layer) were then collected and dried under N_2 gas at 20 °C. The dried extract was stored at –20ºC. When ready to process, the DCM extract was resuspended in a volume of methanol that yielded a final concentration of 250-500 cells μL^{-1} [44].

2.3.6. Neuro-2a cell based assay (CBA-N2a)

The CBA-N2a assay allows estimation of the concentration of CTXs or CTX-like compounds in extracts from fish or phytoplankton [45-49]. The CBA-N2a assay measures bioactive compounds that bind voltage gated-sodium channels, not all of which are ciguatoxins [44]. Previous studies of *Gambierdiscus* and *Fukuyoa* species using LC-MS, and the same dichloromethane extraction protocol as this study, however, have shown CTX or "CTX-like" compound account for a majority of total cellular toxicity [29, 35, 42]. The consistency of these data support CTX or CTX-like compounds as the primary toxins measured in the isolates from this study.

The neuro-2a *Mus musculus* neuroblastoma cell line (N2a) used for the assay was obtained from the American Type Culture Collection (ATCC® CCL-131™). Cells were grown and maintained in Eagle's Minimum Essential Medium (EMEM; ATCC® 30-2003) containing 2 mM L-glutamine, 1 mM sodium pyruvate, 100 µg mL^{-1} streptomycin, 100 units mL^{-1} penicillin, and 10% fetal bovine serum. Growth conditions were kept at 37 °C using a

humidified 5% CO_2-enriched atmosphere. To prepare for toxicity analysis, the N2a cells were harvested with a trypsin-ethylenediaminetetraacetic acid (trypsin-EDTA) solution and seeded into each well of a 96-well microtiter plate at 30,000 cells per 100 µL of growth medium. The cells were subsequently incubated under the same growth conditions as above [36]. The plated N2a cells were allowed to settle and grow 20 - 24 h until they were >90% confluent at the bottom of each well. The standards, controls and samples were then added and the plates were incubated for 24 h. Each plate included control wells containing buffer only or buffer plus 5% methanol, the equivalent of the final methanol concentration when extracts were added. If the assay is working properly, both the buffer only and 5% methanol controls should contain a comparable number of live cells after the 24-hour incubation period. The CTX3C standard curves used in this assay ranged from 0.001-2,000 pg mL^{-1} and were suspended in the same 5% methanol buffer solution as the samples. Aliquots of each sample were added to six wells. Three of these wells contained 100 µM ouabain (O) and 10 µM veratridine (V) (O^+/V^+) to sensitize the CBA-N2a cells to CTX, and the other three contained no O/V (O^-/V^-). The O^-/V^- wells served to identify other non-specific toxins present in the samples. Cell viability in the control wells, standard curve, sample O^-/V^- and the O^+/V^+ wells were assessed after 20-24 hours of toxin exposure at 37 °C using the colorimetric 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) assay [49]. Cell mortality in the O^+/V^+ wells was converted to CTX estimates based on the CTX3C standard curve. The limit of detecton was 0.2 pg CTX3C eq. mL^{-1}.

The resulting toxicity measurements were expressed as both femtogram CTX3C eq. per cell (fg $cell^{-1}$) and attogram per μm^3 cell volume (ag μm^{-3}). The latter normalization employed the average cell volumes determined using the Multisizer when the cells were harvested. This approach determined if the variations in toxicity among isolates and among species were attributable to differences in cell size or toxicity per unit biomass.

For six of the eight species, multiple isolates were examined making it possible to estimate mean, standard deviaiton and coefficiants of variation in toxicity. To determine if the among species toxicity differences were statistically significant, a Kruskal-Wallis nonparametric one factor ANOVA was performed due to unequal variances. *Gambierdiscus excentricus* and

G. silvae were excluded from the analysis because only a single clone was examined [50]. A Dunn's test, which estimates median toxicities, was used to determine if species toxicities fell into distinct groups.

The extent of interspecific variation was also estimated by calculating the ratio between the average toxicitites for each species. In the case of *G. excentricus* and *G. silvae* the single toxicty estimate for each isolate was used to represent the mean value. Still another way to assess variations in toxicity used the mean growth rates and approximate toxicity per cell to estimate toxin production rates as fg CTX3C eq. cell^{-1} d^{-1}. The results were plotted as species versus toxin production rates and the ratio of the least to the most prolific toxin producing species was calculated.

2.4. **Results**

Gambierdiscus excentricus was the most toxic (469 fg CTX3C eq. cell^{-1}) of the species examined (**Table 13**). The next most toxic species were *G. silvae* (19.6 fg CTX3C eq. cell^{-1}) and *Gambierdiscus* ribotype 2 (4.7 to 10.9 fg CTX3C eq. cell^{-1}). The remaining five species exhibited the following range of toxicities: *F. ruetzleri* (0.9 to 24.5 fg CTX3C eq. cell^{-1}), *G. belizeanus* (0.4 to 2.5 fg CTX3C eq. cell^{-1}); *G. caribaeus* (0.2 to 1.3 fg CTX3C eq. cell^{-1}); *G. carolinianus* (non-detectable to 1.0 fg CTX3C eq. cell^{-1}); and *G. carpenteri* (0.3 to 1.4 fg CTX3C eq. cell^{-1}). The within species coefficient of variation in toxicity for the species where multiple isolates were tested ranged from 33% (*Gambierdiscus* ribotype 2) to 162% (*G. carolinianus*) (**Table 13**). Within a species, the highest toxicity isolate was ~2- to 27-fold more toxic than the least toxic isolate (**Table 16**).

The results of a one factor ANOVA (non-parametric Kruskal-Wallis test) using the species for which multiple isolates were available revealed toxicities among *F. ruetzleri*, *G. belizeanus*, *G. caribaeus*, *G. carolinianus*, *G. carpenteri* and *Gambierdiscus* ribotype 2 were significantly different (H = 18.76, p = 0.002) (**Table 14**). A Dunn's test indicated the six species were divided into three groups according to their median toxicities. Group 1 included *F. ruetzleri* and *Gambierdiscus* ribotype 2 (**Table 14**). Group 2 was *G. carpenteri*, *G. caribaeus* and *G. belizeanus* while Group 3 contained only *G. carolinianus*. It should be noted that while each of the species included in the preceding analysis exhibited low toxicity relative to *G. excentricus*, significant differences in toxicity were found among the lower toxicity species (**Figure 37**; **Table 13**). This was also true when the species

were compared on the basis of their toxin production rates (fg CTC3C eq. cell^{-1} d^{-1}) (**Figure 38**). Based on these rates, the difference between the least toxic species, *G. carolinianus* and the most toxic, *G. excentricus* was 613-fold.

Table 14. Results of a Kruskal-Wallis nonparametric one factor ANOVA for differences in CTX toxicity among *Gambierdiscus* and *Fukuyoa* species. *Gambierdiscus excentricus* and *G. silvae* were excluded from the analysis because only a single clone was examined. Abbreviations: n = sample size, M = median toxicity (fg CTX3C eq. cell^{-1}), *H* = Kruskal-Wallis test statistic, *df* = degrees of freedom. Brackets denote result of the Dunn's follow up test. The statistic is designed to estimate median toxicities.

Grouping	Species	n	M	Results
1	*F. ruetzleri*	3	6.50	
1	*Gambierdiscus* ribotype 2	5	5.90	
2	*G. carpenteri*	5	0.93	*H* = 18.76
2	*G. caribaeus*	7	0.62	*df* = 5
2	*G. belizeanus*	6	0.53	*p* = 0.002
3	*G. carolinianus*	5	0.18	

Five of the eight *Gambierdiscus* and *Fukuyoa* species studied had similar average growth rates ranging from 0.16 to 0.17 d^{-1}. *Gambierdiscus* ribotype 2 (0.13 ± 0.01 d^{-1}), *G. silvae* (0.098 d^{-1}) and *G. excentricus* (0.057 d^{-1}) grew more slowly (**Figure 36**; **Table 13**). The observed growth rates were compared to those reported in other studies for the same species and shown in **Table 17**.

A plot of average *Gambierdiscus* growth rate versus average toxicity normalized on both a per cell and per biovolume basis showed the slower growing *Gambierdiscus* species were more toxic (**Figure 37**, **Figure 39**). This increasing toxicity with declining growth rate followed an exponential relationship. Only one *Fukuyoa* species was examined, so it was impossible to say if a similar relationship between growth rate and toxicity exists within this genus. It was apparent that toxicity for the *Fukuyoa* isolates tested was higher on a per cell and a per biomass basis compared to the *Gambierdiscus* species growing at a similar rate (**Figure 37**).

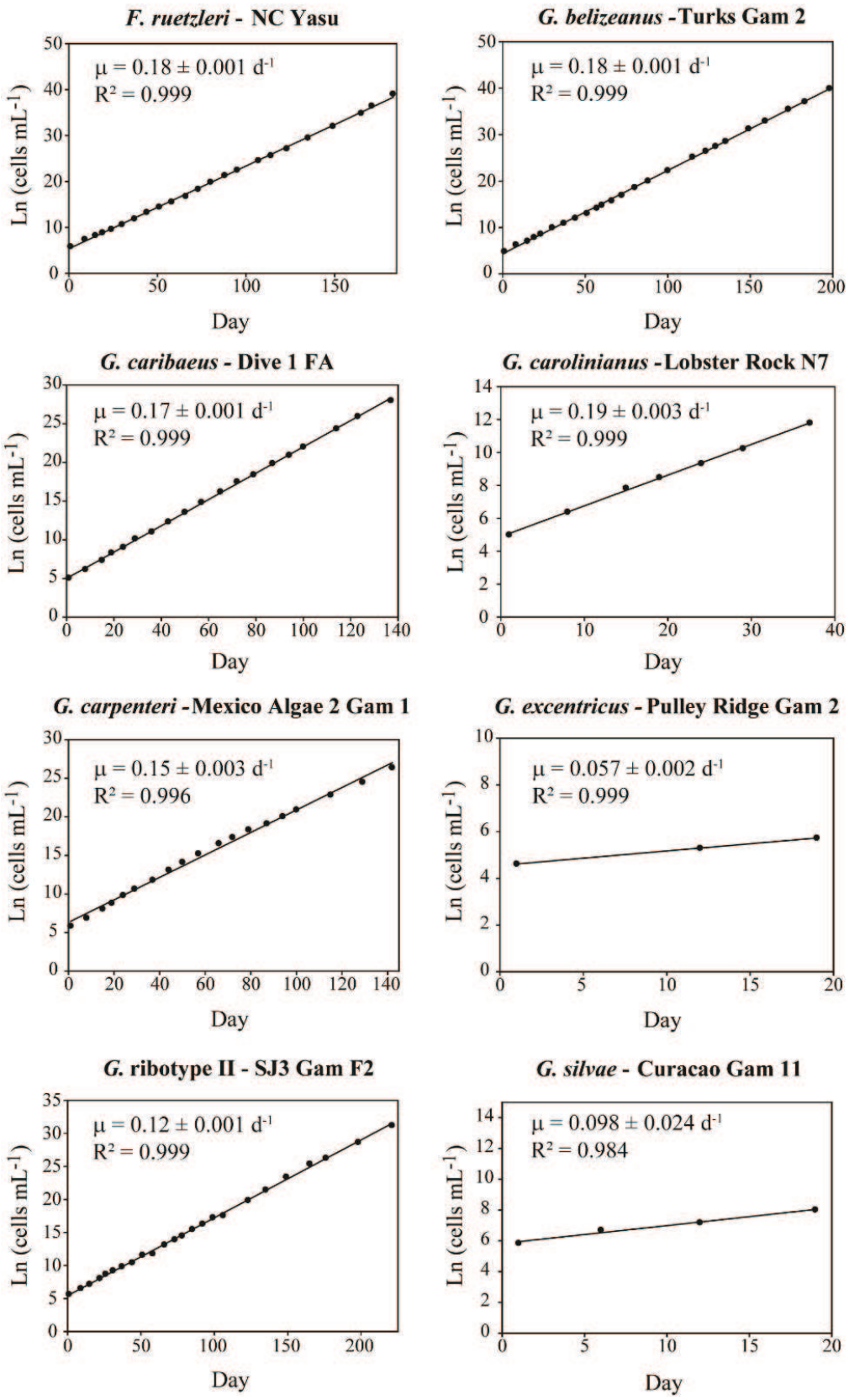

Figure 36. Representative plots showing the long-term steady state growth of the *Gambierdiscus* and *Fukuyoa* isolates achieved in this study. Exponential growth was achieved by acclimating cells to optimal temperature, light and nutrient conditions and maintained in exponential growth phase by periodic dilution with nutrient rich media.

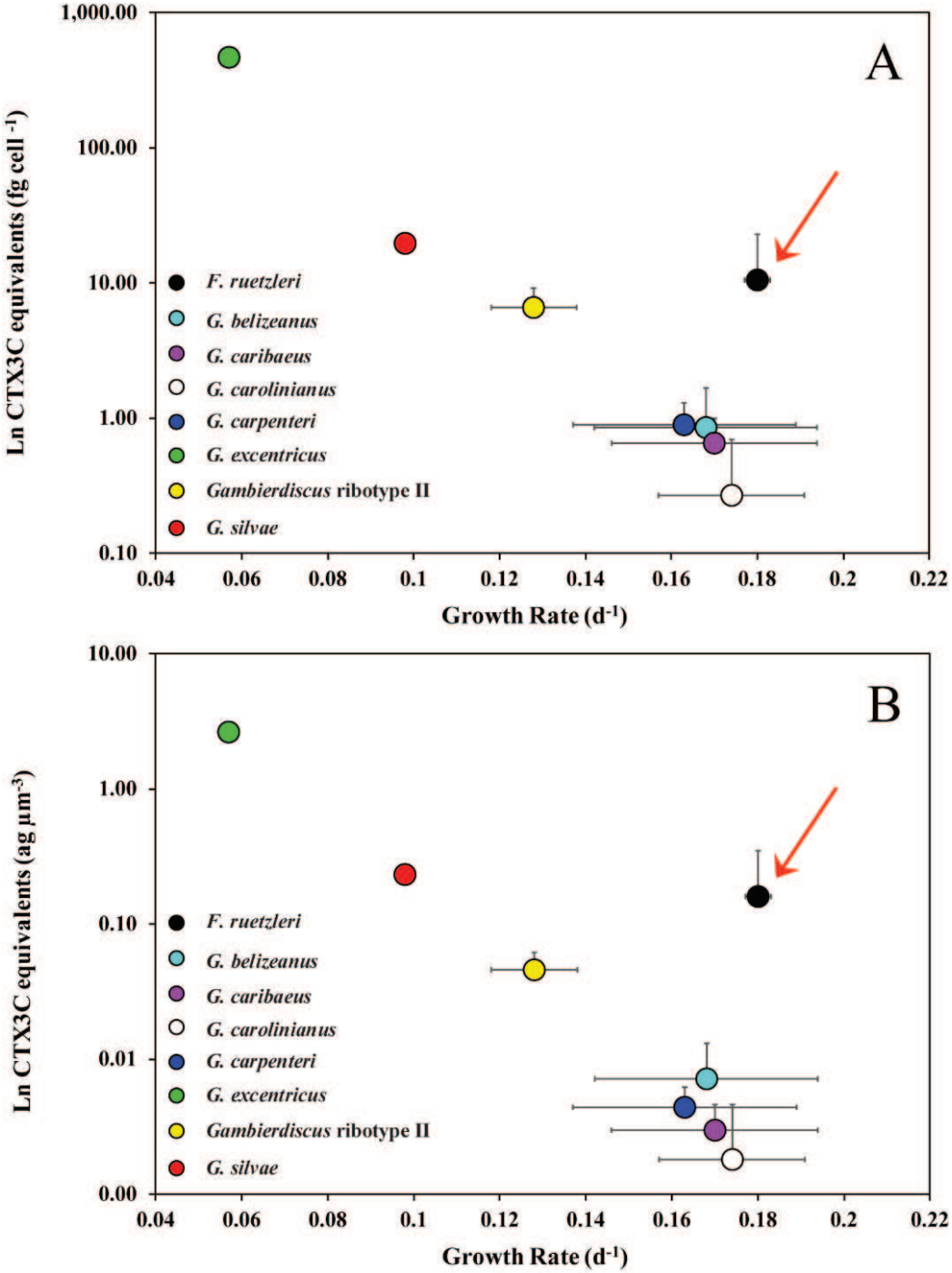

Figure 37. Ciguatoxicity versus growth rate. Natural log of cellular toxicity versus growth rate for each of the *Gambierdiscus* and *Fukuyoa* species normalized (A) to femtograms (fg) CTX3C eq. cell^{-1} and (B) attograms (ag) CTX3C eq. per µm^{-3} biovolume. Error bars = ± 1 standard deviation. The red arrows indicate data for *F. ruetzleri*, which had a higher toxicity than the *Gambierdiscus* species growing at a similar rate.

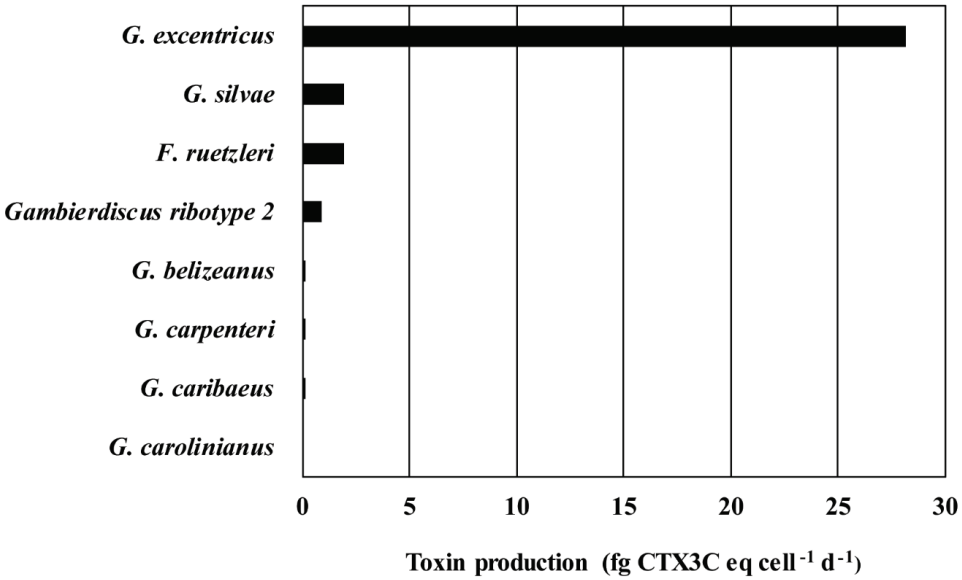

Figure 38. Toxin production rates. This figure shows the estimated toxin production (fg CTX3C eq. cell^{-1} d^{-1}) rate for each species.

2.5. **Discussion**

2.5.1. Relative toxicity of *Gambierdiscus excentricus*

The *Gambierdiscus excentricus* isolate tested in this study was ~44- to >1,740-fold more toxic than the other species examined (~469 fg CTX3C eq. cell^{-1}; **Table 13**, **Table 16**). This result is consistent with the high toxicities reported for *G. excentricus* isolates from the Canary Islands (370 - 1,100 fg CTX1 eq. cell^{-1} and 1,425 fg CTX3C eq. cell^{-1} [47, 49]) and is similar to *G. polynesiensis*, the dominant toxin-producing species in the Pacific [29, 30]. To date, *G. polynesiensis* has not been identified from the eastern Atlantic, Caribbean or Gulf of Mexico (GOM), signifying that *G. excentricus* is the dominant CTX producer in these regions [8, 47, 49] (**Table 13**). In contrast, the range of toxicities exhibited by the other six *Gambierdiscus* and one *Fukuyoa* species examined varied from non-detectible to 24.5 fg CTX3C eq. cell^{-1}.

The extent to which *G. excentricus* may dominate the CTX flux in Caribbean and GOM will depend on both its abundance and distribution. The tenent that environments fostering higher abundances of *G. excentricus* are more likely to produce ciguatoxic fish is put forward as a working hypothesis. Obtaining the data on abundance and distribution necessary to test this hypothesis will depend on quantitative species-specific molecular assays since *Gambierdiscus* species are not readily distinguished using light microscopy [31, 51]. Quantitative species-specific

polymerase chain reaction (qPCR) assays are available for many Caribbean *Gambierdiscus* species, but not *G. excentricus* and the next most toxic species, *G. silvae*. Recently, PCR assays for *G. excentricus* and *G. silvae* were developed in our laboratory, but they have not yet been validated for quantitative (qPCR) estimation of cell abundances (unpublished). However, PCR screening on a limited number of field samples and newly isolated cultures allowed us to begin defining the geographic ranges of these species. *Gambierdiscus excentricus* was found in the Florida Keys, USA and the Bahamas, while *G. silvae* was present in the Bahamas, Saint Croix, and the U.S. Virgin Islands. Combining these data with those from the literature confirmed the minimum geographic range of *G. excentricus* extends from the northwest coast of Africa to southern Florida, USA and the southeast coast of Brazil [52, 53]. *Gambierdiscus silvae* ranges from the Canary Islands through the eastern and western Caribbean [8, 20]. More extensive sampling using species-specific qPCR assays has shown that *F. ruetzleri*, *G. belizeanus*, *G. caribaeus*, *G. carolinianus*, *G. carpenteri* and *Gambierdiscus* ribotype 2 are ubiquitously distributed throughout the Caribbean and GOM [8]. It is likely *G. excentricus* and *G. silvae* share an equally wide distribution. This suggests the contribution of *G. excentricus* to the overall toxin flux depends primarily on their relative abundance. The average toxin rate is 28.1 fg CTX3C eq. cell^{-1} d^{-1} for *G. excentricus* the most toxic species, 1.9 for the next most toxic species, *G. silvae* and *F. ruetzleri*, and 0.05 for *G. carolinianus*, the lest toxic species (**Figure 38**). If a population consisted of only *G. carolinianus* and *G. excentricus*, *G. excentricus* need only make up 0.16% of the total population to produce as much toxin as *G. carolinianus*. If the population contained only *G. silvae*, *F. ruetzleri* and *G. excentricus*, *G. excentricus* would have to make up 6.3% of the population on average to produce as many CTX equivalents as the other two species combined. These calculations indicates that, despite its slow growth rate (0.06 d^{-1}), *G. excentricus* may be the dominant CTX producer even at relatively low abundances (**Table 13**; **Figure 36**).

If *G. excentricus* is confirmed as the primary CTX producing species in the Atlantic, fully investigating its role in causing CFP may require careful chemical characterization of the specific CTX congeners it produces. That characterization would help facilitate development of LC-MS toxin-specific analytical methods capable of answering whether the low toxicity Atlantic

Gambierdiscus and *Fukuyoa* species produce the same analogs in lesser quantities than *G. excentricus*, or only analogs of lower toxicity [26, 47, 49].

2.5.2. Within species versus among species differences in CTX toxicity

A long-standing question in ciguatera research is the extent to which CFP risk is dependent on variations in toxicity among species versus between species [8]. Results of a Kruskal-Wallis nonparametric one factor ANOVA showed significant differences in CTX toxicity exist among the various *Gambierdiscus* species tested (**Table 14**). Between species differences in toxicity are, on average, greater than the toxicity differences among isolates of the same species (**Table 16**). Though the within species variation for *G. excentricus* toxicity was not measured in this study, comparison with estimates in Fraga et al. [47] indicate within species variation is ~3-fold (370 to 1,100 fg P-CTX-1B eq. cell^{-1}, n=3). Other studies using the CBA-N2a assay showed a similar within species variation in toxicity (0.6-2.7 fg CTX3C eq. cell^{-1} (n=3) for *G. australes* [49], 0-19.9 fg P-CTX-1 eq. cell^{-1} (n=4) for *G. balechii* [54], 2.6 - 6.0 fg P-CTX^{-1} eq. cell^{-1} (n=4) for *Gambierdiscus* sp. type 4 [55] and 10.3-12.4 fg CTX3C eq. cell^{-1} (n=2) for *G. silvae* [49]. These data also are consistent with CTX risk being primarily dependent on species composition.

2.5.3. Relationship between growth rate and toxicity

Chinain et al. [29] proposed that slower *Gambierdiscus* cell growth was associated with higher toxin content per cell. Indeed, *G. polynesiensis*, the slowest growing Pacific species tested to date, is by far the most toxic. The trend holds true for the *Gambierdiscus* species measured in this study with the slowest growing species, *G. excentricus* exhibiting the highest toxicity (**Figure 37**; **Table 13**, **Table 17**). These data are consistent with an evolutionary tradeoff between an investment in growth versus the production of defensive compounds as observed in other harmful algal species [40, 56-58]. It is also noteworthy that relationship between toxicity and growth is exponential and not linear (**Figure 37**; [49]).

2.5.4. Estimating CTX fluxes in the environment

Quantifying the contribution of various *Gambierdiscus* and *Fukuyoa* species to the flux of CTXs in the environment requires simultaneous determination of the species abundances and the amount of CTX being produced. Undertaking such studies would be both expensive and technically challenging, especially since the full suite of species and the toxins they produce is unknown. A potentially, more tractable approach to understanding how different species may contribute to overall toxin fluxes is to incorporate the average toxicities into a physiologically-based *Gambierdiscus* growth rate model [15]. This approach would identify regions in the Caribbean and GOM where CTX fluxes may be highest. Model runs could also be adjusted to estimate how different relative abundances of low and high toxicity species would affect the magnitude of toxin flux. Explicit assumptions underlying this approach are that 1) average toxin concentrations represent the toxicity of the population as a whole and, 2) CFP risk is proportional to the toxicities of the *Gambierdiscus* and *Fukuyoa* species themselves.

The use of average toxicities in models is consistent with our knowledge of microalgal population genetics. Numerous studies have shown that algal populations maintain a high diversity of genotypes even during blooms, i.e. they are not dominated by only a few genotypes [59-64]. Averaging the toxicities of different isolates approximates population level toxicities. The relevance of using the toxicity to predict CFP risk is supported by studies showing that as CTX congeners bioaccumulate in the food chain, some remain the same while others are biologically modified to have higher toxicities than their parent compounds [43, 65-67]. As a result, the toxicities remain the same or increase in the food chain so *Gambierdiscus* and *Fukuyoa* toxicities provide minimum estimates of CFP risk.

2.5.5. Management implications

The results of this study have implications for managing CFP risk. Ideally, risk would be routinely assessed in an institutionalized surveillance system by quantitatively measuring a standard suite of CTXs in fish using LC-MS. Unfortunately, this is not practical because of the lack of certified standards and the high cost of analytical methods [46]. Until these

obstacles are overcome, the problem requires a two-tiered approach. The first tier includes monitoring for increased cell abundances to determine elevated CFP risk and understanding the environmental conditions conducive to high *Gambierdiscus* and *Fukuyoa* abundance. The second tier includes the use of qPCR assays to determine the *Gambierdiscus* and *Fukuyoa* species composition with a focus on the relative numbers of *G. excentricus* in the Caribbean.

With respect to the first approach, it is known that CFP events can occur from one month to a year following a significant increase in *Gambierdiscus* cell densities [28, 68-70]. Consequently, genus-level cell counts using light microscopy [71] can provide first order estimates of CFP risk, but cannot predict severity. Despite this limitation, it can provide managers an indication of when and where CFP risk may be elevated [72].

Interpretation of microscopic *Gambierdiscus* and *Fukuyoa* cell abundances can be further informed by understanding the environmental conditions that promote growth. Laboratory and field studies indicate temperature is the primary environmental factor regulating growth of *Gambierdiscus* and *Fukuyoa* species [15, 73, 74]. Modeling studies also have shown that in terms of broad patterns, annual temperature cycles can predict the regions where CFP risk is highest [75]. It is also known that CFP causing dinoflagellate species prefer habitats with low turbulence, appropriate substrate (macrophytes, algal turfs, coral rubble, seagrasses, etc.), nutrients supplied from the benthos or other sources, little or no direct runoff from land, and light levels >10 and < 200-700 µmol photons m^{-2} s^{-1} depending on species [8, 14, 18, 53, 71, 73, 74, 76-79]. The low light requirements of these species mean that habitats down to 50 meters or more may be capable of supporting substantial populations [79]. As GIS databases detailing habitat types throughout the Caribbean and Gulf of Mexico improve, they can be used in combination with the physiologically-based models to predict areas of higher CFP risk.

The second tier approach would use qPCR assays and focus on *G. excentricus* if it is confirmed as the dominant source of CTX in the Caribbean [23, 51]. Quantitative PCR assays are routinely used to monitor harmful algae in many regions of the world [80-83]. Only lack of resources keeps this from being possible throughout the Caribbean. Ultimately, as LC-MS methods become more cost effective, and high CFP risk areas are

identified, the logical course is to use cell-based monitoring to focus on samples that need to be tested for toxins.

2.6. **Conclusions**

Gambierdiscus excentricus was significantly more toxic than the other *Gambierdiscus* and *Fukuyoa* species examined from the Caribbean and GOM. *Gambierdiscus excentricus*, even with its slow growth rate, is likely to contribute disproportionally large fluxes of CTXs in the marine food web. Overall, toxicity was inversely related to growth rate, indicating a tradeoff between investments of cellular resources in growth versus defensive compounds. Monitoring overall *Gambierdiscus* and *Fukuyoa* cell densities using genus-specific light microcopy may provide insight into when CFP risks are of concern, but cannot predict the severity of events. Despite this limitation, a cell-based approach can be used to predict first order risk assessment when no other method is available. If research confirms the hypothesis that one or a relatively few species produce most of the ciguatoxins entering the food web, then monitoring of those species using species-specific qPCR or other molecular assays will support more accurate assessments of CFP risk. Ecological models based on the physiological and ecological preferences of the key toxin producing species, also offer a way to cost effectively identify time periods and locations when CFP risk is the highest and when more expensive testing using LC-?S methods are warranted.

2.7. **Acknowledgements**

Mireille Chinain and Taiana Darius provided critical toxin standards and methodological advice and Mark Vandersea provided constructive criticisms of the manuscript. We also wish to thank the following individuals who have assisted in collecting field samples from numerous locations in the Caribbean and Gulf of Mexico: Christine Addison, John Burke, Dave Cerino, Brian Degan, Michael Dowgiallo, Wilson Freshwater, Brett Harrison, David Johnson, Doug Kesling, William Lee, Roger Mays, James Morris, Roldan Muñoz, Brandon Puckett, Sherry Reed, Bill Sunda, Jenny Vander Plum and Paula Whitfield. We thank the three anonymous reviewers of the manuscript for their constructive edits and suggestions.

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2.8. **Author contributions**

The authors contributed equally to this work. RWL, PAT conceived and designed the experiments and completed literature review for CTX and MTX toxicity presented in supplementary tables: WCH, DRH, FP performed experiments: WCH, DRH, FP developed analytical protocols and performed experiments: RWL, WCH, DRH, PAT, PH analyzed the data and contributed to the writing of the manuscript.

2.9. **Supplementary data**

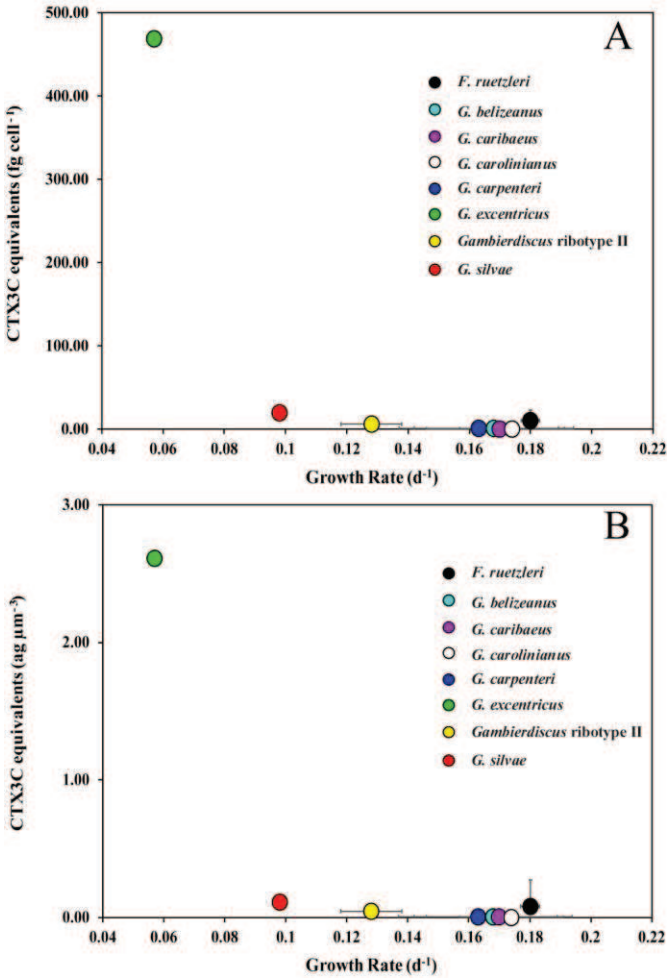

Figure 39. Ciguatoxicity versus growth rate plotted on a linear scale. Cellular toxicity versus growth rate for each of the *Gambierdiscus* and *Fukuyoa* species normalized (A) to femtograms (fg) CTX3C eq. cell^{-1} and (B) attograms (ag) CTX3C eq. per µm^{-3} biovolume. Error bars = ± 1 standard deviation. This graph visually demonstrates the large difference in variation in toxicity of *G. excentricus* relative to the other species.

Table 15. Comprehensive table showing what is known about CTX and MTX production by *Gambierdiscus* and *Fukuyoa* isolates not included in this study. + = either CTX or MTX detected, NT = not tested, - = no measurable toxicity (i.e. below detection limits for the specific method being used), "p-" detonates "putative" congener identification, and ? = extraction method may not have adequately separated CTX and MTX, further investigation is required to confirm the presence of CTX or MTX. For the undescribed species, a "+" in the CTX column = low toxicity (>2,000 cells per mouse unit), ++ = moderate toxicity (~500 to 2000 cells per mouse unit), +++ = high toxicity (<500 cells per mouse unit) as estimated in Holland et al. (2013). For the MTX study done using the hemolytic red blood cell assay: + = EC_{50} >1000 cells, ++ = EC_{50} 100 to 1000 cells, +++ = EC_{50} of 10 to 100 cells (Holland et al., 2013). Other studies with +, ++ and +++ indicate low, intermediate and high toxicities relative to other species measured using the same method. The data from Lewis et al. (2016) indicates the potential number of CTX or MTX congeners present in algal extracts after fractionation and screening of each fraction using a sodium flux assay. In some studies, the presence of a putative CTX or MTX congener could be detected, but below concentrations that would allow accurate quantification. This situation is denoted by "-". NCMA = National Center for Marine Algae and Microbiota formally Center for Culture of Marine Phytoplankton (CCMP).

Isolate	CTX	MTX	Location	Assay employed	Reference
F. ruetzleri					
Gam 1	NT	+++	Southwater Cay, Belize	Hemolytic red blood cell assay	Holland et al. 2013
Gam 1	+ 3	+ 1	Southwater Cay, Belize	Fluorescent calcium flux assay	Lewis et al. 2016
Gam 1	NT	+ p-MTX3	Southwater Cay, Belize	LC-LRMS/MS	Pisapia et al. 2017b
NC Yasu	NT	+	North Carolina, continental shelf, USA	Hemolytic red blood cell assay	Holland et al. 2013
NOAA 3	+	NT	South Water Cay, Belize	LC-MS/MS	Roeder et al. 2010
WH55 Gam4	NT	+ p-MTX3	Flower Garden Banks National Marine Sanctuary (West Bank), Northwestern Gulf of Mexico, USA	LC-LRMS/MS	Pisapia et al. 2017b
F. paulensis					
Dn35EHU	+	+	Formentera Island, Western Mediterranean Sea	Mouse bioassay	Laza-Martinez et al. 2016
F. yasumotoi					
Single isolated not designated	NT	+	Pulau Hantu Island, Singapore	Mouse bioassay	Holmes et al. 1998
F.* cf. *yasumotoi					
CAWD210	- (pg CTX $cell^{-1}$)	- p-MTX3 (pg MTX $cell^{-1}$)	Te Uenga Bay, Bay of Islands, Northland, New Zealand	LC–MS/MS	Rhodes et al. 2014
CAWD211	- (pg CTX $cell^{-1}$)	- p-MTX3 (pg MTX $cell^{-1}$)	Te Uenga Bay, Bay of Islands, Northland, New Zealand	LC–MS/MS	Rhodes et al. 2014
G. australes					
#2V	- (pg CTX $cell^{-1}$)	+ p-MTX-1 5.8 (pg MTX $cell^{-1}$)	North Meyer Island, Kermadec Islands, Pacific	LC-MS/MS	Rhodes et al. 2016

#3–5	- (pg CTX cell^{-1})	+ pMTX-1 2.4 (pg MTX cell^{-1})	North Meyer Island, Kermadec Islands, Pacific	LC-MS/MS	Rhodes et al. 2016
#5a	- (pg CTX cell^{-1})	+ p-MTX-1 3.6 (pg MTX cell^{-1})	North Meyer Island, Kermadec Islands, Pacific	LC-MS/MS	Rhodes et al. 2016
#5h	- (pg CTX cell^{-1})	+ p-MTX-1 8.5 (pg MTX cell^{-1})	North Meyer Island, Kermadec Islands, Pacific	LC-MS/MS	Rhodes et al. 2016
CAWD149	- (pg CTX cell^{-1})	+ p-MTX3 8.3 (pg MTX cell^{-1})	Rarotonga, Cook Islands, Pacific Ocean	LC–MS/MS	Rhodes et al. 2014
CAWD216	- (pg CTX cell^{-1})	- p-MTX3 (pg MTX cell^{-1})	Rarotonga, Cook Islands, Pacific Ocean	LC–MS/MS	Rhodes et al. 2014
CAWD244	- (pg CTX cell^{-1})	+ p-MTX-1 5.9 (pg MTX cell^{-1})	North Meyer Island, Kermadec Islands, Pacific	LC-MS/MS	Rhodes et al. 2016
CAWD245	- (pg CTX cell^{-1})	+ p-MTX-1 0.3 (pg MTX cell^{-1})	North Meyer Island, Kermadec Islands, Pacific	LC-MS/MS	Rhodes et al. 2016
CAWD246	- (pg CTX cell^{-1})	+ p-MTX-1 36.6 (pg MTX cell^{-1})	North Meyer Island, Kermadec Islands, Pacific	LC-MS/MS	Rhodes et al. 2016
CAWD248	- (pg CTX cell^{-1})	+ p-MTX-1 14.7 (pg MTX cell^{-1})	North Meyer Island, Kermadec Islands, Pacific	LC-MS/MS	Rhodes et al. 2016
CAWD254 Mac3-o	- (pg CTX cell^{-1})	- (pg MTX cell^{-1})	Macauley Island, Kermadec Islands, Pacific	LC-MS/MS	Rhodes et al. 2017
CAWD255 Mac1-b	- (pg CTX cell^{-1})	+ 36 (pg MTX cell^{-1})	Macauley Island, Kermadec Islands, Pacific	LC-MS/MS	Rhodes et al. 2017

CAWD256 Mac3-c	- (pg CTX cell^{-1})	+ 31 (pg MTX cell^{-1})	Macauley Island, Kermadec Islands, Pacific	LC-MS/MS	Rhodes et al. 2017
CBaii	- (pg CTX cell^{-1})	+ p-MTX-1 4.7 (pg MTX cell^{-1})	North Meyer Island, Kermadec Islands, Pacific	LC-MS/MS	Rhodes et al. 2016
CBc	- (pg CTX cell^{-1})	+ p-MTX-1 17.7 (pg MTX cell^{-1})	North Meyer Island, Kermadec Islands, Pacific	LC-MS/MS	Rhodes et al. 2016
CBo	- (pg CTX cell^{-1})	- p-MTX-1 (pg MTX cell^{-1})	North Meyer Island, Kermadec Islands, Pacific	LC-MS/MS	Rhodes et al. 2016
CCMP 1653	NT	+ p-MTX3	Tern Island, Hawaii, USA	LC-LRMS/MS	Pisapia et al. 2017b
CCMP 1653	+ 2.7 ± 0.6 (fg CTX3C eq. cell^{-1})	+ 5.0 ± 0.5 (pg MTX eq. cell^{-1})	Tern Island, Hawaii, USA	N2a cytotoxicity assay	Pisapia et al. 2017a
CCMP 1653	+	NT	Tern Island, Hawaii, USA	LC-MS/MS	Roeder et al. 2010
HI9 gam	NT	+	Waikiki Beach, Hawaii, USA	Hemolytic red blood cell assay	Holland et al. 2013
Mac2-a	- (pg CTX cell^{-1})	+ 25 (pg MTX cell^{-1})	Macauley Island, Kermadec Islands, Pacific	LC-MS/MS	Rhodes et al. 2017
Mac2-b	- (pg CTX cell^{-1})	+ 12 (pg MTX cell^{-1})	Macauley Island, Kermadec Islands, Pacific	LC-MS/MS	Rhodes et al. 2017
Mac2-c	- (pg CTX cell^{-1})	+ 22 (pg MTX cell^{-1})	Macauley Island, Kermadec Islands, Pacific	LC-MS/MS	Rhodes et al. 2017
Mac3-a	- (pg CTX cell^{-1})	+ 14 (pg MTX cell^{-1})	Macauley Island, Kermadec Islands, Pacific	LC-MS/MS	Rhodes et al. 2017
Mac3-b	- (pg CTX cell^{-1})	+ 20 (pg MTX cell^{-1})	Macauley Island, Kermadec Islands, Pacific	LC-MS/MS	Rhodes et al. 2017
Mac3-e	- (pg CTX cell^{-1})	+ 19 (pg MTX cell^{-1})	Macauley Island, Kermadec Islands, Pacific	LC-MS/MS	Rhodes et al. 2017

Mac3-i	- (pg CTX cell^{-1}) -	+ 3 (pg MTX cell^{-1})	Macauley Island, Kermadec Islands, Pacific	LC-MS/MS	Rhodes et al. 2017
Mac3-m	- (pg CTX cell^{-1})	+ 19 (pg MTX cell^{-1})	Macauley Island, Kermadec Islands, Pacific	LC-MS/MS	Rhodes et al. 2017
Mac3-n	- (pg CTX cell^{-1})	+ 5 (pg MTX cell^{-1})	Macauley Island, Kermadec Islands, Pacific	LC-MS/MS	Rhodes et al. 2017
Mac4-e	- (pg CTX cell^{-1})	+ 10 (pg MTX cell^{-1})	Macauley Island, Kermadec Islands, Pacific	LC-MS/MS	Rhodes et al. 2017
Mac4-f	- (pg CTX cell^{-1})	+ 9 (pg MTX cell^{-1})	Macauley Island, Kermadec Islands, Pacific	LC-MS/MS	Rhodes et al. 2017
Mac4-fuk	- (pg CTX cell^{-1})	+ 21 (pg MTX cell^{-1})	Macauley Island, Kermadec Islands, Pacific	LC-MS/MS	Rhodes et al. 2017
Mac4-g	- (pg CTX cell^{-1})	+ 27 (pg MTX cell^{-1})	Macauley Island, Kermadec Islands, Pacific	LC-MS/MS	Rhodes et al. 2017
Mac5-a	- (pg CTX cell^{-1})	+ 16 (pg MTX cell^{-1})	Macauley Island, Kermadec Islands, Pacific	LC-MS/MS	Rhodes et al. 2017
Mac5-b	- (pg CTX cell^{-1})	+ 15 (pg MTX cell^{-1})	Macauley Island, Kermadec Islands, Pacific	LC-MS/MS	Rhodes et al. 2017
Mac5-c	- (pg CTX cell^{-1})	+ 8 (pg MTX cell^{-1})	Macauley Island, Kermadec Islands, Pacific	LC-MS/MS	Rhodes et al. 2017
Mac5-e	- (pg CTX cell^{-1})	+ 20 (pg MTX cell^{-1})	Macauley Island, Kermadec Islands, Pacific	LC-MS/MS	Rhodes et al. 2017
Mac5-f	- (pg CTX cell^{-1})	+ 8 (pg MTX cell^{-1})	Macauley Island, Kermadec Islands, Pacific	LC-MS/MS	Rhodes et al. 2017
Mac5-g	- (pg CTX cell^{-1})	+ 7 (pg MTX cell^{-1})	Macauley Island, Kermadec Islands, Pacific	LC-MS/MS	Rhodes et al. 2017

Mac5-j	- (pg CTX cell^{-1})	+ 32 (pg MTX cell^{-1})	Macauley Island, Kermadec Islands, Pacific	LC-MS/MS	Rhodes et al. 2017
Mac5-l	- (pg CTX cell^{-1})	+ 6 (pg MTX cell^{-1})	Macauley Island, Kermadec Islands, Pacific	LC-MS/MS	Rhodes et al. 2017
Mac65-a	- (pg CTX cell^{-1})	+ 18 (pg MTX cell^{-1})	Macauley Island, Kermadec Islands, Pacific	LC-MS/MS	Rhodes et al. 2017
MG-4	- (fg CTX3C eq. cell^{-1})	NT	Rikitea, Mangareva	Receptor binding assay	Chinain et al 2010
MUR-6	17 (fg CTX3C eq. cell^{-1})	NT	Moruroa, French Polynesia	Receptor binding assay	Chinain et al 2010
MUR-14	30 (fg CTX3C eq. cell^{-1})	NT	Moruroa, French Polynesia,	Receptor binding assay	Chinain et al 2010
RAI-5	- (fg CTX3C eq. cell^{-1})	NT	Motu Femme, Raivavae, French Polynesia	Receptor binding assay	Chinain et al 2010
RAV-92	+ 4 (×10^{-4} MU 1,000 cells^{-1})	+ 0.2 (MU 1,000 cells^{-1})	Rairua, Raivavae Island, Australes Archipelago, French Polynesia	Mouse bioassay	Chinain et al. 1999
RAV-92	20 (fg CTX3C eq. cell^{-1})	NT	Rairua, Raivavae Island, Australes Archipelago, French Polynesia	Receptor binding assay	Chinain et al 2010
TB-1	22 (fg CTX3C eq. cell^{-1})	NT	Mataura, Tubuai, French Polynesia	Receptor binding assay	Chinain et al 2010
S080911_1	+ 670 (×10^{-4} MU 1,000 cells^{-1})	+ 67 (×10^{-4} MU 1,000 cells^{-1})	Kutsu, Susaki, Kochi, Japan	Mouse bioassay	Nishimura et al. 2013
S080911_1	NT	+ p-MTX3 22.6 ± 0.5 (pg MTX eq. cell^{-1})	Kutsu, Susaki, Kochi, Japan	LC-LRMS/MS	Pisapia et al. 2017b
VGO1178	NT	+ p-MTX3	Punta Hidalgo, Tenerife, Canary Islands	LC-LRMS/MS	Pisapia et al. 2017b
VGO1178	+ 1.4 ± 0.4 (fg CTX3C eq. cell^{-1})	+ 4.3 ± 1.2 (pg MTX eq. cell^{-1})	Punta Hidalgo, Tenerife, Canary Islands, Spain	N2a cytotoxicity assay	Pisapia et al. 2017a
VGO1181	NT	+ p-MTX3	Punta Hidalgo, Tenerife, Canary Islands, Spain	LC-LRMS/MS	Pisapia et al. 2017b

VGO1181	+ 0.6 ± 0.3 (fg CTX3C eq. cell^{-1})	+ 4.7 ± 2.8 (pg MTX eq. cell^{-1})	Punta Hidalgo, Tenerife, Canary Islands, Spain	N2a cytotoxicity assay	Pisapia et al. 2017a
W B Gam 3	-	+ 2	Waikiki Beach, Hawaii, USA	Fluorescent calcium flux assay	Lewis et al. 2016
G. balechii (*Gambierdiscus* sp. type 6)					
1012M4D02	+ 0.14 ± 0.01 (fg P-CTX-1 eq cell^{-1})	NT	Marakei Island, Kiribati, Pacific	N2a cytotoxicity assay	Dai et al. 2017
1112M1D08	+ 1.13 ± 0.17 (fg P-CTX-1 eq cell^{-1})	NT	Marakei Island, Kiribati, Pacific Marakei Island, Kiribati, Pacific	N2a cytotoxicity assay	Dai et al. 2017
1112M1D09 1	+ 1.11 ± 0.19 (fg P-CTX-1 eq cell^{-1})	NT	Marakei Island, Kiribati, Pacific	N2a cytotoxicity assay	Dai et al. 2017
1112M1M03	+ 19.9 ± 9.55 (fg P-CTX-1 eq cell^{-1})	NT	Marakei Island, Kiribati, Pacific	N2a cytotoxicity assay	Dai et al. 2017
T6PrGd03N	- (fg P-CTX-1 eq cell^{-1})	NT	Rawa Island, Malaysia	N2a cytotoxicity assay	Dai et al. 2017
VGO917	+	+	Manado, Celebes Seat, Pacific Ocean	Mouse bioassay	Fraga et al. 2016
VGO917	NT	+ p-MTX3	Manado, Celebes Seat, Pacific Ocean	LC-LRMS/MS	Pisapia et al. 2017b
VGO917	+ 3.4 ± 1.5 (fg CTX3C eq. cell^{-1})	+ 19.9 ± 2.9 (pg MTX eq. cell^{-1})	Manado, Celebes Seat, Pacific Ocean	N2a cytotoxicity assay	Pisapia et al. 2017a
VGO920	NT	+ p-MTX3	Manado, Celebes Seat, Pacific Ocean	LC-LRMS/MS	Pisapia et al. 2017b
G. belizeanus					
CCMP 399	NT	++	St. Barthelemy, Collectivity of France, Caribbean	Hemolytic red blood cell assay	Holland et al. 2013
CCMP 399	+ 4	+ 4	St. Barthelemy, Collectivity of France, Caribbean	Fluorescent calcium flux assay	Lewis et al. 2016
CCMP 399	NT	+ p-MTX3	St. Barthelemy, Collectivity of France, Caribbean	LC-LRMS/MS	Pisapia et al. 2017b
CCMP 401 (NCMA)	+	NT	St. Barthelemy, Collectivity of France, Caribbean	LC-MS/MS	Roeder et al. 2010
Keys Gam 1	NT	++	Florida Keys, USA	Hemolytic red blood cell assay	Holland et al. 2013
Keys Gam 1	NT	+ p-MTX3	Florida Keys, USA	LC-LRMS/MS	Pisapia et al. 2017b

STB-1	+ 123 (fg CTX3C eq. cell^{-1})	NT	St Barthélémy, Caribbean	Receptor binding assay	Chinain et al 2010
ST1 Gam F4	NT	+	St. Thomas, US Virgin Islands, Caribbean	Hemolytic red blood cell assay	Holland et al. 2013
ST1 Gam F4	NT	+ p-MTX3	St. Thomas, US Virgin Islands, Caribbean	LC-LRMS/MS	Pisapia et al. 2017b
G. caribaeus					
Algae 1 gam 1	NT	++	Cancun, Mexico	Hemolytic red blood cell assay	Holland et al. 2013
Jamaica 1 gam1	NT	+	Ocho Rios, Jamaica	Hemolytic red blood cell assay	Holland et al. 2013
Bill Hi Gam8	NT	+ p-MTX2, 3	Waikiki Beach, Honolulu, Hawaii, USA	LC-LRMS/MS	Pisapia et al. 2017b
Bill Hi Gam8	+ 1.6 ± 1.0 (fg CTX3C eq. cell^{-1})	+ 5.3 ± 1.0 (pg MTX eq. cell^{-1})	Waikiki Beach, Honolulu, Hawaii, USA	N2a cytotoxicity assay	Pisapia et al. 2017a
BB Gam 4	NT	++	Bathtub Beach, Florida , USA	Hemolytic red blood cell assay	Holland et al. 2013
BRP Gam 4	NT	++	Jupiter, Florida, USA	Hemolytic red blood cell assay	Holland et al. 2013
CBC Gam1	NT	++	Carrie Bow Cay, Belize	Hemolytic red blood cell assay	Holland et al. 2013
CCMP 1651 (NCMA)	NT	+	Grand Cayman Island, Caribbean	Hemolytic red blood cell assay	Holland et al. 2013
CCMP 1651 (NCMA)	+	NT	Grand Cayman Island, Caribbean	LC-MS/MS	Roeder et al. 2010
CCMP 1651 (NCMA)	+ 30.7 to 54.3 (fg C-CTX1-eq. cell^{-1})	NT	Grand Cayman Island, Caribbean	N2A cytotoxicity assay	Lartigue et al 2009
CCMP 1651 (NCMA)	NT	+ p-MTX2,3	Grand Cayman Island, Caribbean	LC-LRMS/MS	Pisapia et al. 2017b
CCMP 1652 (NCMA)	+	NT	Mataiva, Tahiti, French Polynesia	LC-MS/MS	Roeder et al. 2010
CCMP 1733 (NCMA)	NT	++	Carrie Bow Cay, Belize	Hemolytic red blood cell assay	Holland et al. 2013
CCMP 1733 (NCMA)	+	NT	Carrie Bow Cay, Belize	LC-MS/MS	Roeder et al. 2010
CCMP 1733 (NCMA)	NT	+ p-MTX2,3	Carrie Bow Cay, Belize	LC-LRMS/MS	Pisapia et al. 2017b
Coral cove Gam 1	NT	++	Jupiter, Florida, USA	Hemolytic red blood cell assay	Holland et al. 2013
Dive 1 fa Gam 1	NT	++	Ft. Pierce, Florida, USA	Hemolytic red blood cell assay	Holland et al. 2013
Dive 1 fa Gam 1	NT	+ p-MTX2,3	Ft. Pierce, Florida, USA	LC-LRMS/MS	Pisapia et al. 2017b
ETB Gam 6	NT	++	Dry Tortugas, Gulf of Mexico	Hemolytic red blood cell assay	Holland et al. 2013
Gam 4	NT	++	Belize	Hemolytic red blood cell assay	Holland et al. 2013
Gam19	NT	+	Belize	Hemolytic red blood cell assay	Holland et al. 2013
Gam 19	-	++ 3	Carrie Bow Cay, Belize	Fluorescent calcium flux assay	Lewis et al. 2016
Keys Jar 7 Gam 7	NT	++	Long Key, Florida Keys, USA	Hemolytic red blood cell assay	Holland et al. 2013

Jamaica Algae 1 Gam 1	NT	++	Belize	Hemolytic red blood cell assay	Holland et al. 2013
Jar 12 Tow 3	NT	++	Belize	Hemolytic red blood cell assay	Holland et al. 2013
Jar 17 gam	NT	+	Belize	Hemolytic red blood cell assay	Holland et al. 2013
Mexico Algae1 Gam1	NT	++ p-MTX2,3	Mexico, western Caribbean	LC-LRMS/MS	Pisapia et al. 2017b
Norval Cay	NT	++	Norval Cay, Belize	Hemolytic red blood cell assay	Holland et al. 2013
Outfish 7-1	NT	++	Dry Tortugas, Gulf of Mexico	Hemolytic red blood cell assay	Holland et al. 2013
Outfish 7-3	NT	++	Dry Tortugas, Gulf of Mexico	Hemolytic red blood cell assay	Holland et al. 2013
Pat Jar 2 Gam	NT	+	Oahu, Hawaii, USA	Hemolytic red blood cell assay	Holland et al. 2013
Pat HI Jar 2 Gam 2	-	+ 3	Big Island, Hawaii, USA	Fluorescent calcium flux assay	Lewis et al. 2016
SW Gam 4	NT	++	South Water Cay, Belize	Hemolytic red blood cell assay	Holland et al. 2013
SW Gam 5	NT	++	South Water Cay, Belize	Hemolytic red blood cell assay	Holland et al. 2013
SJ 3 D7	NT	++	St. John, US Virgin Islands, Caribbean	Hemolytic red blood cell assay	Holland et al. 2013
ST 1 C5	NT	++	St. Thomas, US Virgin Islands, Caribbean	Hemolytic red blood cell assay	Holland et al. 2013
TC tow Gam 3	NT	++	Twin Cays, Belize	Hemolytic red blood cell assay	Holland et al. 2013
WBHR 21 gam 2	NT	+	Flower Garden Banks National Marine Sanctuary, Gulf of Mexico, USA	Hemolytic red blood cell assay	Holland et al. 2013
WBHR 26 gam	NT	+	Flower Garden Banks National Marine Sanctuary, Gulf of Mexico, USA	Hemolytic red blood cell assay	Holland et al. 2013
G. carolinianus					
Algae Gam 1	NT	+	Ocho Rios Jamaica	Hemolytic red blood cell assay	Holland et al. 2013
Big Fish Gam	NT	+	North Carolina, USA	Hemolytic red blood cell assay	Holland et al. 2013
Dive 1 Gam1	NT	++	Carrie Bow Cay, Belize	Hemolytic red blood cell assay	Holland et al. 2013
Dive 1 Gam 1	+ 2	+ 2	Carrie Bow Cay, Belize	Fluorescent calcium flux assay	Lewis et al. 2016
Elbow Cay	NT	++	Elbow Cay, Belize	Hemolytic red blood cell assay	Holland et al. 2013
ETB Exp 28 Gam 10	NT	+	Dry Tortugas, Gulf of Mexico	Hemolytic red blood cell assay	Holland et al. 2013
ETB Exp 28 Gam 10	NT	+ p-MTX3	Dry Tortugas, Gulf of Mexico	LC-LRMS/MS	Pisapia et al. 2017b
Gam 3	NT	+	Crete, Greece	Hemolytic red blood cell assay	Holland et al. 2013
Greece Gam2	NT	+ p-MTX3	Crete, Greece	LC-LRMS/MS	Pisapia et al. 2017b
Greece Gam2	+ 3.3 ± 0.6 (fg CTX3C eq. cell^{-1})	+ 10.6 ± 0.4 (pg MTX eq. cell^{-1})	Crete, Greece	N2a cytotoxicity assay	Pisapia et al. 2017a
Jamaica Algae Gam 1	NT	++	Jupiter, Florida, USA	Hemolytic red blood cell assay	Holland et al. 2013
Jupiter Algae gam	NT	+	Jupiter, Florida, USA	Hemolytic red blood cell assay	Holland et al. 2013

Ken 3	NT	+	Offshore North Carolina , USA	Hemolytic red blood cell assay	Holland et al. 2013
Kenny 6	NT	+	Offshore North Carolina, USA	Hemolytic red blood cell assay	Holland et al. 2013
Kenny 6	-	+ 3	Offshore North Carolina, USA	Fluorescent calcium flux assay	Lewis et al. 2016
Lob Rock N7	NT	+	Offshore North Carolina, USA	Hemolytic red blood cell assay	Holland et al. 2013
Lob Rock N3	NT	+	Offshore North Carolina, USA	Hemolytic red blood cell assay	Holland et al. 2013
Mex Algae Gam 1	NT	++	Mexico	Hemolytic red blood cell assay	Holland et al. 2013
Pat HI Jar 3 Gam 9	-	+ 3	Big Island, Hawaii, USA	Fluorescent calcium flux assay	Lewis et al. 2016
PRG Gam 1	NT	+	Puerto Rico, USA	Hemolytic red blood cell assay	Holland et al. 2013
RROV5	NT	+	Puerto Rico, USA	Hemolytic red blood cell assay	Holland et al. 2013
RROV5	NT	+ p-MTX3	Puerto Rico, USA	LC-LRMS/MS	Pisapia et al. 2017b
ST1 F7	NT	+	St. Thomas, US Virgin Islands, USA	Hemolytic red blood cell assay	Holland et al. 2013
WBHR 21 Gam	NT	++	Flower Garden Banks National Marine Sanctuary, Gulf of Mexico	Hemolytic red blood cell assay	Holland et al. 2013
G. carpenteri					
Algae 2 gam 1	NT	+	Ocho Rios, Jamaica	Hemolytic red blood cell assay	Holland et al. 2013
CCMP 1654	NT	++	Guam, North Pacific, USA	Hemolytic red blood cell assay	Holland et al. 2013
CCMP 1654	+	NT	Guam, North Pacific, USA	LC-MS/MS	Roeder et al. 2010
ETB Exp 24 Gam 1	NT	+	Dry Tortugas, Gulf of Mexico	Hemolytic red blood cell assay	Holland et al. 2013
GT4	NT	++	Carrie Bow Cay, Belize	Hemolytic red blood cell assay	Holland et al. 2013
GT4	-	+ 3	Carrie Bow Cay, Belize	Fluorescent calcium flux assay	Lewis et al. 2016
GT4	NT	+ p-MTX3	Carrie Bow Cay, Belize	LC-LRMS/MS	Pisapia et al. 2017b
Jamaica Algae2 Gam1	NT	+ p-MTX3	Ocho Rios, Jamaica	Hemolytic red blood cell assay	Holland et al. 2013
Jamaica Algae2 Gam1	NT	+ p-MTX3	Ocho Rios, Jamaica	LC-LRMS/MS	Pisapia et al. 2017b
NOAA 12	+	NT	South Water Cay, Belize	LC-MS/MS	Roeder et al. 2010
Pat HI jar 5 Gam 3	NT	+	Oahu, Hawaii, USA	Hemolytic red blood cell assay	Holland et al. 2013
Pat HI Jar 7 Gam 11	NT	+ 3	Waikiki Beach, Honolulu, Hawaii, USA	Fluorescent calcium flux assay	Lewis et al. 2016
Pat HI Jar 7 Gam 11	NT	+ p-MTX3	Waikiki Beach, Honolulu, Hawaii, USA	LC-LRMS/MS	Pisapia et al. 2017b
Pat HI Jar 7 Gam 11	+ 1.4 ± 0.6 (fg CTX3C eq. cell^{-1})	+ 6.3 ± 1.9 (pg MTX eq. cell^{-1})	Waikiki Beach, Honolulu, Hawaii, USA	N2a cytotoxicity assay	Pisapia et al. 2017a

WBHR 21	NT	+	Flower Garden Banks National Marine Sanctuary, Gulf of Mexico, USA	Hemolytic red blood cell assay	Holland et al. 2013
WBHR 21	NT	+ p-MTX3	Flower Garden Banks National Marine Sanctuary, Gulf of Mexico	LC-LRMS/MS	Pisapia et al. 2017b
G. cheloniae					
CAWD232	-	- p-MTX3 0.320 (0.221–0.426) (mg/kg)	Highland Paradise, Rarotonga, Cook Islands	Mouse bioassay, LD50 by intraperitoneal injection	Smith et al 2016
CAWD236	-	- p-MTX3 1.58 (1.11–2.09) (mg/kg)	Papua Passage, Rarotonga, Cook Islands	Mouse bioassay, LD50 by intraperitoneal injection	Smith et al 2016
G. excentricus					
Pulley Ridge Gam 2	NT	+ MTX4 22.9 (pg MTX eq. cell^{-1})	Southern Gulf of Mexico	LC-LRMS/MS	Pisapia et al. 2017b
UNR-07	NT	++ MTX4 16.0 ± 2.3 (pg MTX eq. cell^{-1})	Armação dos Búzios, Rio de Janeiro, Brazil	LC-LRMS/MS	Pisapia et al. 2017b
UNR-08	NT	++ MTX4 19.8 ± 6.4 (pg MTX eq. cell^{-1})	Armação dos Búzios, Rio de Janeiro, Brazil	LC-LRMS/MS	Pisapia et al. 2017b
VGO790	+++ 1.10 ± 0.19 (pg CTX 1B eq. (cell^{-1})	+++ 1.38 ± 0.31 (ng MTX eq. cell-^{1})	Punta Hidalgo, Tenerife, Canary Islands, Spain	N2a cytotoxicity assay	Fraga et al. 2011
VGO790	NT	++ MTX4 23.2 (pg MTX eq. cell^{-1})	Punta Hidalgo, Tenerife, Canary Islands, Spain	LC-LRMS/MS	Pisapia et al. 2017b
VGO791	+++ 1.05 ± 0.18 (pg CTX 1B eq. cell^{-1})	+++ 0.60 ± 0.24 (ng MTX eq. cell-^{1})	Punta Hidalgo, Tenerife, Canary Islands, Spain	CBA-N2a cytotoxicity assay	Fraga et al. 2011
VGO791	NT	+++ MTX4 72.8 ± 8.5 (pg MTX eq. cell^{-1})	Punta Hidalgo, Tenerife, Canary Islands, Spain	LC-LRMS/MS	Pisapia et al. 2017b

VGO791	+++ 1426 ± 55 (fg CTX3C eq. cell^{-1})	+++ 85.7 ± 41.5 (pg MTX eq. cell^{-1})	Punta Hidalgo, Tenerife, Canary Islands, Spain	N2a cytotoxicity assay	Pisapia et al. 2017a
VGO792	+++ 0.37 ± 0.17 (pg CTX1B eq. cell^{-1})	+++ 0.48 ± 0.16 (ng MTX eq. cell^{-1})	Punta Hidalgo, Tenerife, Canary Islands, Spain	CBA-N2a cytotoxicity assay	Fraga et al. 2011
VGO792	NT	++ MTX4 20.0 ± 2.9 (pg MTX eq. cell^{-1})	Punta Hidalgo, Tenerife, Canary Islands, Spain	LC-LRMS/MS	Pisapia et al. 2017b
VGO1035	NT	++ MTX4 13 (pg MTX eq. cell^{-1})	Playa Las Cabras, La Palma, Canary Islands, Spain	LC-LRMS/MS	Pisapia et al. 2017b
G. honu					
CAWD233	-	+ p-MTX3 0.20 (0.15–0.24) (mg kg^{-1})	Betela, Cook Islands, Pacific	Mouse bioassay, LD_{50} by intraperitoneal injection	Rhodes et al 2017
CAWD242	- (pg CTX cell^{-1})	- p-MTX3 (pg MTX cell^{-1})	North Meyer Island, Kermadec Islands, Pacific	LC-MS/MS	Rhodes et al. 2016
CAWD242	- (pg CTX cell^{-1})	- p-MTX3 (pg MTX cell^{-1}) 0.20 (0.07–0.23) (mg kg^{-1})	North Meyer Island, Kermadec Islands, Pacific	Mouse bioassay, LD_{50} by intraperitoneal injection	Rhodes et al. 2017
G. lapillus					
HG4	? 150 (mg kg-1)	+	Heron Island, Great Barrier Reef, Australia	Mouse bioassay, median lethal doses by intraperitoneal injection	Kretzschmar et al. 2017
HG6	? 0.78 (0.40 - 1.60) (mg kg^{-1})	+	Heron Island, Great Barrier Reef, Australia	Mouse bioassay, median lethal doses by intraperitoneal injection	Kretzschmar et al. 2017

HG7	? 12.5 (10.1 - 15.3) (mg kg^{-1})	+	Heron Island, Great Barrier Reef, Australia	Mouse bioassay, median lethal doses by intraperitoneal injection	Kretzschmar et al. 2017
G. pacificus					
3S0509-27	+ 0.011 (fg P-CTX-1 eq. cell^{-1})	NT	Marakei, Republic of Kiribati, Pacific ocean	CBA-N2a cytotoxicity assay	Xu et al. 2014
CAWD213	- (pg CTX cell^{-1})	- p-MTX3 (pg CTX cell^{-1})	Rarotonga Lagoon, Cook Islands, Pacific	LC–MS/MS	Rhodes et al. 2014
CCMP 1650 (NCMA)	NT	+	Moorea, Society Islands, Pacific Ocean	Hemolytic red blood cell assay	Holland et al. 2013
CCMP 1650 (NCMA)	+	NT	Moorea, Society Islands, Pacific Ocean	LC-MS/MS	Roeder et al. 2010
CCMP 1650 (NCMA)	NT	+ p-MTX2,3	Moorea, Society Islands, Pacific Ocean	LC-LRMS/MS	Pisapia et al. 2017b
CCMP 1650 (NCMA)	+ 12.1 ± 0.0 (fg CTX3C eq. cell^{-1})	++ 20.1 ± 5.9 (pg MTX eq. cell^{-1})	Moorea, Society Islands, Pacific Ocean	N2a cytotoxicity assay	Pisapia et al. 2017a
G10DC	+ Tentatively 2,3-dihydroxyCTX3C, 51-hydroxyCTX3C M-/L-*seco*-CTX3C	+	Malaysia	CBA-N2a cytotoxicity assay LC-MS/MS	Caillaud et al. 2011
ET-2	- (fg CTX3C eq. cell^{-1})	NT	Mahu, Tubuai, French Polynesia	Receptor binding assay	Chinain et al 2010
G10DC	NT	+ p-MTX2,3	Malaysia	LC-LRMS/MS	Pisapia et al. 2017b
HO-91	+ 9 (×10^{-4} MU 1,000 cells^{-1})	+ 0.7 (×10^{-4} MU 1,000 cells^{-1})	Otepa, Hao Island, French Polynesia	Mouse bioassay	Chinain et al. 1999
HO-91	- (fg CTX3C eq. cell^{-1})	NT	Otepa, Hao, French Polynesia	Receptor binding assay	Chinain et al 2010
MUR-4	- (fg CTX3C eq. cell^{-1})	NT	Moruroa, French Polynesia	Receptor binding assay	Chinain et al 2010
G. polynesiensis					
CAWD212	+ 1,820 (fg CTX cell^{-1})	- (fg MTX cell^{-1})	Rarotonga, Cook Islands, Pacific ocean	LC–MS/MS	Rhodes et al. 2014

MG-7	+ 17 (fg CTX3C eq. cell^{-1})	NT	Rikitea, Mangareva, French Polynesia	Receptor binding assay	Chinain et al 2010
RAI-1	+ 4,400 (fg CTX3C eq. cell^{-1})	NT	Motu Femme, Raivavae, French Polynesia	Receptor binding assay	Chinain et al 2010
RG-92	+++ 800 (×10^{-4} MU 1,000 cells−1)	+ 0.06 (MU 1,000 cells^{-1})	Avatoru, Rangiroa Island, Tuamotu Archipelago, French Polynesia	Mouse bioassay	Chinain et al. 1999
RG-92	+ 2,800 (fg CTX3C eq. cell^{-1})	NT	Avatoru, Rangiroa Island, Tuamotu Archipelago, French Polynesia	Receptor binding assay	Chinain et al 2010
TB-92	+++ 1500 (×10^{-4} MU 1,000 cells^{-1})	+ 0.1 (MU 1,000 cells^{-1})	Mataura, Tubuai Island, French Polynesia	Mouse bioassay	Chinain et al. 1999
TB-92	+ 4,300 (fg CTX3C eq. cell^{-1})	NT	Mataura, Tubuai Island, French Polynesia	Receptor binding assay	Chinain et al 2010
***Gambierdiscus* ribotype 2**					
CCMP 1655 (NCMA)	NT	+++	Martinique, Caribbean, insular region of France	Hemolytic red blood cell assay	Holland et al. 2013
CCMP 1655 (NCMA)	+ 1.3 to 8.7 (fg C-CTX1-eq. cell^{-1})	NT	Martinique, Caribbean, insular region of France	N2a cytotoxicity assay	Lartigue et al 2009
Mixed PR Gam 3	+ 1	+ 2	Puerto Rico, USA	Fluorescent calcium flux assay	Lewis et al. 2016
Mixed PR Gam 3	NT	+ p-MTX3	Puerto Rico, USA	LC-LRMS/MS	Pisapia et al. 2017b
Mixed PR Gam 4	NT	+++	Puerto Rico, USA	Hemolytic red blood cell assay	Holland et al. 2013
ST3 Gam F2	NT	+++	St. Thomas, US Virgin Islands, USA	Hemolytic red blood cell assay	Holland et al. 2013
St. Maarten Gam 6	+ 2	+ 2	St. Maarten, Caribbean, insular region of France	Fluorescent calcium flux assay	Lewis et al. 2016
St. Maarten Gam 10	NT	+ p-MTX3	St. Maarten, Collectivity of France, Caribbean	LC-LRMS/MS	Pisapia et al. 2017b
SW Algae Gam1	NT	+ p-MTX3	South Water Cay, Belize	LC-LRMS/MS	Pisapia et al. 2017b
G. scabrosus					
KW070922_1	20 (×10^{-4} MU 1,000 cells^{-1})	67 (×10^{-4} MU 1,000 cells^{-1})	Kashiwa-jima Island, Otsuki, Kochi, Japan	Mouse bioassay	Nishimura et al. 2013

KW070922_1	++ 27.9 ± 3.8 (fg CTX3C eq. cell^{-1})	+ 1.5 ± 0.2 (pg MTX eq. cell^{-1})	Kashiwa-jima Island, Otsuki, Kochi, Japan	N2a cytotoxicity assay	Pisapia et al. 2017a
KW070922_1	NT	-	Kashiwa-jima Island, Otsuki, Kochi, Japan	LC-LRMS/MS	Pisapia et al. 2017b
G. silvae					
UNR-30	NT	+ p-MTX3	Brazil	LC-LRMS/MS	Pisapia et al. 2017b
VGO1167	NT	+ p-MTX3	Punta Hidalgo, Tenerife, Canary Islands	LC-LRMS/MS	Pisapia et al. 2017b
VGO1167	+ 10.3 ± 2.7 (fg CTX3C eq. cell^{-1})	+ 2.2 ± 0.3 (pg MTX eq. cell^{-1})	Punta Hidalgo, Tenerife, Canary Islands	N2a cytotoxicity assay	Pisapia et al. 2017a
VGO1180	NT	+ p-MTX3	Punta Hidalgo, Tenerife, Canary Islands	LC-LRMS/MS	Pisapia et al. 2017b
VGO1180	+ 12.4 ± 3.6 (fg CTX3C eq. cell^{-1})	+ 3.3 ± 0.9 (pg MTX eq. cell^{-1})	Punta Hidalgo, Tenerife, Canary Islands	N2a cytotoxicity assay	Pisapia et al. 2017a
***Gambierdiscus* sp.** (not all same species)					
RAI-2	102 (fg CTX3C eq. cell^{-1})	NT	Motu Femme, Raivavae, French Polynesia	Receptor binding assay	Chinain et al 2010
Viet Nam	+	NT	Cau Island, Binh Thuan, South China Sea, Viet Nam	LC-MS/MS	Roeder et al. 2010
Viet Nam	NT	+ p-MTX3	Cau Island, Binh Thuan, South China Sea, Viet Nam	LC-LRMS/MS	Pisapia et al. 2017b
Viet Nam	++ 40.8 ± 19.6 (fg CTX3C eq. cell^{-1})	+++ 70.9 ± 44.8 (pg MTX eq. cell^{-1})	Cau Island, Binh Thuan, South China Sea, Viet Nam	N2a cytotoxicity assay	Pisapia et al. 2017a
***Gambierdiscus sp.* type 2**					
M080828_2	- (×10^{-4} MU 1,000 cells^{-1})	- (×10^{-4} MU 1,000 cells^{-1})	Muroto Promontory, Muroto City, Kochi, Japan	Mouse Bioassay	Nishimura et al. 2013
T070411_1	- (×10^{-4} MU 1,000 cells^{-1})	NT (×10^{-4} MU 1,000 cells^{-1})	Tei Promontory, Konan City, Kochi, Japan	Mouse Bioassay	Nishimura et al. 2013
***Gambierdiscus sp.* type 3**					
WI9G	- (×10^{-4} MU 1,000 cells^{-1})	- (×10^{-4} MU 1,000 cells^{-1})	Itsumo, Kushimoto Town, Wakayama, Japan	Mouse Bioassay	Nishimura et al. 2013

WI11G	- ($\times 10^{-4}$ MU 1,000 cells^{-1})	++ 67 ($\times 10^{-4}$ MU 1,000 cells^{-1})	Itsumo, Kushimoto Town, Wakayama, Japan	Mouse Bioassay	Nishimura et al. 2013
***Gambierdiscus* sp. type 4**					
IS00-01	+ 2.6 (fg CTX-1 eq. cell^{-1})	NT	Marakei, Republic of Kiribati, Pacific Ocean	CBA-N2a cytotoxicity assay	Xu et al. 2014
IS00-04	+ 4.4 (fg P-CTX-1 eq. cell^{-1})	NT	Marakei, Republic of Kiribati, Pacific Ocean	CBA-N2a cytotoxicity assay	Xu et al. 2014
ID0509-16	+ 4.1 (fg P-CTX-1 eq. cell^{-1})	NT	Marakei, Republic of Kiribati, Pacific Ocean	CBA-N2a cytotoxicity assay	Xu et al. 2014
ID0510-22	+ 6.0 (fg P-CTX1 eq. cell^{-1})	NT	Marakei, Republic of Kiribati, Pacific Ocean	CBA-N2a cytotoxicity assay	Xu et al. 2014
***Gambierdiscus* sp. type 5**					
DS0511-03	+ 0.010 (fg P-CTX1 eq. cell^{-1})	NT	Marakei, Republic of Kiribati, Pacific Ocean	CBA-N2a cytotoxicity assay	Xu et al. 2014
M4SWC11	- (fg P-CTX1 eq cell^{-1})	NT	Marakei Island, Kiribati	N2a cytotoxicity assay	Dai et al. 2017
G. toxicus					
GTT-91	- ($\times 10^{-4}$ MU 1,000 cells^{-1})	0.7 (MU 1,000 cells−1)	Teahupoo, Tahiti, French Polynesia	Mouse bioassay	Chinain et al., 1999
GTT-91	- (fg CTX3C eq. cell^{-1})	NT	Teahupoo, Tahiti, French Polynesia	Receptor binding assay	Chinain et al 2010
GTT-91	NT	+ p-MTX3	Teahupoo, Tahiti, French Polynesia	LC-LRMS/MS	Pisapia et al. 2017b
HIT-0	NT	+ p-MTX3	Hitiaa, Tahiti, French Polynesia	LC-LRMS/MS	Pisapia et al. 2017b
HIT-0	- (pg CTX3C eq. cell^{-1})	NT	Hitiaa, Tahiti, French Polynesia	Receptor binding assay	Chinain et al 2010
HIT-25	+ 22 (fg CTX3C eq. cell^{-1})	NT	Hitiaa, Tahiti, French Polynesia	Receptor binding assay	Chinain et al 2010
HIT-25	NT	+ p-MTX3	Hitiaa, Tahiti, French Polynesia	LC-LRMS/MS	Pisapia et al. 2017b

PAP-1	+ 28 (fg CTX3C eq. cell^{-1})	NT	Papara, Tahiti, French Polynesia	Receptor binding assay	Chinain et al 2010
REN-1	- (×10^{-4} MU 1,000 cells^{-1})	1.7 (MU 1,000 cells^{-1})	Saint-Leudville fringing reef, La Réunion Island, Indian Ocean	Mouse bioassay	Chinain et al., 1999
REN-1	- (fg CTX3C eq. cell^{-1})	NT	Saint-Leudville fringing reef, La Réunion Island, Indian Ocean	Receptor binding assay	Chinain et al 2010
TUR	- (×10^{-4} MU 1,000 cells^{-1})	0.6 (MU 1,000 cells^{-1})		Mouse bioassay	Chinain et al., 1999
Unidentified *Gambierdiscus* isolates*					
135	?	++	Bermuda, Atlantic Ocean	Mouse bioassay	Bomber et al. 1989
157	?	+	Drift algae, Caribbean, Caribbean	Mouse bioassay	Bomber et al. 1989
158	?	++	Drift algae	Mouse bioassay	Bomber et al. 1989
162	?	++	Bahamas, Atlantic Ocean	Mouse bioassay	Bomber et al. 1989
163	?	+++	Bahamas, Atlantic Ocean	Mouse bioassay	Bomber et al. 1989
165	?	+++	Drift algae, Caribbean	Mouse bioassay	Bomber et al. 1989
169	?	++	Drift algae, Caribbean	Mouse bioassay	Bomber et al. 1989
170	?	+++	Bahamas, Atlantic Ocean	Mouse bioassay	Bomber et al. 1989
171	?	++	Bahamas, Atlantic Ocean	Mouse bioassay	Bomber et al., 1989
172	?	++	Bahamas, Atlantic Ocean	Mouse bioassay	Bomber et al., 1989
175	?	+++	Martinique, Caribbean	Mouse bioassay	Bomber et al., 1989
177	?	+++	Hawaii, USA	Mouse bioassay	Bomber et al., 1989
196	?	++	Florida Keys, USA	Mouse bioassay	Bomber et al., 1989
199	?	+++	Florida Keys, USA	Mouse bioassay	Bomber et al., 1989

200	?	++	Florida Keys, USA	Mouse bioassay	Bomber et al., 1989
300	?	+++	Florida Keys, USA	Mouse bioassay	Bomber et al., 1989
350	?	+++	Atlantic, Virgin Gorda, Caribbean	Mouse bioassay	Bomber et al., 1989
94-13	-	-	Southwest Puerto Rico, USA	Mouse bioassay	Tosteson et al. 1989
101-16	-	-	Southwest Puerto Rico, USA	Mouse bioassay	Tosteson et al. 1989
105-13	-	+	Southwest Puerto Rico, USA	Mouse bioassay	Tosteson et al. 1989
106-13	-	-	Southwest Puerto Rico, USA	Mouse bioassay	Tosteson et al. 1989
107-24	-	-	Southwest Puerto Rico, USA	Mouse bioassay	Tosteson et al. 1989
B30	?	+	Bahamas, Atlantic Ocean	Mouse bioassay	Bomber et al. 1989
B6	?	+	Bahamas, Atlantic Ocean	Mouse bioassay	Bomber et al., 1989
Cd4	?	+++	Knight Key, Florida, USA	Mouse bioassay	Babinchak et al. 1986
Cd8	?	++	Knight Key, Florida, USA	Mouse bioassay	Babinchak et al. 1986
Cd9	?	+	Knight Key, Florida, USA	Mouse bioassay	Babinchak et al. 1986
Cd10	?	++	Knight Key, Florida, USA	Mouse bioassay	Babinchak et al. 1986
Cd13	?	+	Knight Key, Florida, USA	Mouse bioassay	Babinchak et al. 1986
Cd20	?	+++	Knight Key, Florida, USA	Mouse bioassay	Babinchak et al. 1986
CGT-a	+	+	Gambier Island, French Polynesia	Mouse bioassay	Bagnis et al. 1980
CGT-b	++	+	Gambier Island, French Polynesia	Mouse bioassay	Bagnis et al. 1980
Clone 1	+++	+	Gambier Island, French Polynesia	Mouse bioassay	Durand-Clément 1987
Clone 2	++	+	Gambier Island, French Polynesia	Mouse bioassay	Durand-Clément 1987

Clone 3	++	+	Gambier Island, French Polynesia	Mouse bioassay	Durand-Clément, 1987
Clone 4	++	+	Gambier Island, French Polynesia	Mouse bioassay	Durand-Clément 1987
Clone 5	++	+	Gambier Island, French Polynesia	Mouse bioassay	Durand-Clément 1987
Culture 1	+	+	Gambier Island, French Polynesia	Mouse bioassay	Yasumoto et al. 1979
FP	-	NT	Gambier Island, French Polynesia	Mouse bioassay	Holmes et al. 1991
GT178	-	NT	Kahala Bay, Hawaii, USA	Mouse bioassay	Holmes et al. 1991
GT18/1	-	NT	Kahala Bay, Hawaii, USA	Mouse bioassay	Holmes et al. 1991
GT360	-	NT	South Sound, US Virgin Islands, USA	Mouse bioassay	Holmes et al. 1991
HIMB	+	++	Northwest Hawaiian Islands, USA	Mouse bioassay	Withers 1984
HR1	-	NT	Hoffman Rocks, Queensland, Australia	Mouse bioassay	Holmes et al. 1991
HR7	-	NT	Hoffman Rocks, Queensland, Australia	Mouse bioassay	Holmes et al. 1991
MQ-1	?	+	Florida Keys, USA	Mouse bioassay	Bomber et al. 1988b
NQ1	-	NT	Hastings Reef, Queensland, Australia	Mouse bioassay	Holmes et al. 1991
NQ1	-	NT	Hastings Reef, , Queensland, Australia	Mouse bioassay	Holmes et al. 1991
NQ1	-	NT	Hastings Reef, Queensland, Australia	Mouse bioassay	Holmes et al. 1991
NQ2/7	+	NT	Arlington reef, Queensland, Australia	Mouse bioassay	Holmes et al. 1991
SQ3/3	-	NT	Flinders Reef, Queensland, Australia	Mouse bioassay	Holmes et al. 1991
Strain A	++	+	Gambier Island, French Polynesia	Mouse bioassay	Durand-Clément 1987
Strain B	+++	+	Gambier Island, French Polynesia	Mouse bioassay	Durand-Clément 1987
T39	?	+++	Tern Island, Hawaii, USA	Mouse bioassay	Babinchak et al. 1986
WC1/1	+	NT	Platypus Bay, Station 1, Queensland, Australia	Mouse bioassay	Holmes et al. 1991

WC1/1	+	NT	Platypus Bay, Station 1, Queensland, Australia	Mouse bioassay	Holmes et al. 1991
WC1/18	-	NT	Platypus Bay, Station 1, Queensland, Australia	Mouse bioassay	Holmes et al. 1991
WC2/1	-	NT	Platypus Bay, Station 2, Queensland, Australia	Mouse bioassay	Holmes et al. 1991
WC3/1	-	NT	Platypus Bay, Station 1, Queensland, Australia	Mouse bioassay	Holmes et al. 1991
T39	?	+++	Tern Island, Hawaii, USA	Mouse bioassay	Babinchak et al. 1986

Table 16. (A) Ratio of the highest toxicity isolate divided by the lowest isolate in fg CTX3C eq. cell^{-1}. One of the *G. carolinianus* isolates did not have detectable toxin so value of 0.1, which is below the limit of detection was used to estimate the ratio. **(B)** Ratio of mean among species toxicity (fg CTX3C eq. cell^{-1}). In case of *G. excentricus* and *G. silvae* the estimate for the single clone measured was used to represent the mean value.

(A)

	min	*max*	*Ratio max/min*
F. ruetzleri	0.9	24.5	27.2
G. belizeanus	0.4	2.5	6.3
G. caribaeus	0.2	1.3	6.5
*G. carolinianus**	0.05	1	20.0
G. carpenteri	0.3	1.4	4.7
Gambierdiscus ribotype 2	4.7	10.9	2.3

(B)

	A	*F. ruetzleri*	*G. belizeanus*	*G. caribaeus*	*G. carpenteri*	*G. excentricus*	*Gambierdiscus* ribotype 2	*G. silvae*
		Ratio A/B	Ratio A/B	Ratio A/B	Ratio A/B	Ratio A/B	Ratio A/B	Ratio A/B
B	Mean	10.60	0.85	0.66	0.89	469.00	6.62	19.60
F. ruetzleri	10.6	1.00	0.08	0.06	0.08	44.25	0.62	1.85
G. belizeanus	0.85		1.00	0.78	1.05	551.76	7.79	23.06
G. caribaeus	0.66			1.00	1.35	710.61	10.03	29.70
G. carolinianus	0.27				3.30	1737.04	24.52	72.59
G. carpenteri	0.89				1.00	526.97	7.44	22.02
G. excentricus	469					1.00	0.01	0.04
Gambierdiscus ribotype 2	6.62						1.00	2.96
G. silvae	19.6							1.00

Table 17. Comparison of the *Gambierdiscus* and *Fukuyoa* growth rate estimates determined in this study *versus* rates published in other studies. The table is divided into upper section, where growth rates were estimated using direct cell counts, and lower section, where growth rates were estimated using relative changes in chlorophyll *a* fluorescence.

Growth Rates (d^{-1}) ± standard deviation								
Study	***G. belizeanus***	***G. caribaeus***	***G. carolinianus***	***G. carpenteri***	***Gambierdiscus* ribotype 2**	***G. silvae***	***G. excentricus***	***F. ruetzleri***
Growth estimated based on direct cell counts								
This study	0.167 ± 0.026	0.172 ± 0.024	0.174 ± 0.017	0.163 ± 0.026	0.128 ± 0.010	0.09	0.05	0.176 ± 0.003
Lartigue et al. 2009		0.14			0.11			
Parsons et al. 2010		0.110 – 0.156						
Chinain et al. 2010	0.140							
Growth estimated by changes in Chl *a* fluorescence								
Kibler et al. 2015	0.146	0.194	0.159		0.104			0.208
Xu et al. 2016	0.191 – 0.235	0.204 – 0.229	0.152 – 0.191	0.132 – 0.201		~ 0.124		

2.10. **References**

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Chapter 3: In search of bioactive compounds produced by *Gambierdiscus excentricus*

1. Maitotoxin-4, a novel MTX analog produced by *Gambierdiscus excentricus*

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1.1. Abstract

Maitotoxins (MTXs) are among the most potent toxins known. These toxins are produced by epi-benthic dinoflagellates of the genera *Gambierdiscus* and *Fukuyoa* and may play a role in causing the symptoms associated with Ciguatera Fish Poisoning. A recent survey revealed that, of the species tested, the newly described species from the Canary Islands, *G. excentricus*, is one of the most maitotoxic. The goal of the present study was to characterize MTX-related compounds produced by this species. Initially, lysates of cells from two Canary Island *G. excentricus* strains VGO791 and VGO792 were partially purified by (i) liquid-liquid partitioning between dichloromethane and aqueous methanol followed by (ii) size-exclusion chromatography. Fractions from chromatographic separation were screened for MTX toxicity using both the neuroblastoma neuro-2a (N2a) cytotoxicity and Ca^{2+} flux functional assays. Fractions containing MTX activity were analyzed using liquid chromatography coupled to high-resolution mass spectrometry (LC-HRMS) to pinpoint potential MTX analogs. Subsequent non-targeted HRMS analysis permitted the identification of a novel MTX analog, maitotoxin-4 (MTX4, accurate mono-isotopic mass of 3292.4860 Da, as free acid form) in the most toxic fractions. HRMS/MS spectra of MTX4 as well as of MTX are presented. In addition, crude methanolic extracts of five other strains of *G. excentricus* and 37 other strains representing one *Fukuyoa* species and ten species, one ribotype and one undetermined strain/species of *Gambierdiscus* were screened for the presence of MTXs using low resolution tandem mass spectrometry (LRMS/MS). This targeted analysis indicated the original maitotoxin (MTX) was only present in one strain (*G. australes* S080911_1).

Putative maitotoxin-2 (p-MTX2) and maitotoxin-3 (p-MTX3) were identified in several other species, but confirmation was not possible because of the lack of reference material. Maitotoxin-4 was detected in all seven strains of *G. excentricus* examined, independently of their origin (Brazil, Canary Islands and Caribbean), and not detected in any other species. MTX4 may therefore serve as a biomarker for the highly toxic *G. excentricus* in the Atlantic area.

Keywords: *Gambierdiscus excentricus*; maitotoxin-4; bioguided fractionation; size-exclusion chromatography (LH-20); neuro-2a (N2a) assay; LC-HRMS/MS (Q-Tof 6550); LC-LRMS/MS (API4000 QTrap)

1.2. Introduction

Maitotoxin (MTX) (**Figure 40**) is among the most potent marine toxins identified to date, with an intraperitoneal (i.p.) lethal dose 50 (LD_{50}) in mice of 0.050 μg kg^{-1} [1]. Its oral potency, however, is much lower [2], probably due to low intestinal absorption caused by its high molecular weight and hydrophilicity. Consequently, MTX is primarily found in the tissues associated with the digestive tract of fish and is believed to play a role in ciguatera fish poisoning (CFP) if gut and liver tissues are consumed [3].

Maitotoxin was first detected in 1976 in the viscera of the bristletooth surgeonfish *Ctenochaetus striatus* (in Tahitian “maito”, hence its name) collected in Tahiti (French Polynesia) and it was initially suspected of contributing to the diversity of ciguatera symptoms [4, 5]. Eleven years later, the toxin was isolated from the dinoflagellate *Gambierdiscus* by Yasumoto, et al. [6] confirming the source of the toxin isolated from contaminated fish. Purified MTX exists as a white amorphous solid that is soluble in polar solvents (e.g., water, methanol and dimethylsulfoxide) and it is relatively stable in alkaline but not in acidic conditions. In aqueous solution, pure MTX tends to adhere to both glass and plastic surfaces [7, 8]. When dissolved in methanol-water, MTX exhibits a single UV absorbance maximum at 230 nm [9] due to the presence of a conjugated diene at one extremity of the molecule (C_2-C_3-C_4-C_{144}, **Figure 40**).

Experiments using purified MTX showed it causes a rapid influx of external Ca^{2+} and a steep increase of intracellular Ca^{2+} ($_iCa^{2+}$) concentration in a wide variety of cells [10, 11]. The Ca^{2+} influx elicited by

MTX leads to numerous secondary events, including: depolarization in neuronal cells [12], phosphoinositide breakdown [13], smooth muscle contraction [14-16], induction of acrosome reaction in sperm [17-19], secretion of neurotransmitters (e.g., dopamine [20], noradrenaline [21, 22], GABA [23]), hormones (e.g., insulin [24, 25]) and inflammatory intermediates (e.g., arachidonic acid [26] and histamine [27]), formation or activation of large cytolytic/oncotic pores [28-30].

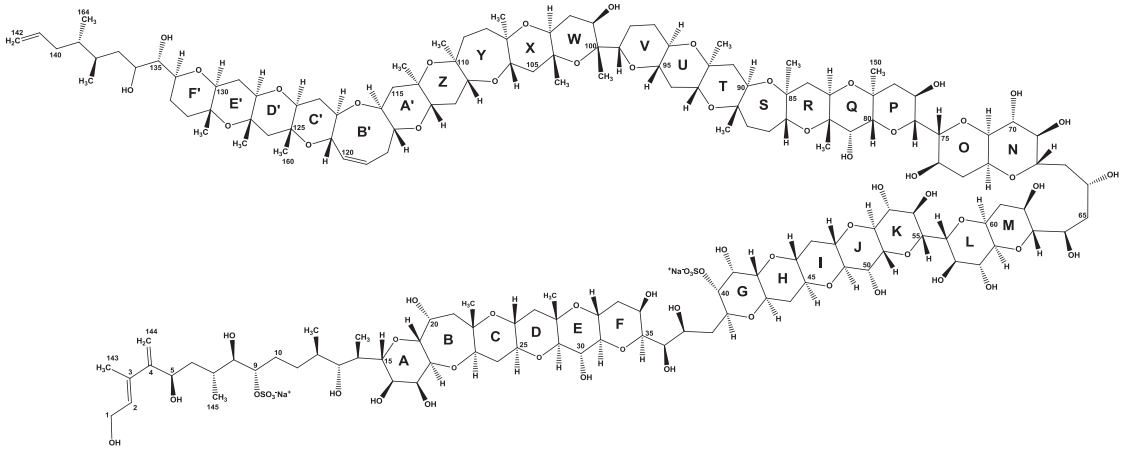

Figure 40. Absolute stereochemistry of maitotoxin (MTX) according to Sasaki, et al. [31] and Nonomura, et al. [32].

The complete chemical structure of MTX was elucidated in 1993 following purification from the *Gambierdiscus* strain (GII-1) isolated from Gambier Islands (French Polynesia) [1]. That analysis showed MTX is the largest non-polymeric marine toxin identified to date, consisting of a ladder-shaped cyclic polyether that is composed of 32 fused ether rings, 28 hydroxyl groups, 21 methyl groups, two sulfates and 98 chiral centers (molecular formula: $C_{164}H_{256}O_{68}S_2Na_2$, mono-isotopic mass = 3423.5811 Da for the di-sodium salt). In 1996, the stereochemistry of the entire molecule was also assigned [31-33] (**Figure 40**). Gallimore and Spencer [34] contested the stereochemistry of the junction between J and K rings according to a mechanistic hypothesis for the biosynthesis of marine ladder-shaped polyethers. Subsequent studies by Nicolaou and Frederick [35] and Nicolaou, et al. [36] supported the originally assigned structure based on NMR spectroscopic data, computational studies and providing chemical synthesis and NMR analysis of the GHIJK ring system. X-ray crystal structure of MTX is needed to solve this controversy.

Though the effects of MTX at the cellular level are well characterized, its actual mode of action has not been fully elucidated. Initially, MTX was

considered to be a specific activator of voltage-gated calcium channels [37-39]. In actuality, MTX increases $_{i}Ca^{2+}$ by activating a voltage-independent Ca^{2+} entry mechanism in the plasma membrane, without directly promoting the release of Ca^{2+} from intracellular storage compartments [40-42]. So far, MTX has been shown to activate non-selective ion channels, probably involving TRPC1 (transient receptor potential canonical 1) [40, 41, 43, 44]. The activation of the sodium-calcium exchanger in reverse mode has also been observed in rat aortic smooth muscle cells [45]. The activation of the sodium-hydrogen exchanger equally appears to play a role in MTX cytotoxic activity in cortical neurons [46] and it may be a consequence of MTX-induced intracellular acidification, probably involving voltage-gated sodium channels [47]. Maitotoxin is also likely to convert the Ca^{2+}-ATPase (PCMA) pump into a Ca^{2+}-permeable non-selective ion channel, as demonstrated in PCMA-overexpressed *Spodoptera frugiperda* (Sf9) insect cells and human embryonic kidneys (HEK-293 cells) [48]. To date, it is unclear whether MTX directly interacts with any of these targets. Several research groups have postulated that MTX may bind a still undescribed MTX-receptor [35, 42, 49, 50]. Since the specific molecular target of MTX is still unknown, its structure-activity relationship can only be inferred based on its NMR structural features and analogies with other ladder-shaped polyether toxins. Konoki, et al. [51] first hypothesized that the hydrophobic side of MTX (rings R through F') penetrates the phospholipid bilayer of cell membranes and the hydrophilic portion of the molecule, presenting the polyhydroxy- groups and the two sulfate ester groups (rings A through Q), remains outside the cell (**Figure 40**). Modeling studies conducted by Reyes et al. [11] corroborate this hypothesis.

Sulfate ester groups seem to be critical for the biological activity of MTXs [52, 53]. A study conducted by Murata et al. [52] in particular showed that desulfatation or hydrogenation of MTX significantly decreased its ability to induce Ca^{2+} influx or phosphoinositide breakdown in insulinoma or glioma cells. Murata, et al. [54] also hypothesized that a self-assemblage of four or more molecules could occur to form a pore on cell membranes for non-selective ion influx; however, this hypothesis has not been confirmed.

During the 1990s, two other MTX analogs, MTX2 and MTX3, were isolated by Holmes et al. [53, 55]. Maitotoxin-2 (MTX2) was obtained from a single Australian *Gambierdiscus* strain from Queensland (NQ1) [55]. It had an i.p. LD_{50} in mice of 0.080 µg kg^{-1} [53], i.e., 1.6-fold less toxic than MTX

[1]. When dissolved in acetonitrile-water, MTX2 had a single UV absorbance maximum at 230 nm [53], identical to that reported for MTX in methanol-water [9]. The molecular structure of MTX2 has not been elucidated yet. Lewis, et al. [56] conducted LC-LRMS analyses of material isolated from strain NQ1 in ionspray ionization in positive ion acquisition mode (IS^+), ionspray ionization in negative ion acquisition mode (IS^-) and fast atom bombardment ionization in negative ion acquisition mode (FAB^-) and suggested that MTX2 is mono-sulfated with a molecular weight (MW) of 3298 Da (as mono-sodium salt).

Maitotoxin-3 (MTX3) was isolated from the Australian *Gambierdiscus* strain WC1/1 [53]. Maitotoxin-3 was found to be toxic in mice, inducing similar symptoms than those observed for MTX and MTX2, but scarcity of the purified compound did not permit the determination of MTX3 potency (i.p. LD_{50} in mice) [53]. On a reversed-phase column, MTX3 elutes earlier than MTX2 and later than MTX when using a linear gradient of acetonitrile/water [53]. When dissolved in acetonitrile-water, MTX3 had a UV spectrum composed of two peaks, a minor peak at 200 nm and a major peak at 235 nm, slightly higher than MTX and MTX2 [53]. Lewis et al. [56] conducted LC-LRMS analyses of material isolated from strain WC1/1 in IS^+ acquisition mode. Their results suggested that MTX3 is di-sulfated with a MW = 1060.5 Da (as di-sodium salt). The actual molecular structure of MTX3 has yet to be determined.

Since the molecular structures of MTX2 and MTX3 are still unknown, the only structural feature that is known to be common to the MTX class of toxins is the presence of (at least) one sulfate ester group. Desulfatation experiments conducted by Holmes and Lewis [53] on the three MTX analogs again suggested that at least one of the sulfate ester groups is critical for the bioactivity.

A schematic summary of the properties of three MTXs known to date with relevant chemical information is listed in **Table 18**. A recent study conducted by Lewis et al. [57] indicated that several strains of *Gambierdiscus*/*Fukuyoa* produce multiple MTX congeners, in one case more than four (*G. belizeanus* CCMP399), suggesting broader chemical diversity than what is known so far within MTX group. These analogs are not listed here since no further information beyond activity was provided in that study.

Table 18. List of the three MTXs known to date with relevant chemical information. *Gambierdiscus* sp. GII-1 was isolated from Gambier Islands (French Polynesia); *Gambierdiscus* sp. NQ1 from Queensland (Australia) and *Gambierdiscus* sp. WC1/1 from Australia. FAB^-: fast atom bombardment ionization in negative ion acquisition mode. IS^+: ionspray ionization in positive ion acquisition mode. IS^-: ionspray ionization in negative ion acquisition mode. UNKN: unknown.

Name	Abbr.	Formula	Mass (Da)	Structural Studies	Toxicity (i.p. LD_{50} in Mice, $\mu g\ kg^{-1}$)	Source	Ref.
Maitotoxin	MTX	$C_{164}H_{256}O_{68}S_2Na_2$	3423.5811	IR, UV (λ_{max} = 230 nm) LRMS/MS: FAB^- NMR with complete stereochemistry	0.050	GII-1	[1, 31-33]
Maitotoxin-2	MTX2	UNKN (mono-sodiated salt of a molecule containing one sulfate ester)	3298	UV (λ_{max} = 230 nm) LRMS: IS^+, IS^-, FAB^-	0.080	NQ1	[55]
Maitotoxin-3	MTX3	UNKN (di-sodiated salt of a molecule containing two sulfate esters)	1060.5	UV (λ_{max} = 200, 235 nm) LRMS: IS^+	UNKN	WC1/1	[53]

Fifteen *Gambierdiscus* and three *Fukuyoa* species have been described in the past few decades [58-66]. As previously suggested [58], ongoing taxonomic studies being conducted by Tunin-Ley, et al. [67] also indicate that the biological diversity within these genera could be much higher than expected to date. The functional MTX toxicity for many of these species has been examined using erythrocyte lysis assay [68, 69] or neuroblastoma SH-SY5Y Ca^{2+} assay [57]. Of the species tested, *G. excentricus*, which was described from isolates obtained in the Canary Islands, exhibited much higher maito- and cigua (CTX)-toxicity than any other species known to occur in the Atlantic [61, 69]. Its MTX- and CTX-toxicity were comparable to *G. polynesiensis*, the most toxic species isolated to date from the Pacific [70, 71]. Due to its high MTX-toxicity, the aim of the present study was to characterize the MTX congeners produced by *G. excentricus* and to determine if they were the same or different from those previously identified. The approach was based on bioguided fractionation of the aqueous methanol fraction containing MTXs using size-exclusion chromatography (LH-20). Individual fractions were screened for MTX activity using the N2a cytotoxicity and Ca^{2+} flux functional assays. The MTX positive fractions were subjected to chemical analyses (LC-HRMS, LC-HRMS/MS and LC-LRMS/MS) to identify potential MTX congeners. Unfractionated methanolic extracts of a total of 44 strains representing one species of *Fukuyoa* and 11 species, one ribotype and one strain of *Gambierdiscus* whose species identity has yet to be determined were also

screened at the same time. The results indicated *G. excentricus* produces a novel MTX congener, MTX4, which was not found in any of the other species tested.

1.3. **Results**

1.3.1. Toxicity of the aqueous methanol fractions (MSFs) from *G. excentricus* strains VGO791 and VGO792

The toxicity of the aqueous MeOH soluble fractions (MSFs) of strains VGO791 and VGO792 from the Canary Islands were assessed using the N2a cytotoxicity assay performed at the Phycotoxins Laboratory (Ifremer, Nantes, France). The assay was calibrated using a purified MTX standard, which induced mortality of the N2a cells in a concentration-dependent manner, with an EC_{50} of 158.5 ± 5.4 (SD, n = 3) ng MTX mL^{-1} (**Figure 48**, **section 1.6**). Strain VGO791 exhibited a toxin content of 0.65 ± 0.13 ng MTX equivalents (eq) $cell^{-1}$ and VGO792 a toxin content of 0.19 ± 0.05 ng MTX eq $cell^{-1}$. Strain VGO791 was therefore 3.4-fold more toxic than VGO792. The results for VGO791 were in accordance with a previous study conducted by Fraga et al. [61], which estimated the toxicity of this strain at 0.60 ± 0.24 MTX eq $cell^{-1}$. In contrast, the toxicity estimated for VGO792 in the present study was 2.5-fold lower than that of 0.48 ± 0.16 ng MTX eq $cell^{-1}$ obtained by Fraga et al. [61].

1.3.2. Screening of fractionated *G. excentricus* extracts using neuroblastoma N2a assays

1.3.2.1. N2a cytotoxicity assay

The fractionation of the extracted MSF sample from strain VGO791 was accomplished using size-exclusion chromatography (SEC, LH-20). The fractions containing MTX activity consistently eluted within an elution volume (V_e) range of 12.5–27.5 mL. The most toxic fractions were found in V_e = 15.0–17.5 mL (up to 58.8% total cytotoxicity) (**Figure 41a**). Similarly, toxic fractions from strain VGO792 eluted in the range of V_e = 13.0–26.0 mL, with the most toxic fractions being fraction V_e = 15.0–16.0 mL (up to 29.8% total cytotoxicity) and V_e = 16.0–17.0 mL (up to 23.3% total

cytotoxicity) (**Figure 41b**). Fractions of VGO792 corresponding to V_e = 23.0–26.0 mL showed slight cytotoxic activity (N2a cell survival ~80–90%) only when the highest concentration of cell extracts was tested (150 *Gambierdiscus* cell eq per well). Serial dilutions of the latter fractions contained no detectable toxicity as measured using the N2a assay (N2a cell survival >90%). Consequently, sigmoidal dose-response curves could not be plotted and EC_{50} values could not be calculated for quantification purposes for the low toxicity fractions.

The N2a cytotoxicity assay showed that toxic compound(s) eluted right after the total exclusion volume of the LH-20 column (i.e., approximately 30% of bed volume, 12.4 mL). LH-20 chromatography separates compounds with MW ≤ 5000 Da according to their size (i.e., smaller compounds elute later than bigger ones) meaning the toxic compound(s) from these strains are likely to fall in the range of ~3000–3500 Da, consistent with the molecular weight of MTX.

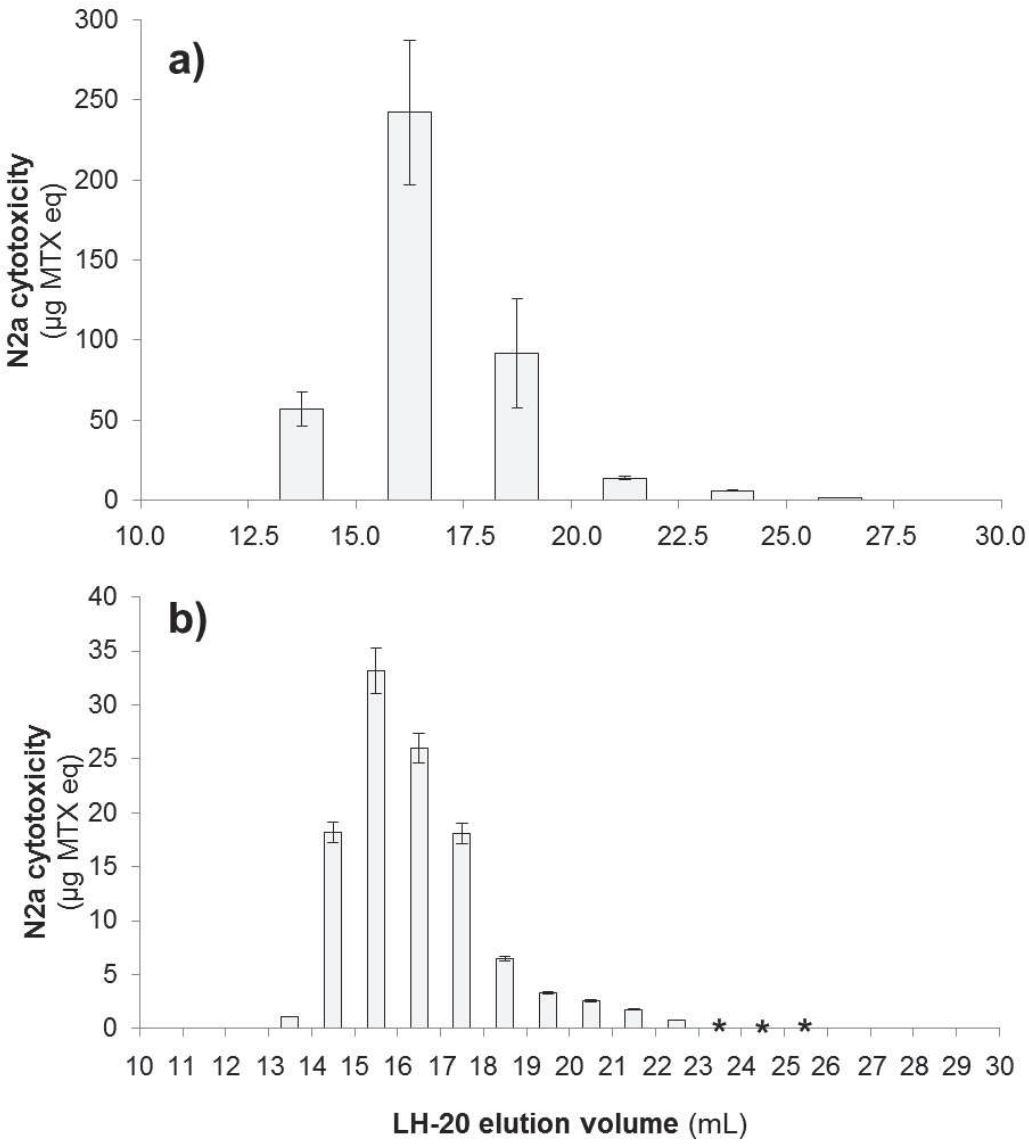

Figure 41. Estimated maitotoxin equivalents (µg MTX eq) in the LH-20-fractionated MSFs of *G. excentricus* strains VGO791 and VGO792 measured using the N2a cytotoxicity assay. (**a**) MTX eq of the individual 2.5 mL-fractions for *G. excentricus* VGO791 (2.20 million cells extracted in original MSF, approximately half of which was loaded on the LH-20 column). (**b**) MTX eq of the individual 1 mL-fractions for *G. excentricus* VGO792 (2.16 million cells extracted in original MSF, approximately half of which was loaded on the LH-20 column). The x-axis is expressed as the total elution volume (mL) that has passed through the LH-20 column when each fraction was collected. Error bars represent assay variability (standard deviation, SD) measured by running extracts in three separate assays. In each assay, three separate wells were used for assaying each fraction. Cytotoxicity was observed only in LH-20 fractions within an elution volume (V_e) range of (**a**) V_e = 12.5–27.5 mL and (**b**) V_e = 13.0–26.0 mL. Asterisks (*) in (**b**) indicate detection of non-quantifiable cytotoxicity in fractions with V_e = 23.0–26.0 mL. Fractions corresponding to V_e < 10.0 mL and V_e > 30.0 mL are not shown because no cytotoxicity was observed in those fractions.

1.3.2.2. N2a calcium flux assay

The N2a-based high-content screening (HCS) assay for calcium (Ca^{2+}) flux was performed at the ANSES Laboratory (Fougères, France). A Ca^{2+} flux assay was used in this study to measure changes in internal Ca^{2+} concentration in N2a cells. The assay works by loading cells with a

fluorescent dye, in this case Fluo-4-AM, whose fluorescence changes as a function of intracellular Ca^{2+} ($_{i}Ca^{2+}$) concentration. Maitotoxin standard elicited an increase of $_{i}Ca^{2+}$ in N2a cells in a concentration-dependent manner (**Figure 49**, **section 1.6**). Since MTX induces influx of Ca^{2+} into cells, a significant increase in fluorescence is consistent with the presence of MTX (**section 1.5.5.2**). Results were expressed as a fold of intensity compared to control treatment (5% MeOH in FCS-free N2a medium).

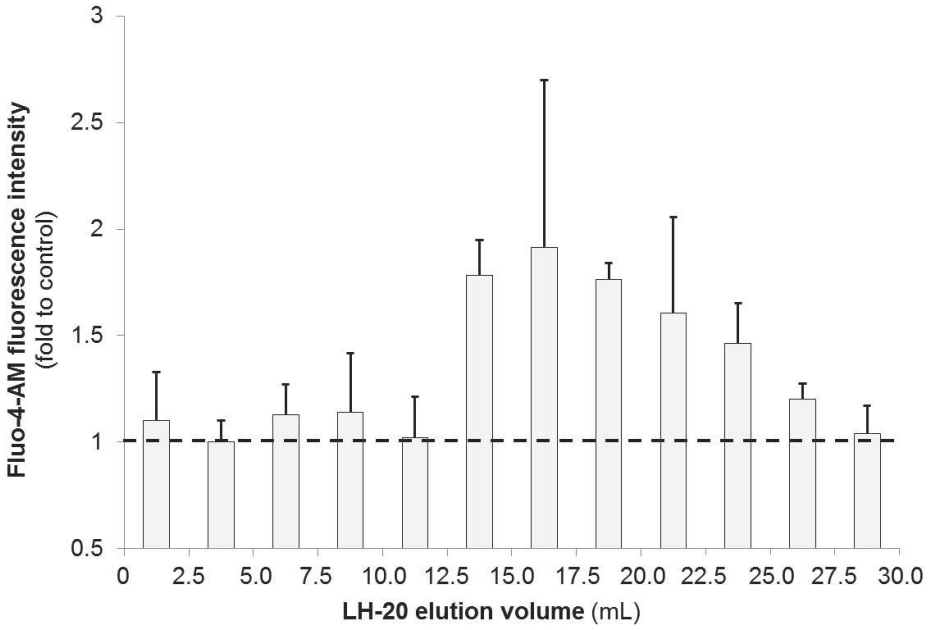

Figure 42. Calcium (Ca^{2+}) influx into N2a cells induced by LH-20 fractions of *G. excentricus* VGO791 (MSF sample). Ca^{2+} flux was measured using fluorescence of Fluo-4-AM (488 nm). Fluo-4-AM fluorescence was expressed as a fold of intensity compared to the control wells (horizontal dashed line). Error bars represent assay variability (standard deviation, SD) measured by running extracts in three separate assays. In each assay, three separate wells were used for assaying each fraction. Ca^{2+} influx was observed only in LH-20 fractions corresponding to an elution volume (V_e) of 12.5–27.5 mL. Fractions eluting after a V_e of 30 mL were omitted because they exhibited no MTX-induced fluorescence changes.

The crude extract (CE) and the MSF of *G. excentricus* VGO791 increased $_{i}Ca^{2+}$ levels in N2a cells up to the saturation level (data not shown), indicating the presence of compounds exhibiting activity similar to MTX. Only the six LH-20 fractions of MSF falling within a V_e range of 12.5–27.5 mL showed an increase in $_{i}Ca^{2+}$ levels of between 1.2- and 1.8-fold (**Figure 42**), suggesting that MTX-related compound(s) mostly eluted in these fractions. These findings are consistent with the results obtained by the N2a cytotoxicity assay (**section 1.3.2.1**).

1.3.3. Liquid chromatography coupled to full-scan high resolution mass spectrometry and discovery of a new maitotoxin analog

The Molecular Feature Extraction (MFE) algorithm of the Agilent MassHunter Qualitative Analysis software allows for retreatment of raw data of non-targeted HRMS analysis (Q-Tof 6550). Extracted compound chromatograms acquired in negative ionization mode (negative ECCs) of *G. excentricus* VGO791 confirmed that liquid-liquid partitioning and size-exclusion chromatography purification steps considerably reduced data complexity. More than one thousand features were detected in the crude extract, while only 40 features (3%) were present in the most toxic LH-20 fraction (V_e = 15.0–17.5 mL) (**Figure 43**). Similarly, MFE results for *G. excentricus* VGO792 also exhibited a reduction of data complexity from more than five thousands features to only 239 (4.3%) in the most toxic LH-20 fraction of MSF (V_e = 15.0–16.0 mL), data not shown. These results indicate that fractionation via liquid-liquid partitioning and size-exclusion chromatography (LH-20) is a suitable strategy for *Gambierdiscus* toxin purification. In particular, LH-20 is an efficient clean-up step for high molecular weight compounds as it allows for sufficient purification for individual compounds to be identified as potential MTX congeners.

Among the occurring negative ions present in the negative mode ECCs of the most toxic LH-20 fractions of *G. excentricus* VGO791 and VGO792, only one bi-charged anion presented, like MTX, an *m/z* ratio >1500. This compound possesses a retention time close to that of MTX (ΔRT = +0.49 min). Interestingly, it was detected only in the most toxic LH-20 fractions of both strains (as well as in their MSF and crude extract) and was not detected in non-toxic fractions. Thus, the presence of this compound in toxic fractions only, along with similar MS and chromatographic behavior as MTX, suggested that the compound could be a novel MTX analog and hence it was named maitotoxin-4 (MTX4).

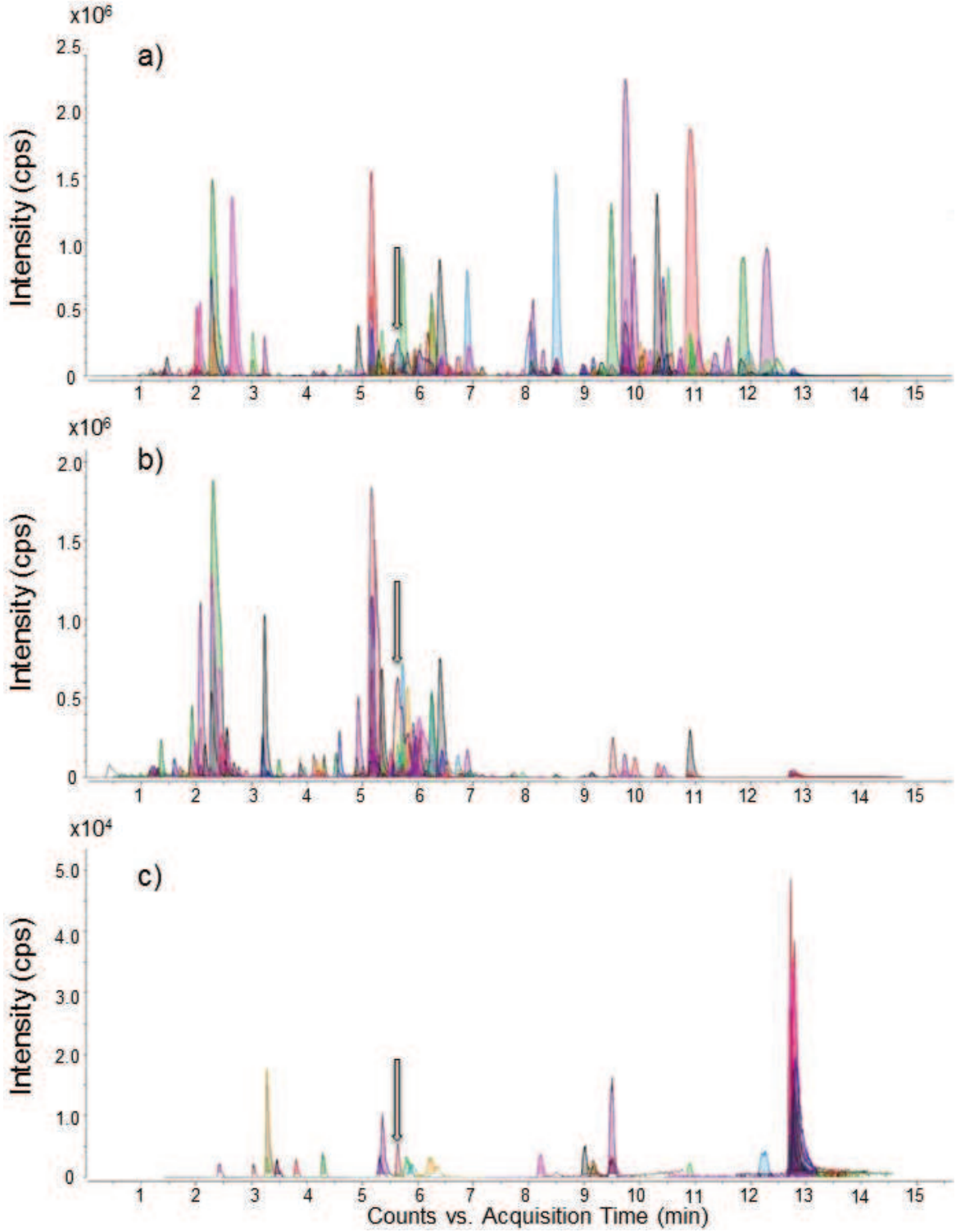

Figure 43. Negative Extracted Compound Chromatograms (ECCs) of *G. excentricus* VGO791 samples: (**a**) crude extract; (**b**) aqueous methanol soluble fraction (MSF); (**c**) the most toxic LH-20 fraction (V_e = 15.0–17.5 mL) of MSF. The different color peaks represent unique features. The highlighted grey arrow indicates the peak corresponding to maitotoxin-4 (MTX4).

1.3.4. Mass spectral comparison of maitotoxin-4 with aitotoxin

1.3.4.1. Negative mode HRMS spectra

Maitotoxin-4 (MTX4) had a spectral profile similar to MTX. Maitotoxin-4 spectra originate from the pre-purified LH-20 fraction (V_e = 15.0–16.0 mL) of *G. excentricus* VGO792. The assigned negative HRMS ion species (accurate mono-isotopic *m/z*) for MTX and MTX4 are listed in **Table 19**.

Table 19. List of the assigned negative HRMS ion species for MTX and MTX4. MTX4 spectra originate from the pre-purified LH-20 fraction (V_e = 15.0–16.0 mL) of *G. excentricus* VGO792. The *m/z* values in the table correspond to the accurate mono-isotopic *m/z*. ND: not detected. UNKN: unknown.

		MTX	**MTX4**
Elemental formula (free acid form)		$C_{164}H_{258}O_{68}S_2$	UNKN
Retention time (*RT*, min)		4.09 min	4.58 min
Ion species (accurate mono-isotopic *m/z*)	$[M-2H]^{2-}$	1688.8027 (Δppm: −0.8)	1645.2357
	$[M+Na-3H]^{2-}$	1699.7914 (Δppm: +0.5)	1656.2256
	$[M+2Na-4H]^{2-}$	1710.7814 (Δppm: +1.1)	1667.2075
	$[M-3H]^{3-}$	1125.5334 (Δppm: −1.4)	1096.4889
	$[M-4H]^{4-}$	843.8989 (Δppm: −2.1)	ND

Both MTX and MTX4 presented: (i) bi-charged molecular anions $[M-2H]^{2-}$, with accurate mono-isotopic *m/z* of 1688.8027 for MTX (Δppm: −0.8) and 1645.2357 for MTX4 and (ii) tri-charged molecular anions $[M-3H]^{3-}$, with accurate mono-isotopic *m/z* of 1125.5334 for MTX (Δppm: −1.4) and 1096.4889 for MTX4 (**Figure 44a,b**). Hence, MTX4 presents a lower mass than the MTX standard: 3292.4860 (MTX4 accurate calculated mass, free acid form) versus 3379.6172 (MTX theoretical exact mass, free acid form), *ΔM* = 87.1312. For MTX only, it was also possible to observe the quadri-charged molecular anion $[M-4H]^{4-}$, with accurate mono-isotopic *m/z* of 843.8989 (Δppm: −2.1) (**Figure 44a**). Further, the following adducts (accurate mono-isotopic *m/z*) could be assigned: $[M+Na-3H]^{2-}$ = 1699.7914 for MTX (Δppm: +0.5) and 1656.2256 for MTX4; $[M+2Na-4H]^{2-}$ = 1710.7814 for MTX (Δppm: +1.1) and 1667.2075 for MTX4 (**Figure 44a,b**). In the negative mode HRMS spectrum of MTX4, two additional peaks were observed, with accurate mono-isotopic *m/z* of 1356.6470 (*z* = 2, bi-charged anion) and 904.0954 (*z* = 3, tri-charged anion), suggesting either that some fragmentation occurred in the ESI source or that co-elution occurred during the chromatographic separation (**Figure 44b**).

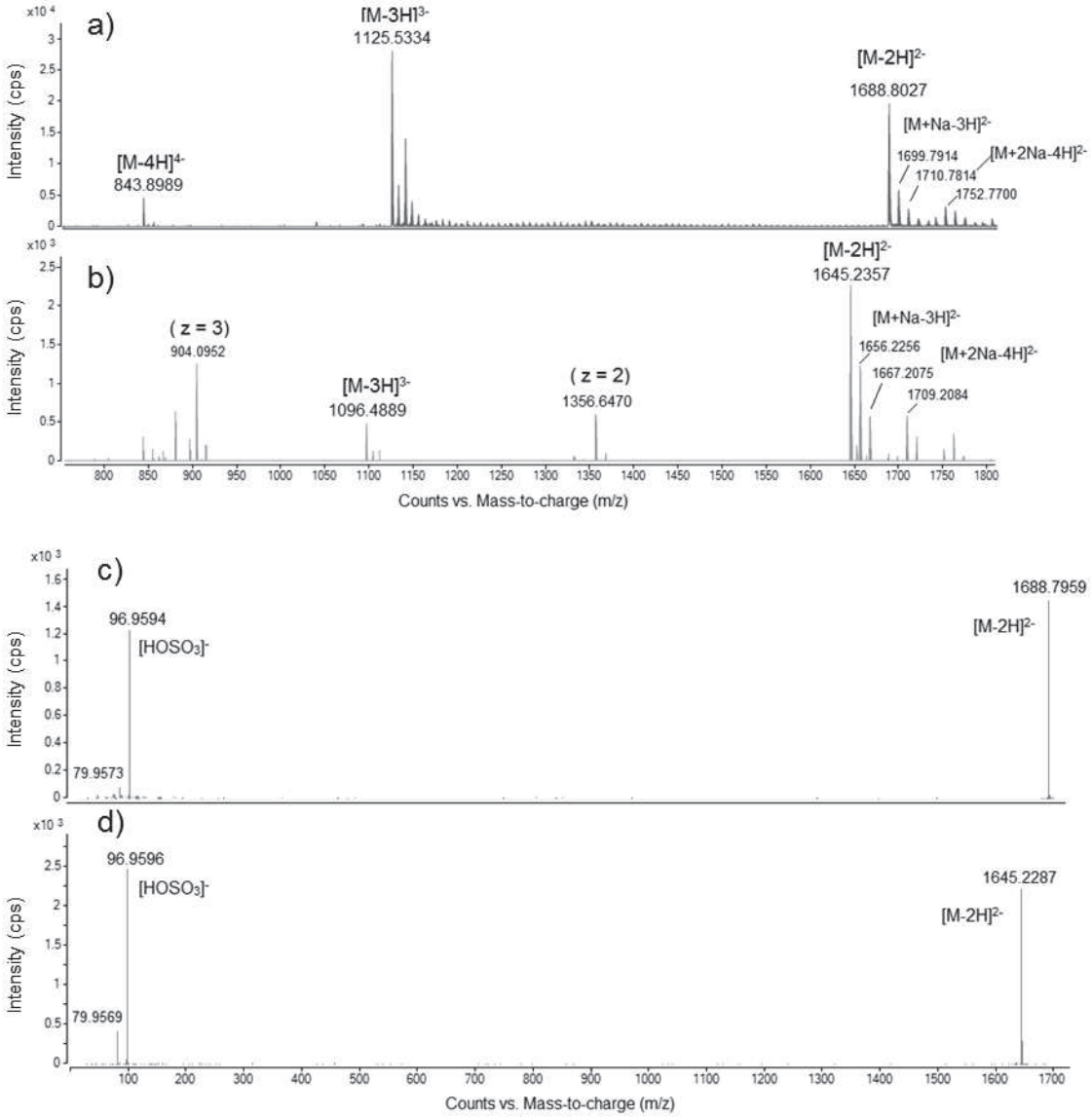

Figure 44. Raw spectra in full scan, negative ion acquisition mode HRMS of (**a**) maitotoxin (MTX) and (**b**) maitotoxin-4 (MTX4) acquired over an *m/z* range from 100 to 3200, focused on the *m/z* range from 800 to 1800. Maitotoxin-4 spectra originate from the pre-purified LH-20 fraction (V_e = 15.0–16.0 mL) of *G. excentricus* VGO792. Note the presence of bi-charged and tri-charged ion clusters for both MTX and MTX4. Negative mode ESI product ion spectra of bi-charged molecular anions of (**c**) MTX and (**d**) MTX4 at an average of three collision energies (CE): 50, 100 and 200 eV over an *m/z* range from 25 to 3200, focused on the *m/z* range from 25 to 1700. Note the same product ion $[HOSO_3]^-$ for both MTX and MTX4. Nota bene: the *m/z* values highlighted in the figure correspond to the accurate, i.e., measured mono-isotopic *m/z*.

1.3.4.2. Negative Mode HRMS/MS spectra

Maitotoxin-4 (MTX4) was further analyzed using Collision Induced Dissociation in Q-Tof targeted MS/MS mode (HRMS/MS). Maitotoxin-4 spectra originate from the pre-purified LH-20 fraction (V_e = 15.0–16.0 mL) of *G. excentricus* VGO792. Accurate mass data and isotopic distributions

for the precursor and product ions of MTX4 were compared to spectral data of the reference compound, MTX, obtained in identical experimental conditions.

HRMS/MS fragmentation of the bi-charged molecular anion ($[M-2H]^{2-}$) of MTX and MTX4 showed that the two molecules share the same product ion at an average *m/z* of 96.9593 ± SD 0.0003 (*n* = 4) when using a collision energy (CE) ≥ 100 eV (**Figure 44c,d**). This fragment corresponds to the hydrogenated sulfate anion $[HOSO_3]^-$ (Δppm: +8.3). No other characteristic fragment ion has been found at any of the collision energies tested.

1.3.5. Quantification of MTX4 via LC-LRMS/MS

The limit of detection (LOD) and the limit of quantification (LOQ) of the MRM transition $[M-2H]^{2-} \rightarrow [M-2H]^{2-}$ of MTX chosen for quantification purpose, were, respectively, 0.64 µg mL^{-1} and 2.12 µg mL^{-1}. LOD and LOQ of MTX4 were assumed being the same of MTX. The LOD and the LOQ of the MRM transition $[M-3H]^{3-} \rightarrow [M-3H]^{3-}$ of MTX were, respectively, 0.12 µg mL^{-1} and 0.39 µg mL^{-1}. As shown in **Figure 44a**, the tri-charged molecular anion of MTX gave a more intense response than the bi-charged molecular anion in our analytical conditions. Nevertheless, that was not the case for MTX4 (**Figure 44b**). Although the higher LOD and LOQ, the MRM transition $[M-2H]^{2-} \rightarrow [M-2H]^{2-}$ was chosen for quantification instead of the MRM transition $[M-3H]^{3-} \rightarrow [M-3H]^{3-}$.

Table 20 presents the results of (i) the amount of MTX4 present in crude extracts from approximately 2.2 million cells of *G. excentricus* VGO791 and VGO792 and (ii) the amount of MTX4 remaining after the subsequent liquid-liquid partitioning and LH-20 chromatography purification steps. The quantities of MTX4 were estimated using multiple reaction monitoring (MRM) mode on LC-LRMS/MS as described in **section 1.5.6.2**. The amounts of MTX4 present were expressed as µg MTX eq, assuming an equimolar response of MTX4 and MTX in MS. The efficiency of each purification step was expressed in percent recovery relative to the amount of MTX in the crude extract. The combination of the two purification steps allowed for a percent recovery of 72% for VGO791 and 70% for VGO792 if all the toxic fractions with a concentration of MTX4 > LOQ were combined (**Table 20**).

Table 20. Amounts of MTX4 present in crude extract from approximately 2.2 million cells of *G. excentricus* VGO791 and VGO792 and the amounts of toxin remaining after the subsequent liquid-liquid partitioning and LH-20 chromatography purification steps. The MTX4 estimates were obtained using multiple reactions monitoring (MRM) mode on LC-LRMS/MS. Quantification of MTX4 was based on the MRM transition 1646.2 → 1646.2 *m/z* ($[M-2H]^{2-}$ → $[M-2H]^{2-}$) using MTX as the reference standard, quantified using the MRM transition 1689.8 → 1689.8 *m/z* ($[M-2H]^{2-}$ → $[M-2H]^{2-}$). Amounts of MTX4 were therefore expressed in µg MTX eq, assuming an equimolar response of MTX4 and MTX in MS. * MSF: aqueous methanol soluble fraction.

Strain Name	Sample Name	MTX4 (µg MTX eq)	% Recovery (Liquid-Liquid Partitioning)	% Recovery (LH-20)
Gambierdiscus excentricus VGO791	Crude extract	160.1	100%	
	Liquid-liquid partitioning of crude extract			
	MSF *	137.4	85.8%	
	Size-exclusion chromatography (LH-20) of MSF sample			
	V_e = 12.5–15.0 mL	19.1		13.9%
	V_e = 15.0–17.5 mL	69.2		50.4%
	V_e = 17.5–20.0 mL	19.0		13.8%
	V_e = 20.0–22.5 mL	4.5		3.3%
	V_e = 22.5–25.0 mL	1.5		1.1%
	V_e = 25.0–27.5 mL	1.2		0.9%
Gambierdiscus excentricus VGO792	Crude extract	43.1	100%	
	Liquid-liquid partitioning of crude extract			
	MSF *	34.2	79.4%	
	Size-exclusion chromatography (LH-20) of MSF sample			
	V_e = 13.0–14.0 mL	0.6		1.7%
	V_e = 14.0–15.0 mL	4.6		13.5%
	V_e = 15.0–16.0 mL	10.3		30.1%
	V_e = 16.0–17.0 mL	6.2		18.0%
	V_e = 17.0–18.0 mL	3.3		9.6%
	V_e = 18.0–19.0 mL	1.8		5.3%
	V_e = 19.0–20.0 mL	1.2		3.4%
	V_e = 20.0–21.0 mL	0.8		2.5%
	V_e = 21.0–22.0 mL	0.6		1.9%
	V_e = 22.0–23.0 mL	0.6		1.7%
	V_e = 23.0–24.0 mL	<LOQ		
	V_e = 24.0–25.0 mL	<LOQ		
	V_e = 25.0–26.0 mL	<LOQ		

1.3.6. Relationship between N2a cytotoxicity and MTX4 content

The MTX contents of MSFs estimated via N2a cytotoxicity assay were: 1427 ± 289 µg MTX eq for *G. excentricus* VGO791 (2.20 million cells) and 408 ± 110 µg MTX eq for *G. excentricus* VGO792 (2.16 million cells). This estimation is, respectively, 10.4- and 11.8-fold higher than the MTX4 content estimated via LC-LRMS/MS (137.4 and 34.2 µg MTX eq, respectively, **section 1.3.5**).

Six LH-20 fractions of VGO791 (V_e = 12.5–27.5 mL) and ten LH-20 fractions of VGO792 (V_e = 13.0–23.0 mL) contained measurable quantities of (i) MTX equivalent toxicity (µg MTX eq, starting with an initial crude extract of ~2.2 million cells) as measured using the N2a cytotoxicity assay

(**section 1.3.2.1**) and (ii) MTX4 (µg MTX eq) using LC-LRMS/MS (**section 1.3.5**). Toxin content estimated with the N2a cytotoxicity assay was plotted against MTX4 quantification performed using LC-LRMS/MS. The linear correlation between the amount of MTX equivalents (N2a cytotoxicity assay) and the MTX4 content (LC-LRMS/MS) among the 16 toxic LH-20 fractions (slope: 3.498, R^2: 0.986, n = 16) suggests that MTX4 is a major contributor agent for the MSF-toxicity of both *G. excentricus* strains (**Figure 45**).

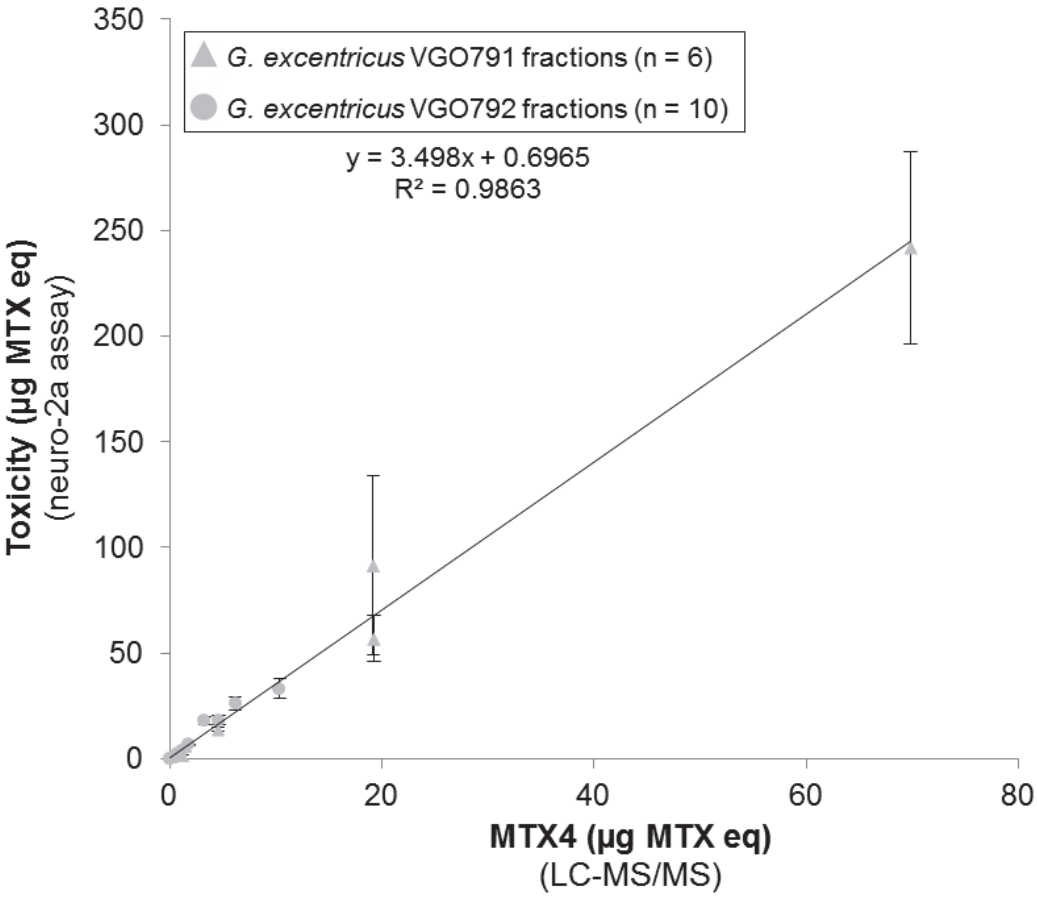

Figure 45. Linear correlation between the MTX equivalents (N2a cytotoxicity assay) and the MTX4 content (LC-LRMS/MS) in all LH-20 fractions containing quantifiable amounts of the two strains of *G. excentricus* VGO791 (n = 6) and VGO792 (n = 10). Results are expressed in µg MTX eq, assuming an equimolar response of MTX4 and MTX in MS. Error bars represent assay variability (standard deviation, SD) measured by running extracts in three separate assays. In each of these assays, three separate wells were used for assaying each fraction. The MTX4 LC-LRMS/MS determinations were not replicated.

1.3.7. Diversity of MTX analogs among *Gambierdiscus* and *Fukuyoa* strains

A total of two strains of *Fukuyoa* (one species, *F. ruetzleri*) and 42 strains of *Gambierdiscus* representing 11 species, one ribotype and one strain of *Gambierdiscus* whose identity has yet to be determined, were

screened for the presence of MTX analogs using the LC-LRMS/MS conditions described in **section 1.5.6.2**.

The LC-LRMS/MS method consisted of searching for several MRM transitions of different parent ions pointing towards the hydrogenated sulfate anion $[HOSO_3]^-$ (96.9 *m/z*). The parent ions for MTX and MTX4 were chosen from the negative ionization mode HRMS analysis conducted in this study (**section 1.3.4.1**). More precisely, the third isotopic peak (M + 2) was chosen because it was the most intense (i.e., 1689.8 *m/z* and 1126.2 *m/z* for MTX and 1645.2 *m/z* and 1097.1 *m/z* for MTX4). In the absence of MS/MS data on MTX2 and MTX3, parent ions were selected from the literature [56, 72]. For MTX2, bi-charged and tri-charged adducts with Na^+ and K^+ have been reported in a previous study [56] and these have been used for LRMS/MS analysis. Furthermore, the bi-charged and tri-charged molecular anions have been searched assuming similar MS behavior of MTX4 compared to MTX. Thus, both molecular ($[M-2H]^{2-}$ and $[M-3H]^{3-}$) and pseudomolecular anions (i.e., Na^+ and K^+ adducts) have been searched for. Maitotoxin-3 is assumed to be di-sulfated [56] and was tentatively detected in previous studies using the loss of sulfate from the mono-charged mono-sodium adduct, i.e., the MRM transition $[M+Na-2H]^- \rightarrow [HOSO_3]^-$ (*m/z* 1037.6 → 96.9) [59, 64, 66, 71-74]. With the aim of increasing selectivity, in this study the mono-charged and bi-charged molecular anions, as well as the mono-charged di-sodium adduct have been searched for, assuming similar MS behavior as that of MTX and MTX4 (**section 1.5.6.2**).

Results are summarized in **Table 21**. Limits of detection (LODs) in **Table 21** have been calculated from the number of cells extracted, specified in **Table 22**. Maitotoxin was found only in one strain, *G. australes* S080911_1, at a concentration of 22.6 ± 0.5 pg MTX $cell^{-1}$. Maitotoxin-4 was found in all seven *G. excentricus* strains examined (13–72.8 pg MTX eq $cell^{-1}$), independently of their geographical origin (**Table 21**).

MRM transitions of bi-charged anions (1637.5, 1648.2, 1656.0 *m/z*) of MTX2 towards the $[HOSO_3]^-$ (96.9 *m/z*) were not found in any of the strains examined. Putative MTX2 identified using 1091.5 and/or 1103.8 tri-charged anions as parent ions was found in *G. caribaeus*, *G. excentricus*, *G. pacificus* and *Gambierdiscus* sp. Viet Nam. HRMS analyses conducted on the most concentrated samples (*G. excentricus* strains, *G. pacificus* G10-DC and *Gambierdiscus* sp. Viet Nam) unveiled that peaks found in

LRMS/MS for MTX2 were actually false positives: 1091.5 was indeed a mono-charged ion species, so it could not be the molecular tri-charged anion $[M-3H]^{3-}$ estimated for MTX2. HRMS analyses also revealed very similar *RT* and MS/MS spectra for the peak with a nominal mass of 1091.5 in the two strains *G. pacificus* G10-DC and *Gambierdiscus* sp. Viet Nam, even though they had a different accurate mass. Additionally, the compound with a similar nominal mass of 1091.5 had a different *RT* and HRMS/MS fragmentation pathway in the *G. excentricus* strain Pulley Ridge Gam2 (**Figure 46**).

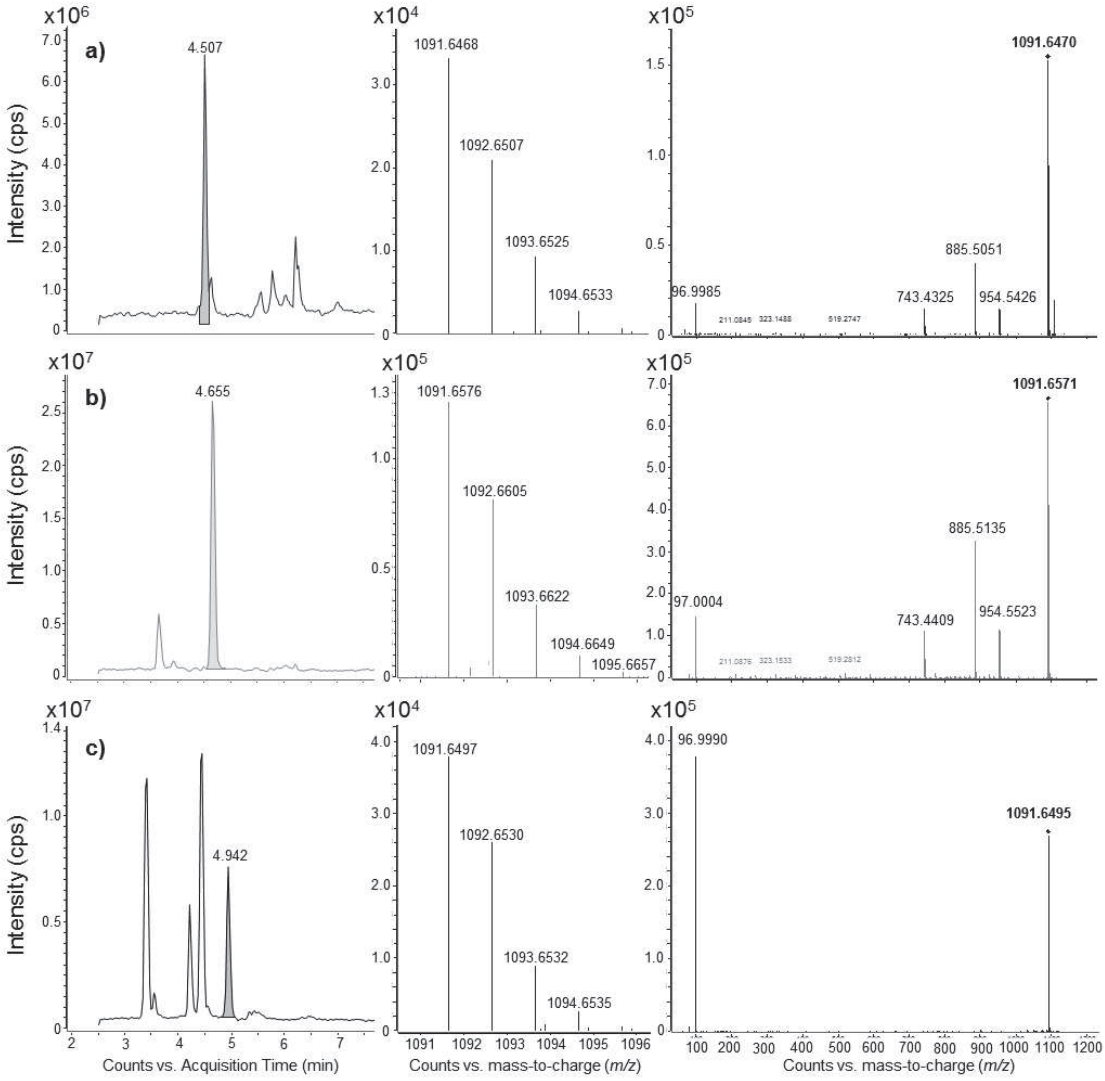

Figure 46. False positives for MTX2. Negative electrospray chromatogram, HRMS spectra and averaged HRMS/MS spectra (CEs = 25, 50 and 75 eV) of 1091.5 *m/z* found in crude extracts of: (**a**) *G. pacificus* G10-DC, (**b**) *Gambierdiscus* sp. Viet Nam and (**c**) *G. excentricus* Pulley Ridge Gam2.

Putative MTX3 analogs were found in all *Fukuyoa* and *Gambierdiscus* strains examined. LRMS/MS already suggested at least two different compounds with *m/z* = 1037.6, i.e., one compound present in all *G. excentricus* strains (*RT* = 4.0–4.1 min) and one in some strains of other species (*RT* = 4.8–4.9 min) (**Table 21**). HRMS analyses confirmed the differences between *G. excentricus* strains and all the other strains (*G. australes* VGO1178, CCMP1653 and S080911_1; *G. balechii* VGO917 and VGO920; *G. caribaeus* CCMP1733 and Bill Hi Gam8; *G. carpenteri* GT4 and WHBR21; *G. pacificus* CCMP1650*; G. scabrosus* KW070922_1) (**Figure 47**).

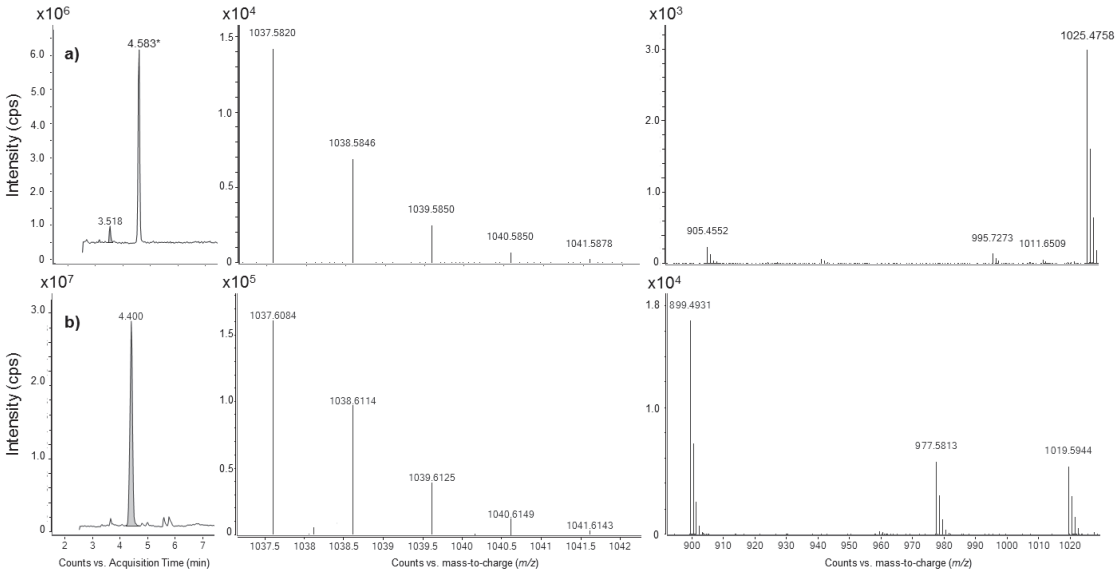

Figure 47. Putative candidates for MTX3. Negative electrospray chromatogram, HRMS spectra and averaged HRMS/MS spectra (CEs = 50, 100 and 150 eV) of 1037.5 *m/z* found in crude extracts of (**a**) *G. excentricus* VGO792, used as example for all *G. excentricus* strains; (**b**) *G. australes* S080911_1, used as example for all *G. australes*, *G. balechii*, *G. caribaeus*, *G. carpenteri*, *G. pacificus*, *G. scabrosus* strains. HRMS/MS spectra are presented with a zoom on an *m/z* range from 900 to 1020. * = peak only observed due to large mass window in quadrupole filter of HRMS/MS.

For *G. excentricus* species, LH-20 fractionation of VGO791 and VGO792 showed no correlation between the cytotoxicity observed and the peak intensities corresponding to p-MTX2 (V_e = 21.0–34.0 mL) and p-MTX3 (V_e = 16.0–29.0 mL) analogs but only with MTX4 (**section 1.3.6**).

Table 21. Screening for the presence of MTX analogs in crude extracts of a total of 44 strains of *Gambierdiscus* and *Fukuyoa* using LC-LRMS/MS analysis. MTX4 content is expressed in pg MTX eq cell^{-1}, assuming an equimolar response of MTX4 and MTX in MS. *RT*: retention time. ND: not detected. *: only one replicate available. LOD for MTX and MTX4 per sample is indicated in parenthesis and it is expressed in pg MTX eq cell^{-1}. LODs were calculated according to the number of cells extracted for each strain (**Table 22**).

Species	Strain	MTX	p-MTX2		p-MTX3		MTX4
		pg MTX cell^{-1} ± SD (n = 3) *RT* = 4.64 min	1091.5 → 96.9	1103.8 → 96.9	1015.5 → 96.9	1037.6 → 96.9	pg MTX eq cell^{-1} ± SD (n = 3) RT = 5.05 min
F. ruetzleri	Gam1	ND (< 2.97)	ND	ND	ND	RT = 4.89 & 5.74	ND (< 2.97)
	WH55-Gam4	ND (< 4.97)	ND	ND	ND	RT = 4.88	ND (< 4.97)
G. australes	CCMP1653 (NOAA 24) (T39)	ND (< 1.54)	ND	ND	ND	RT = 4.86	ND (< 1.54)
	S080911_1	22.6 ± 0.5	ND	ND	ND	RT = 4.86	ND (< 0.80)
	VGO1178	ND (< 2.46)	ND	ND	ND	RT = 4.87	ND (< 2.46)
	VGO1181	ND (< 2.22)	ND	ND	ND	RT = 4.87	ND (< 2.22)
G. balechii	VGO917	ND (< 1.66)	ND	ND	RT = 4.10 & 4.21	RT = 4.80	ND (< 1.66)
	VGO920	ND (< 2.02)	ND	ND	RT = 3.94	RT = 4.86	ND (< 2.02)
G. belizeanus	CCMP399 (NOAA2) (SB03)	ND (< 3.51)	ND	ND	ND	RT = 4.80	ND (< 3.51)
	Keys Gam1	ND (< 7.79)	ND	ND	ND	RT = 4.80	ND (< 7.79)
	ST1-F4	ND (< 6.20)	ND	ND	ND	RT = 4.83	ND (< 6.20)
G. caribaeus	CCMP1733 (NOAA11)	ND (< 2.27)	RT = 3.64 & 4.28	RT = 3.64 & 4.28	ND	RT = 4.84	ND (< 2.27)
	Bill Hi Gam8	ND (< 5.79)	RT = 4.05 & 4.30	RT = 4.05 & 4.30	ND	RT = 4.83	ND (< 5.79)
	CCMP1651 (NOAA20)	ND (< 10.27)	RT = 4.27	RT = 4.27	ND	RT = 4.82	ND (< 10.27)
	Dive 1 fa Gam1	ND (< 1.69)	RT = 4.26	RT = 4.26	ND	RT = 4.83	ND (< 1.69)
	Mexico Algae1 Gam1	ND (< 5.19)	RT = 4.27	RT = 4.27	ND	RT = 4.80	ND (< 5.19)
G. carolinianus	ETB Exp28 Gam10	ND (< 10.49)	ND	ND	RT = 4.55, 6.11 & 6.21	RT = 4.89, 6.11 & 6.21	ND (< 10.49)
	Greece Gam2	ND (< 7.73)	ND	ND	RT = 4.54 & 6.21	RT = 4.86, 6.21 & 7.17	ND (< 7.73)
	RROV5	ND (< 5.47)	ND	ND	RT = 5.12 & 6.25	RT = 5.12 & 6.25	ND (< 5.47)
G. carpenteri	GT4	ND (< 7.27)	ND	ND	ND	RT = 4.86	ND (< 7.27)
	Jamaica Algae2 Gam1	ND (< 6.51)	ND	ND	ND	RT = 4.81	ND (< 6.51)
	Pat Hi Jar7 Gam11	ND (< 10.72)	ND	ND	ND	RT = 4.86	ND (< 10.72)
	WHBR21	ND (< 6.62)	ND	ND	ND	RT = 4.84	ND (< 6.62)
G. excentricus	Pulley Ridge Gam 2	ND (< 0.39)	RT = 5.68	ND	ND	RT = 4.01	22.9*
	UNR-07	ND (< 0.63)	RT = 5.64	ND	ND	RT = 4.05	16.0 ± 2.3
	UNR-08	ND (< 1.45)	RT = 5.68	ND	ND	RT = 4.05	19.8 ± 6.4
	VGO1035	ND (< 2.64)	RT = 5.68	ND	ND	RT = 4.01	13.0*
	VGO790	ND (< 3.73)	RT = 5.67	ND	ND	RT = 4.01	23.2*
	VGO791	ND (< 0.29)	RT = 5.67	ND	ND	RT = 3.71 & 4.01	72.8 ± 8.5
	VGO792	ND (< 0.30)	RT = 5.68	ND	ND	RT = 3.68 & 3.99	20.0 ± 2.9

Species	Strain	MTX	p-MTX2		p-MTX3		MTX4
		pg MTX cell^{-1} ± SD (n = 3) RT = 4.64 min	1091.5 → 96.9	1103.8 → 96.9	1015.5 → 96.9	1037.6 → 96.9	pg MTX eq cell^{-1} ± SD (n = 3) RT = 5.05 min
G. pacificus	CCMP1650 (NOAA 9) (MR1)	ND (< 1.02)	RT = 5.33, 6.06, 6.24 & 6.82	RT = 5.33, 6.06, 6.24 & 6.82	ND	RT = 4.89	ND (< 1.02)
	G10DC	ND (< 0.66)	RT = 5.06	ND	RT = 3.90 & 4.15	-	ND (< 0.66)
Gambierdiscus sp. ribotype 2	CCMP1655 (MQ2)	ND (< 5.87)	ND	ND	ND	RT = 4.83	ND (< 5.87)
	Mixed PR	ND (< 6.49)	ND	ND	ND	RT = 4.90	ND (< 6.49)
	St Maartens Gam10	ND (< 5.52)	ND	ND	ND	RT = 4.90	ND (< 5.52)
	SW Algae Gam1	ND (< 8.38)	ND	ND	ND	RT = 4.81	ND (< 8.38)
G. scabrosus	KW070922_1	ND (< 0.83)	ND	ND	ND	RT = 4.80	ND (< 0.83)
G. silvae	UNR-30	ND (< 1.76)	ND	ND	RT = 5.93 & 6.13	RT = 3.22, 4.76, 5.35, 5.93, 6.13, 6.98	ND (< 1.76)
	VGO1167	ND (< 1.27)	ND	ND	-	RT = 4.83	ND (< 1.27)
	VGO1180	ND (< 1.52)	ND	ND	RT = 5.92 & 6.14	RT = 4.68, 5.92 & 6.14	ND (< 1.52)
Gambierdiscus sp.	Viet Nam	ND (< 0.37)	RT = 5.14	ND	RT = 7.02	RT = 7.64	ND (< 0.37)
G. toxicus	GTT-91	ND (< 5.49)	ND	ND	RT = 2.94	RT = 4.89	ND (< 5.49)
	HIT-0	ND (< 6.00)	ND	ND	RT = 2.92	RT = 4.89	ND (< 6.00)
	HIT-25	ND (< 8.56)	ND	ND	RT = 2.90	RT = 4.88	ND (< 8.56)

1.4. **Discussion**

1.4.1. *Gambierdiscus excentricus* and the discovery of maitotoxin-4

Recent studies showed *G. excentricus* as one of the most toxic species known to date, both for CTXs and MTXs [61, 69, 75]. The species was first described in the Canary Islands [61], a subtropical region (North-Eastern Atlantic Ocean) from which Ciguatera Fish Poisoning (CFP) has recently been reported [76, 77]. Subsequently, it has also been found in Brazil [78, 79] and in the Caribbean Sea [75]. The aim of the present study was to identify maitotoxin or analogs produced by this species using high resolution mass spectrometry (HRMS).

Several difficulties had to be surmounted in the present study to identify such analogs. While the N2a cytotoxicity assay allowed for MTX detection at ng mL^{-1} levels, comparable to what had been reported by Caillaud, et al. [80], Q-Tof LC-HRMS (negative ion acquisition mode) had dramatically poorer sensitivity, with an LOD for MTX at 1.88 µg mL^{-1}. The LOD of MTX using HRMS was relatively high compared to the LOD reported for LRMS [3]. However, as MTX itself was not present, an untargeted approach based on full-scan HRMS was necessary to screen for any potential analogs present, and the lower sensitivity of this technique had to be accepted. Compared to the need for µg quantities of toxin for LC-HRMS analysis, maitotoxic species only produce up to ca. 80 pg MTX eq $cell^{-1}$ [69]. Hence, it was necessary to have a substantial biomass for purification purposes in order to obtain detectable amounts of toxin. *Gambierdiscus excentricus* is difficult to cultivate compared to other algae, including other *Gambierdiscus* species [69, 75]. This is challenging because *Gambierdiscus* species grow extremely slowly (0.08–0.10 divisions day^{-1}) compared to most other microalgae [69, 75, 81, 82]. Hence, large-scale cultures of the Canary Island strains VGO791 and VGO792 were necessary to obtain sufficient material (>2 million cells) for LC-HRMS analysis.

Once sufficient biomass was obtained, toxicity screening (N2a-based assays) was applied to identify toxic fractions following liquid-liquid partitioning and LH-20 chromatography. Results of both N2a cytotoxicity and Ca^{2+} flux assays on LH-20 fractions suggested that *G. excentricus*

strains produce compound(s) with relatively high molecular weight (early elution on LH-20) and Ca^{2+}-related activity (N2a assays), similar to MTX. Chemical analyses using LC-HRMS (full scan mode) and LC-HRMS/MS (targeted mode) led to the discovery of a novel MTX analog, named maitotoxin-4 (MTX4), a sulfated compound with an accurate mono-isotopic mass of 3292.4860 Da (for the free acid form). No MTX2 was detected in any of the seven strains of *G. excentricus*, and, additionally, toxicity in LH-20 fractions was not correlated to putative MTX3. Therefore, and even though MTX4 was not yet completely purified, the correlation between MTX4 content in pre-purified fractions and their N2a cytotoxicity (**Figure 45**) supports the hypothesis that MTX4 is a major contributor to the toxicity of the MSF fraction of *G. excentricus*.

MTX equivalents measured in LH-20 fractions of both strains of *G. excentricus*, using the N2a cytotoxicity assay (**section 1.3.6**), were 3.5-fold higher than the MTX4 content measured via LC-LRMS/MS for an equivalent number of extracted cells. This factor of 3.5 does not necessarily indicate that the toxin content is overestimated using the cytotoxicity assay as the following assumptions were made: (i) MTX4 exhibits the same toxicity as MTX and (ii) MTX and MTX4 have the same behavior in MS, i.e., that they have an equimolar response. The relative toxicity between the two molecules is not yet known, and neither are their ionization and fragmentation yields known in MS. Furthermore, as suggested by Lewis et al. [57] for several other *Gambierdiscus* species, additional analogs of MTX4 may be present in the same LH-20 fractions, albeit at lower concentration as we were not able to identify such analogs by HRMS.

1.4.2. Screening for the presence of other MTX analogs

Another aim of the present study was to assess the diversity of previously reported MTX analogs produced by different strains and species of the genera *Gambierdiscus* and *Fukuyoa*. In order to achieve this goal, an LC-LRMS/MS method (API 4000 QTrap) was developed to screen for the presence of four MTX analogs in the extracts of two strains of *F. ruetzleri* and 42 strains representing 11 species, one ribotype and one strain of undetermined species (*Gambierdiscus* sp. Viet Nam) of *Gambierdiscus* spp. (**section 1.5.6.2**).

Previous LC-LRMS/MS studies conducted by a group from the Cawthron Institute [59, 64, 66, 71-74] evaluated the presence of MTX and

putative MTX3 in a total of 32 strains of *Gambierdiscus* and *Fukuyoa*, i.e., two strains of *F.* cf. *yasumotoi*, 13 strains of *G. australes*, one strain of *G. belizeanus*, one strain of *G. carpenteri*, two strains of *G. cheloniae*, two strains of *G. honu*, six strains of *G. lapillus*, four strains of *G. pacificus* and one strain of *G. polynesiensis*. These authors reported that MTX was only present in 11 out of 13 *G. australes* strains (0.3–36.6 pg MTX $cell^{-1}$), one originating from Cook Islands [71] and the other ten from Kermadec Islands [74]. In our present study, MTX was only detected in the one strain of *G. australes* (S080911_1) from Japan, at a concentration of 22.6 pg MTX $cell^{-1}$, also confirmed by LC-HRMS. The other three strains of *G. australes* examined in this study (CCMP1653, from Hawaii; VGO1178 and VGO1181, from Canary Islands) did not contain detectable (>LOD) levels of MTX. This absence of MTX in the other strains may derive from comparatively high LODs around 1/10th of the concentration of MTX detected in the *G. australes* strain from Japan. Still, this finding is consistent with previous studies in suggesting that MTX itself has been mostly confirmed in strains of *G. australes*, at least since the most recent taxonomic separation into species and phylotypes. Again, chemical diversity in *G. australes* may also be larger than reported until now since Lewis et al. [57] reported at least two compounds with Ca^{2+} flux activity in a strain of *G. australes*.

Maitotoxin-4 was produced by all strains of *G. excentricus* examined in this study (13.0–72.8 pg MTX eq $cell^{-1}$) (**Table 21**), independently of their geographical origin, albeit all seven from the Atlantic Ocean (Brazil, Canary Islands and Caribbean Sea), and was not detected in any other species examined. These findings suggest that the production of certain MTX analogs is likely to be species-specific within *Gambierdiscus* and *Fukuyoa* genera.

While results were clear and consistent with previous studies for MTX and MTX4, further examination is needed concerning MTX2 and MTX3. Maitotoxin-2 was described only once, in 1990, from an Australian strain of *Gambierdiscus* sp. (NQ1) by Holmes et al. [55]. To our knowledge, no LC-MS/MS method has been described for the detection of MTX2, set aside one study by Lewis et al. [56], which reported negative ionspray MS data based on infusion (using an orifice voltage of -120 V) of MTX2 dissolved in MeOH:H_2O (1:1, *v/v*). The assigned negative ion species for the most intense MS peaks were: 1648.2 *m/z* for $[M+Na-3H]^{2-}$, 1656.0 *m/z* for $[M+K-3H]^{2-}$, 1098.6 *m/z* for $[M+Na-4H]^{3-}$ and 1103.8 *m/z* for $[M+K-4H]^{3-}$.

MTX2 is assumed to be a mono-sulfated compound [53, 56]. In the absence of MS fragmentation data for maitotoxin-2 (MTX2), the MRM transitions chosen in this study were based on bi-charged and tri-charged molecular anions and their respective sodium and potassium adducts pointing towards the hydrogenated sulfate anion $[HOSO_3]^-$. Therefore, analysis additionally included 1091.5 *m/z* for $[M-3H]^{3-}$ and 1637.5 *m/z* for $[M-2H]^{2-}$, derived from analogy with spectral behavior observed for MTX and MTX4. None of the strains presented a chromatographic peak with both bi- and tri-charged anion clusters in the mass spectrum. All strains of several species (*G. caribaeus*, *G. excentricus*, *G. pacificus* and *Gambierdiscus* sp. Viet Nam) were positive for the anion cluster of the nominal mass 1091.5 (which could potentially correspond to $[M-3H]^{3-}$ of MTX2). HRMS unveiled that these compounds were false positives of MTX2: the parent ion 1091.5 is actually a mono-charged ion species in *G. excentricus* strains, *G. pacificus* G10-DC and *Gambierdiscus* sp. Viet Nam (not sufficient sample material for *G. caribaeus*). Therefore, low resolution MS may misidentify these compounds for MTX2 in several species. Moreover, different retention time, accurate mass and HRMS/MS fragmentation pattern also revealed that the sulfated compound with an *m/z* 1091.5 found in *G. excentricus* strains was different from those found in *G. pacificus* G10-DC and *Gambierdiscus* sp. Viet Nam (**Figure 46**).

Maitotoxin-3 was isolated in 1994 from an Australian strain of *Gambierdiscus* sp. (WC1/1) by Holmes, et al. [53]. The only existing MS data are from positive ionspray MS analysis at different orifice potentials [56]. The authors described MTX3 as a disulfated compound (MW = 1060.5 Da for the disodium salt), with the most intense MS peak at 1039.5 *m/z* for the mono-sodiated adduct $[M+Na]^+$ [56]. The only existing LC-LRMS/MS method for MTX3 detection has been developed by Kohli at the Cawthron Institute (Nelson, New Zealand) in 2013 and uses negative ionization mode [72]. This method identified a putative MTX3 (p-MTX3) based on the MRM transition *m/z* 1037.6 → 96.8. The parent ion at 1037.6 *m/z* had been incorrectly assigned to $[M-H]^-$ by Kohli [72]; indeed, according to the original study [56], it actually corresponds to $[M+Na-2H]^-$. All but one (*G. carpenteri* Merimbula) out of the 32 strains examined by the group of the Cawthron Institute were positive for the presence of p-MTX3 [59, 64, 66, 71-74]. All the strains examined in this present study were also positive for the presence of p-MTX3, suggesting that p-MTX3 is ubiquitous within

Gambierdiscus and *Fukuyoa* genera. Interestingly, the parent ion chosen for p-MTX3 ($[M+Na-2H]^-$) has the same nominal *m/z* as that for 2,3-dihydroxyCTX3C in negative ionization MS after loss of one molecule of water (*m/z* 1037.5 for $[M-H-H_2O]^-$). 2,3-dihydroxyCTX3C is considered to be an oxidation product of CTX3C and has extensively been found in *Gambierdiscus* [80, 83]. In one study, conducted by Roeder et al. [83], however, it had been the only analog present in ten out of a total of eleven *Gambierdiscus* strains, and this study also used low resolution negative ionization MS. It is surprising that CTX3C had not been detected in that study along with 2,3-dihydroxyCTX3C, except for one strain (*Gambierdiscus* sp. Viet Nam). Therefore, we presume that this study by Roeder et al. [83] may have misidentified 2,3-dihydroxyCTX3C, as it is likely that the actual compound present was the ubiquitous p-MTX3. Therefore, we recommend that when attempting to detect 2,3-dihydroxyCTX3C in negative mode LRMS/MS to verify that the compound does not have a loss of sulfate since this points towards p-MTX3 rather than 2,3-dihydroxyCTX3C. In our study, the tentative identification of p-MTX3 involved different possible parent ions (in some cases 1037.6, on other occasions 1015.5, and sometimes both) with different retention times, therefore different compounds were present that could be tentatively related to MTX3. HRMS/MS analyses for the most concentrated samples confirmed presence of sulfate ester group(s) in these compounds which is coherent with these compounds responding to the transition of the sulfate loss in low resolution tandem MS. At least one of the compounds, i.e., the one identified in all *G. excentricus* strains, does not correlate with cytotoxicity which suggests that this potential MTX analog is not important in accounting for toxicity.

It should also be noted that *Gambierdiscus* species produce other polyether compounds besides MTX containing sulfate ester group(s). Consequently, the presence of sulfate ester group(s) alone does not constitute definitive evidence for the presence of MTX. For example, a recent study conducted by Rodríguez, et al. [84] reported on the isolation and structural characterization (HRMS and NMR) of gambierone ($C_{51}H_{76}O_{19}S$, with an exact mass of 1024.47015 Da) from *G. belizeanus* CCMP401. This molecule is a ladder-shaped polyether toxin and presents a sulfate ester group, but, contrarily to MTX, it behaves as a sodium channel activator such as CTXs, albeit with significantly smaller activity.

Similarly, Watanabe, et al. [85] reported on the structural elucidation (HRMS and NMR) of gambieroxide ($C_{60}H_{90}O_{22}S$, with an exact mass of 1194.5644), another sulfate-containing polyether compound isolated from *G. toxicus* GTP-2 (French Polynesia). Its chemical structure is very similar to that of yessotoxin (YTX); its biological activity has not yet been described.

Care should be taken in compound identification when operating in low-resolution mass spectrometry, especially when the standard is not commercially available and when searching for non-specific MRM transitions. In the present study, purification of MTX4 has not been completed and it was not possible to elucidate the full structure due to compound scarcity. Still, the evidence presented here for MTX4 as a MTX analog is based on (i) ion cluster similarity of MTX4 with MTX, (ii) targeted HRMS (loss of sulfate), (iii) bioguided fractionation behavior (both partitioning and size-exclusion chromatography), and (iv) the high cytotoxicity and Ca^{2+} influx activity. These data taken together strongly suggest that the novel molecule is a MTX analog. As MTX4 is a compound correlated with high cytotoxicity and could serve as a biomarker for the highly toxic *G. excentricus* species in the Atlantic area, further studies will focus on HRMS specific fragmentation pathways of MTX and MTX4, in both positive and negative ionization modes, and eventually nuclear magnetic resonance once sufficient compound is available.

1.5. **Materials and Methods**

1.5.1. Reference toxins and chemicals

Maitotoxin (MTX) was purchased from Wako Chemicals USA, Inc. (Richmond, VA, USA) and was used as the reference standard for cellular bioassays and chemical analyses. MTX was dissolved and stored in MeOH:H_2O (1:1, *v/v*). The stock solution was prepared at a concentration of 20 µg mL^{-1}.

HPLC grade methanol and dichloromethane for extraction were purchased from Sigma Aldrich (Saint Quentin Fallavier, France). Milli-Q water was supplied by a Milli-Q integral 3 system (Millipore, Saint-Quentin-Yvelines, France). Water (Optima quality), acetonitrile (Optima quality), formic acid (Puriss quality) and ammonium formate (Purity for MS) were

used to prepare mobile phases. These chemicals were purchased from Sigma Aldrich (Saint Quentin Fallavier, France).

Eagle's Minimum Essential Medium (EMEM, ATCC® 30-2003) for culture of mouse neuroblastoma neuro-2a (N2a) cells at the Phycotoxins Laboratory was purchased from the American Type Culture Collection (ATCC). Roswell Park Memorial Institute 1640 medium supplemented with glutamine (RPMI-1640-GlutaMAX™) for culture of N2a cells at the ANSES Laboratory was purchased from Thermofisher Scientific (Waltham, MA, USA). The following additives to the N2a medium were purchased from Sigma Aldrich (Saint Quentin Fallavier, France): sodium pyruvate, streptomycin, penicillin and fetal bovine serum (Phycotoxins Laboratory) or fetal calf serum (ANSES Laboratory). N2a assay reagents were also purchased from Sigma Aldrich (Saint Quentin Fallavier, France): trypsin-(ethylenediaminetetraacetic acid) (trypsin-EDTA) and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT). Fluo-4-AM and Hoechst 33342 probes for the N2a Ca^{2+} flux high-content screening (HCS) assay were purchased from Thermofisher Scientific (Waltham, MA, USA).

1.5.2. *Gambierdiscus* and *Fukuyoa* strains examined in this study

The 44 strains of *Gambierdiscus* and *Fukuyoa,* which were examined in this study, and their location of origin, culture conditions and number of cells extracted are listed in **Table 22**. Strains were cultivated either at the Phycotoxins Laboratory (PHYC, Ifremer, Nantes, France) [69], or at the Center for Coastal Fisheries Habit Research Laboratory (CCFHR, NOAA, Beaufort, NC, USA) [75], or at the University of Rio (UNIRIO, Federal University of Rio de Janeiro State, RJ, Brazil) [79].

Table 22. List of the two *Fukuyoa* and 42 *Gambierdiscus* strains examined in this study along with their species designation, geographical origin, culture collection of origin, culture conditions, number of cells extracted for toxin analysis and the references where the strains have been previously cited. CCFHR: National Oceanographic and Atmospheric Administration (NOAA), National Ocean Service, National Centers for Coastal Ocean Science, Center for Coastal Fisheries Habit Research (CCFHR), Beaufort, NC, USA; CCVIEO: Culture Collection of Harmful Microalgae of IEO (CCVIEO), Centro de Vigo, Vigo, Spain; IRTA: Investigación y tecnología agroalimentarias, Department of Agriculture, Government of Catalonia, Sant Carles de la Ràpita, Spain; KU: Kochi University (KU), Kochi, Japan; NCMA: Provasoli—Guillard National Center for Marine Algae and Microbiota (NCMA), Bigelow Laboratory for Ocean Sciences, East Boothbay, Maine, USA; UNIRIO: Rio de Janeiro State, Federal University (UNIRIO), Rio de Janeiro, RJ, Brazil; VNIO: Viet Nam National Institute of Oceanography (VNIO, VAST), Vinh Nguyen, Nha Trang, Viet Nam.

Species / Phylotype	Strain	Geographical origin	Culture collection	Culture conditions	Number of cells extracted	Reference
F. ruetzleri	Gam1	Southwater Cay, Belize	CCFHR	CCFHR	215,190	[57, 68]
	WH55-Gam4	Flower Garden Banks National Marine Sanctuary (West Bank), Northwestern Gulf of Mexico, United States of America	CCFHR	CCFHR	128,800	[75]
G. australes	CCMP1653 (NOAA 24) (T39)	Tern Island, Hawaii, United States of America	NCMA	PHYC	416,220	[58, 86]
	S080911_1	Kutsu, Susaki, Kochi, Japan	KU	PHYC	798,285	[87]
	VGO1178	Punta Hidalgo, Tenerife, Canary Islands, Spain	CCVIEO	PHYC	260,388	[62, 69]
	VGO1181	Punta Hidalgo, Tenerife, Canary Islands, Spain	CCVIEO	PHYC	288,608	[69]
G. balechii	VGO917	Manado, Celebes Sea, Indonesia	CCVIEO	PHYC	384,650	[60, 88]
	VGO920	Manado, Celebes Sea, Indonesia	CCVIEO	PHYC	317,600	[60]
G. belizeanus	CCMP399 (NOAA2) (SB03)	St. Barthélemy Island, Caribbean, Territorial collectivity of Saint-Barthélemy	NCMA	PHYC	182,495	[57, 58]
	Keys Gam1	Florida Keys, Florida, United States of America	CCFHR	CCFHR	82,125	[68]
	ST1-F4	St. Thomas, US Virgin Islands, United States of America	CCFHR	CCFHR	103,300	[68]
G. caribaeus	CCMP1733 (NOAA11)	Carrie Bow Cay, Belize, Caribbean, United States of America	NCMA	PHYC	282,450	[58]
	Bill Hi Gam8	Waikiki Beach, Honolulu, Hawaii, United States of America	CCFHR	CCFHR	110,600	[69]
	CCMP1651 (NOAA20)	Grand Cayman Island, Caribbean, Territory of United Kingdom	NCMA	CCFHR	62,300	[58]
	Dive 1 fa Gam1	Ft. Pierce, Florida, United States of America	CCFHR	CCFHR	378,000	[68]
	Mexico Algae1 Gam1	Cancun, Mexico	CCFHR	CCFHR	123,395	[68]
G. carolinianus	ETB Exp28 Gam10	Dry Tortugas, United States of America	CCFHR	CCFHR	61,005	[68]
	Greece Gam2	Crete, Greece	CCFHR	CCFHR	82,775	[69]
	RROV5	Puerto Rico, United States of America	CCFHR	CCFHR	116,985	[68]
G. carpenteri	GT4	Carrie Bow Cay, Belize	CCFHR	CCFHR	88,000	[57, 68]
	Jamaica Algae2 Gam1	Ocho Rios, Jamaica	CCFHR	CCFHR	98,250	[68]
	Pat Hi Jar7 Gam11	Waikiki Beach, Honolulu, Hawaii, United States of America	CCFHR	CCFHR	59,680	[57, 69]
	WHBR21	Flower Garden Banks National Marine Sanctuary (West Bank), Northwestern Gulf of Mexico, United States of America	CCFHR	CCFHR	96,720	[68]

Species / Phylotype	Strain	Geographical origin	Culture collection	Culture conditions	Number of cells extracted	Reference
G. excentricus	Pulley Ridge Gam 2	Pulley Ridge, Florida, United States of America	CCFHR	CCFHR	1,630,000	[75]
	UNR-07	Armação dos Búzios, Rio de Janeiro, Brazil	UNIRIO	UNIRIO	1,013,833	[79]
	UNR-08	Armação dos Búzios, Rio de Janeiro, Brazil	UNIRIO	UNIRIO	441,490	[79]
	VGO1035	Playa Las Cabras, La Palma, Canary Islands, Spain)	CCVIEO	PHYC	242,050	[61]
	VGO790	Punta Hidalgo, Tenerife, Canary Islands, Spain	CCVIEO	PHYC	171,711	[61]
	VGO791	Punta Hidalgo, Tenerife, Canary Islands, Spain	CCVIEO	PHYC	2,200,160	[61]
	VGO792	Punta Hidalgo, Tenerife, Canary Islands, Spain	CCVIEO	PHYC	2,159,997	[61]
G. pacificus	CCMP1650 (NOAA 9) (MR1)	Moorea, Society Islands, French Polynesia	NCMA	PHYC	630,175	[58]
	G10DC	Malaysia	IRTA	PHYC	965,040	[80]
Gambierdiscus sp. ribotype 2	CCMP1655 (MQ2)	Martinique, Caribbean, insular region of France	NCMA	PHYC	109,080	[90]
	Mixed PR	Puerto Rico, United States of America	CCFHR	CCFHR	98,560	[75]
	St Maartens Gam10	St. Maarteens, Kingdom of the Netherlands	CCFHR	CCFHR	116,000	[75]
	SW Algae Gam1	Southwater Cay, Belize	CCFHR	CCFHR	76,410	[75]
G. scabrosus	KW070922_1	Kashiwa-jima Island, Otsuki, Kochi, Japan	KU	PHYC	771,711	[63, 87]
G. silvae	UNR-30	Brazil	UNIRIO	UNIRIO	362,877	Unpublished strain
	VGO1167	Punta Hidalgo, Tenerife, Canary Islands, Spain	CCVIEO	PHYC	502,080	[62]
	VGO1180	Punta Hidalgo, Tenerife, Canary Islands, Spain	CCVIEO	PHYC	421,430	[62]
Gambierdiscus sp.	Viet Nam	Cau Island, Binh Thuan, South China Sea, Viet Nam	VNIO	PHYC	1,726,949	[67, 83, 90]
G. toxicus	GTT-91	Teahupoo, Tahiti, French Polynesia	CCFHR	CCFHR	116,625	[70]
	HIT-0	Hitiaa, Tahiti, French Polynesia	CCFHR	CCFHR	106,650	[70]
	HIT-25	Hitiaa, Tahiti, French Polynesia	CCFHR	CCFHR	74,800	[70]

1.5.3. Cell pellet extraction

Cell pellets of *Gambierdiscus* and *Fukuyoa* strains were extracted three times with MeOH (30 mL per 1 million cells) using a 3 mm diameter probe sonicator (Q-Sonica, Q700, Newtown, CT, USA) at 30% of the total power (500 W). The sonication was conducted in an ice bath (0 °C) for 15 min in pulse mode (10 s ON, 5 s OFF). At the end of each sonication step, the supernatant (crude extract) was collected by centrifugation (4 °C, 10 min, 4000 *g*). Crude extracts were filtered through a Nanosep MF 0.2 µm filter and stored at −20 °C until LC-MS analyses.

1.5.4. Fractionation of *Gambierdiscus excentricus* VGO791 and VGO792

After three weeks of semi-continuous culture [69], cells were first filtered on a 25 µm sieve, then harvested by centrifugation (20 min, 3000 *g*, 4 °C) in 50 mL Falcon® tubes. A total of 2.20 million cells (3.307 g wet pellet, 4.5 L of culture, 15 flasks) and 2.16 million cells (3.076 g wet pellet, 4.5 L of culture, 15 flasks) were harvested for *G. excentricus* VGO791 and VGO792, respectively. Cell pellets were stored at −20 °C until further extraction for toxicity screening and chemical analyses. After the cells had been harvested in log phase growth, they were extracted as described above (**section 1.5.3**). An aliquot of crude extract volume was filtered through a Nanosep MF 0.2 µm filter and stored at −20 °C until cellular bioassays and LC-MS analyses. The remnant part was evaporated under N_2 at 40 °C and stored at −20 °C.

1.5.4.1. Liquid-liquid partitioning

The residues of crude extracts (0.263 g for *G. excentricus* VGO791 and 0.251 g for *G. excentricus* VGO792) were suspended in dichloromethane (50 mL per 1 million cells) and partitioned twice with MeOH:H_2O (3:2, *v/v*) (25 mL per 1 million cells) as previously described by Satake, et al. [91]. Maitotoxins are supposed to partition into the aqueous methanol soluble fraction (MSF). Once the MSF was isolated, it was blown dry under N_2 gas at 40 °C and stored at −20 °C. The dried MSF residue was re-dissolved in 1.5 mL MeOH:H_2O (1:1, *v/v*) and filtered through a Nanosep MF 0.2 µm filter.

1.5.4.2. Size-exclusion chromatography (SEC): Sephadex™ LH-20

Prior to use, Sephadex™ LH-20 powder (10 g) was swollen in methanol (MeOH) over one night, then gently packed in a glass open column (intern diameter: 1 cm) in one continuous motion and finally rinsed with MeOH. Bed height was 52.7 cm, hence bed volume was calculated to be 41.4 mL. An aliquot of MSFs of *G. excentricus* VGO791 (0.745 mL, 1.082 million cells, 32.5 mg MSF residue) and *G. excentricus* VGO792 (0.700 mL, 0.998 million cells, 29.9 mg MSF residue) were separately deposited on the top of the LH-20 column; compounds were then eluted with MeOH under atmospheric pressure (flow rate: 0.4 mL min^{-1}). Eluting fractions were collected as follows: 50 fractions of 2.5 mL each for *G. excentricus* VGO791; 1st fraction of 10 mL, 45 fractions of 1 mL and two last fractions of 30 mL for *G. excentricus* VGO792.

1.5.5. Neuroblastoma neuro-2a (N2a) assays

1.5.5.1. N2a cytotoxicity assay

The N2a cytotoxicity assay was performed at the Phycotoxins Laboratory (Ifremer, Nantes, France) using the protocol for MTX detection described by Caillaud, et al. [92].

The N2a cell line was obtained from the American Type Culture Collection (ATCC, CCL 131). N2a cells were grown and maintained as described by Hardison, et al. [93]. The assay was carried out in 96-well flat-bottom Falcon® tissue culture plates with vacuum gas plasma treatment for cell adhesion (Dominique DUTSCHER SAS, Brumath, France). Plates were seeded with 30,000 N2a cells per well and were incubated for 24 h until they were >90% confluent at the bottom of each well. The MTX standard, controls and *G. excentricus* samples were added next and incubated for 2.5 h. The 6-point MTX standard curve for this assay ranged from 0.29 to 29,127 ng mL^{-1} per well. Controls included buffer wells to provide maximum survival estimates and wells with the addition of 3% MeOH (final concentration in well) to identify any cell mortality caused by the presence of MeOH used to dissolve the samples. The following sample aliquots (3 µL additions) were tested: MSF extracts of *G. excentricus* VGO791 and

VGO792 and their respective fractions obtained by SEC (LH-20). Total well volume was hence 103 µL.

For each sample, six 10-fold serial dilutions were tested in three separate experiments and three replicate wells for each dilution were run for each experiment. Sigmoidal dose-response curves were plotted using the four-parameter logistic model (4PL) and EC_{50} values (cell eq mL^{-1}) were calculated for each sample using SigmaPlot® 12. Quantitation of MTX eq in the samples using the N2a cytotoxicity assay was calculated by converting EC_{50} values (cell eq mL^{-1}) into toxin equivalent per cell (pg MTX eq $cell^{-1}$) taking into account the EC_{50} value obtained from the MTX standard curve. Results were then converted in µg MTX eq multiplying for the initial number of cells extracted.

Cell viability was assessed after 2.5 h incubation using the quantitative colorimetric 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) assay [94] using a Tecan Infinite® M200 plate reader (Tecan Austria GmbH, Grödig, Austria) at 544 nm. The viability of cells treated with MTX standard or algal extracts was estimated relative to control wells in solvent vehicle (3% MeOH in N2a medium).

1.5.5.2. N2a calcium flux assay

The high-content screening (HCS) assay employed an automated epifluorescence microscopy and image analysis of cells in a microtiter plate format [95, 96]. In the present study, HCS assay was used to measure Ca^{2+} influx induced by MTXs using the N2a cell line. The assay was performed at the Toxicology of Contaminants Unit, ANSES Laboratory (Fougères, France).

N2a cells (ATCC, CCL 131) were grown and maintained as described by Sérandour et al. [97]. The assay was carried out in Nunc 96-well thin bottom microplates (Thermo Scientific, Waltham, MA, USA). Each well was seeded with 33,000 N2a cells and plates were incubated for 24 h until they were >90% confluent at the bottom of each well. Next, two fluorescent dyes, Hoechst 33342 (3 µg mL^{-1}) for cell nuclei staining and Fluo-4-AM (5 µM) for ${}_{i}Ca^{2+}$ staining, were added to each well and the plates were placed back in the incubator for 40 min.

Vehicle control solution for the assay consisted of N2a culture medium without fetal calf serum (FCS) containing 5% MeOH. The MTX standard curve for the assay ranged from 26.7 to 102,776 pg mL^{-1} in well (from 7.81

to 30,000 pM) and was prepared in FCS-free N2a culture medium containing 5% MeOH. The crude extract and partially purified extracts from *G. excentricus* VGO791 were prepared as follows: 0.05 mL of each sample were diluted in 0.95 mL of FCS-free N2a culture medium resulting in a solution with final MeOH content of 5%.

Six wells per plate were dedicated to vehicle control exposure to identify any non-specific flux of Ca^{2+} due to the presence of 5% MeOH. For each sample, three separate experiments were performed, and each experiment was run in three replicate wells. N2a medium was discarded from the wells, one-by-one, and immediately replaced with 100 µL of vehicle control, MTX standard or *G. excentricus* VGO791 samples just before the fluorescence measurement. Fluorescence (388 nm for cell nuclei, 488 nm for $_{i}Ca^{2+}$) was followed in real-time with one image per 1.5 min frame rate using an ArrayScan VTI HCS Reader (Thermo Scientific, Waltham, MA, USA) with 10× objective. Intracellular calcium signal was assessed after 4.5 min exposure and expressed as a fold of intensity compared to control treatment (5% MeOH in FCS-free N2a culture medium).

1.5.6. LC-MS analyses

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

LC-HRMS analyses were performed at the Phycotoxins Laboratory (Ifremer, Nantes, France) using a UHPLC system 1290 Infinity II (Agilent Technologies, Santa Clara, CA, USA) coupled to a high resolution time-of-flight mass spectrometer Q-Tof 6550 iFunnel (Agilent Technologies, Santa Clara, CA, USA) equipped with a Dual Jet Stream® electrospray ionization (ESI) interface operating in negative mode. Toxins were separated using a reversed-phase C_{18} Kinetex column (100 Å, 2.6 µm, 50 × 2.1 mm, Phenomenex, Le Pecq, France) with water (A) and 95% acetonitrile/water (B). The column oven and the sample tray temperatures were set at 40 °C and 4 °C, respectively. The flow rate was set at 0.4 mL min^{-1}, the injection volume was set at 3 µL. Separation was achieved using the following mobile phase gradient: from 10% to 95% B in 10 min, plateau at 95% B for 2 min, return to the initial condition (10% B) in 0.1 min and a re-equilibration period (10% B) for 3.9 min. The chromatographic run lasted 16 min per

analysis. The MTX standard used for LC-HRMS experiments was at a concentration of 20 µg mL^{-1} MeOH:H_2O (1:1, *v/v*).

The conditions of the ESI source were set as follows: source temperature, 200 °C; drying gas, N_2; flow rate, 11 mL min^{-1}; sheath gas temperature, 350 °C; sheath gas flow rate, 11 mL min^{-1}; nebulizer, 45 psig; capillary voltage, −3.5 kV; nozzle voltage, 500 V. The instrument was mass calibrated in negative ionization mode before each analysis, using the Agilent tuning mix. A mixture solution of reference mass compounds (purine, 2 mL L^{-1}; HP-0921, 1 mL L^{-1}; HP-1221, 1 mL L^{-1}; HP-1821, 2 mL L^{-1}; HP-2421, 2 mL L^{-1}) in MeOH:H_2O (95:5, *v/v*) was infused with an isocratic pump to a separate ESI sprayer in the dual spray source at a constant flow rate of 1.5 µL min^{-1}. Purine and HP-0921 allowed for correction of the measured *m/z* throughout the batch.

Mass spectrum detection was carried out in full scan and targeted MS/MS mode in negative ion acquisition. The full scan acquisition operated at a mass resolution of 45,000 Full Width at Half Maximum (FWHM) over a mass-to-charge ratio (*m/z*) range from 100 to 3200 with a scan rate of 2 spectra s^{-1}. The LOD (S/N ratio > 3) calculated for MTX (negative mode EIC of 1688.8013 *m/z*) was 1.88 µg mL^{-1}. The targeted MS/MS mode was performed in a Collision Induced Dissociation cell using a mass resolving power of 45,000 FWHM over the scan range *m/z* from 50 to 3200 with a MS scan rate of 10 spectra s^{-1} and a MS/MS scan rate of 3 spectra s^{-1}. Three different collision energies were applied to the precursor ions to obtain a good fragmentation pathway.

All the acquisition and analysis data were controlled by MassHunter software (Agilent Technologies, Santa Clara, CA, USA). Raw data were processed using the Molecular Feature Extraction (MFE) algorithm of the Agilent MassHunter Qualitative Analysis software, version B.07.00, service pack 1 (Agilent Technologies, Santa Clara, CA, USA). The algorithm performs all tasks related to “peak-picking” and thus allowed for identification of all sample components down to the lowest-level abundance (abundance cut-off set at 500 counts) and to extract all relevant spectral and chromatographic information. Data-mining was carried out to manage data complexity and to correlate MS data to toxicity.

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

LC-LRMS/MS experiments to monitor specific MTX congeners in the methanolic extracts obtained from the strains listed in **Table 22** were performed at the Phycotoxins Laboratory (Ifremer, Nantes, France) using a LC system (UFLC XR Nexera, Shimadzu, Japan) coupled to a hybrid triple quadrupole/ion-trap mass spectrometer API 4000 QTrap (SCIEX, Redwood City, CA, USA) equipped with a turboV® ESI source. Toxins were separated using the same chromatographic conditions as described above (**section 1.5.6.1**). The injection volume was set at 5 µL. Mass spectrum detection was carried out in negative ion acquisition mode using multiple reactions monitoring (MRM).

The MTX standard calibration range for the LC-LRMS/MS experiments consisted of nine concentrations ranging from 0.1 to 20 µg mL^{-1} MeOH:H_2O (1:1, *v/v*). MRM experiments were established using the following source settings: curtain gas set at 25 psi, ion spray at −4.5 kV, a turbogas temperature of 300 °C, gas 1 and 2 set, respectively, at 40 and 60 psi, an entrance and declustering potential of −10 V and −210 V, respectively. The limit of detection (LOD) and the limit of quantification (LOQ) were determined with the ordinary least-squares regression data method [98, 99] using the lowest 3 points from the calibration curves. The LOD was calculated as 3 times the standard deviation of the y-intercepts over the slope of the calibration curve; the LOQ was calculated as 10 times the standard deviation of the y-intercepts over the slope of the calibration curve [98, 99].

The fragment ion monitored for all the MRM transition of the MTX-group of toxins was the hydrogenated sulfate anion $[HOSO_3]^-$ (*m/z* 96.9). The precursor ions were chosen according to data available in literature or provided in this study. Seven and six MRM transitions (*m/z*), were monitored for MTX and MTX4, respectively, to permit the best toxin identification (**Table 23**) with a dwell time of 80 msec. Quantification of MTX and MTX4 was operated using the MRM transition $[M-2H]^{2-} \rightarrow [M-2H]^{2-}$. MTX4 was quantified over the MTX calibration curve, assuming equal molar response and applying the same LOD and LOQ calculated for MTX. In the absence of MS/MS data on MTX2, precursor ions (*m/z*) were selected according to Lewis et al. [56]. Bi-charged and tri-charged molecular anions were also calculated and added to the MRM method (**Table 23**). For MTX3, the lack of MS data in negative ion acquisition mode

did not allow the selection of precursor ions (*m/z*) which were already described in literature. In the present study, a MRM transition involving $[M+Na-2H]^-$ as precursor ion was selected according to a previous study conducted by Kohli et al. [72]. MRM transitions in this study also involved the corresponding molecular mono-charged anion $[M-H]^-$ (calculated *m/z* 1015.5), its di-sodium adduct (calculated *m/z* 1057.5) and the molecular bi-charged anion $[M-2H]^{2-}$ (calculated *m/z* 507.3) as precursor ions (**Table 23**).

Table 23. List of the MRM transitions (*m/z*) for the four MTXs known to date (LC-LRMS/MS, API 4000 QTrap). MRM transitions of MTX and MTX4 were chosen according to HRMS data provided in this study. Quantification of MTX and MTX4 was operated using the MRM transition $[M-2H]^{2-} \rightarrow [M-2H]^{2-}$. In the absence of MS/MS data on maitotoxin-2 (MTX2) and maitotoxin-3 (MTX3), putative MRM transitions were chosen based on their MS spectral peaks reported in literature and assuming that they share the same fragmentation behavior as that of MTX and MTX4. * = M refers to the free acid form, i.e. without the sodium salts on sulfate ester group(s), e.g. M = $C_{164}H_{258}O_{68}S_2$ for MTX.

Compound	MRM Transitions (*m/z*) *		CE (eV)	CXP (eV)
MTX	**$[M-2H]^{2-}/[M-2H]^{2-}$**	**1689.8/1689.8**	−40	−15
	$[M-2H]^{2-}/[HOSO_3]^-$	1689.8/96.9	−125	−21
	$[M+Na-3H]^{2-}/[HOSO_3]^-$	1700.8/96.9	−125	−21
	$[M+2Na-3H]^{2-}/[HOSO_3]^-$	1711.8/96.9	−125	−21
	$[M-3H]^{3-}/[M-3H]^{3-}$	1126.2/1126.2	−40	−15
	$[M-3H]^{3-}/[HOSO_3]^-$	1126.2/96.9	−125	−21
	$[M-4H]^{4-}/[HOSO_3]^-$	844.4/96.9	−125	−21
MTX2	$[M-2H]^{2-}/[HOSO_3]^-$	1637.5/96.9	−125	−21
	$[M+Na-3H]^{2-}/[HOSO_3]^-$	1648.2/96.9	−125	−21
	$[M+K-3H]^{2-}/[HOSO_3]^-$	1656.0/96.9	−125	−21
	$[M-3H]^{3-}/[HOSO_3]^-$	1091.5/96.9	−125	−21
	$[M+Na-4H]^{3-}/[HOSO_3]^-$	1098.6/96.9	−125	−21
	$[M+K-4H]^{3-}/[HOSO_3]^-$	1103.8/96.9	−125	−21
MTX3	$[M-H]^-/[HOSO_3]^-$	1015.5/96.9	−125	−21
	$[M+Na-2H]^-/[HOSO_3]^-$	1037.6/96.9	−125	−21
	$[M+2Na-3H]^-/[HOSO_3]^-$	1057.5/96.9	−125	−21
	$[M-2H]^{2-}/[HOSO_3]^-$	507.3/96.9	−125	−21
MTX4	**$[M-2H]^{2-}/[M-2H]^{2-}$**	**1646.2/1646.2**	−40	−15
	$[M-2H]^{2-}/[HOSO_3]^-$	1646.2/96.9	−125	−21
	$[M+Na-3H]^{2-}/[HOSO_3]^-$	1657.2/96.9	−125	−21
	$[M+2Na-3H]^{2-}/[HOSO_3]^-$	1668.2/96.9	−125	−21
	$[M-3H]^{3-}/[M-3H]^{3-}$	1097.1/1097.1	−40	−15
	$[M-3H]^{3-}/[HOSO_3]^-$	1097.1/96.9	−125	−21

1.6. **Supplementary materials**

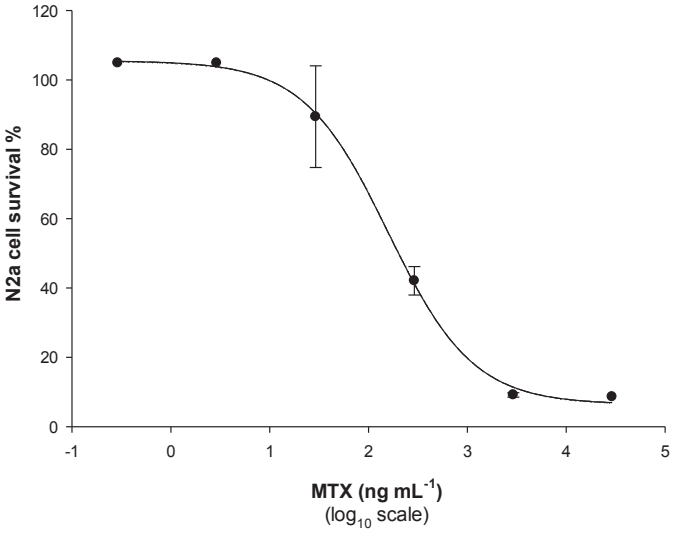

Figure 48. Sigmoidal dose-response curve of MTX standard on the neuroblastoma N2a cytotoxicity assay after 2.5 h exposure. Error bars represent assay variability (standard deviation, SD) measured by running extracts in three separate assays. In each assay, three separate wells were used for assaying each fraction.

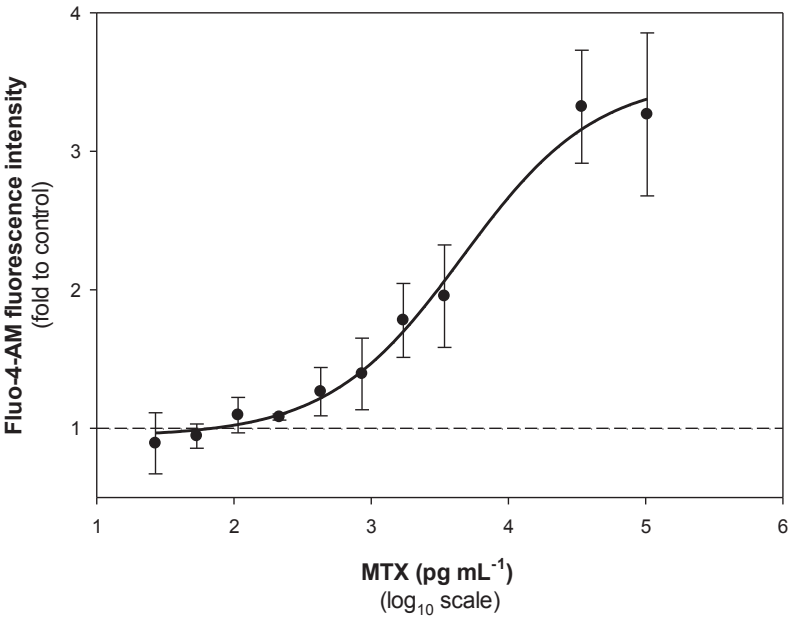

Figure 49. Sigmoidal dose-response curve of MTX standard on the N2a-based HCS assay for Ca^{2+} flux after 4.5 min exposure. Ca^{2+} flux was estimated measuring the fluorescence of the Fluo-4-AM dye at 488 nm. Fluo-4-AM fluorescence was expressed as a fold of intensity compared to vehicle control condition (5% MeOH in FCS-free N2a medium). Error bars represent assay variability (standard deviation, SD) measured by running extracts in three separate assays. In each assay, three separate wells were used for assaying each fraction.

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1.8. Author contributions

F.P., M.S., C.H. and P.H. conceived and designed the experiments; F.P. and G.G. performed: cultures of *Gambierdiscus*, cell pellet extraction, size-exclusion chromatography and cytotoxicity screening via N2a cytotoxicity assay; F.P. and K.L. performed the cultures of N2a cells; F.P., V.F. and P.-J.F. designed and performed the N2a Ca^{2+} flux high-content screening (HCS) assay experiments; F.P., M.S., P.H. and C.H. performed and analyzed the HRMS/MS and LRMS/MS data; S.F., S.M.N. and W.C.H. contributed some of the strains and culture material used in this study, V.F., C.R. contributed reagents, materials and data analysis; F.P. wrote the paper; V.F., C.R., R.W.L., C.H. and P.H. corrected and revised the paper.

1.9. Conflict of interest

The authors declare no conflict of interest. The funding sponsors had no role in the design of the study; in the collection, analyses, or interpretation of data; in the writing of the manuscript, and in the decision to publish the results.

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2. Towards the characterization of maitotoxin-4 (MTX4)

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2.1. Introduction

The epi-benthic dinoflagellate genera *Gambierdiscus* and *Fukuyoa* are the primary producers of the toxins responsible for Ciguatera Fish Poisoning (CFP), the most common non-bacterial food poisoning due to consumption of fish [1].

Recent studies revealed that *G. excentricus* is one of the most toxic species known to date, both for ciguatoxin (CTX) and maitotoxin (MTX) production [2-4]. Ciguatoxins (CTXs) are the causative agents of CFP [5]. Maitotoxins (MTXs) are extremely potent toxins [6] and may play a role in causing the symptoms associated with CFP [7]. To date, four MTX congeners have been described: maitotoxin (MTX) [8-10], maitotoxin-2 (MTX2) [11], maitotoxin-3 (MTX3) [12] and maitotoxin-4 (MTX4) [13].

Maitotoxin (MTX) is the largest non-polymeric marine toxin identified to date, consisting of a ladder-shaped cyclic polyether that is composed of 32 fused ether rings, 28 hydroxyl groups, 21 methyl groups, two sulfates and 98 chiral centers (elemental formula: $C_{164}H_{256}O_{68}S_2Na_2$, accurate mono-isotopic mass of 3423.5811 Da for the di-sodium salt) [6, 14, 15] (**Figure 50**). Production of MTX has been mostly confirmed in strains of *G. australes*, at least since the most recent taxonomic separation into species and phylotypes [13, 16-19].

The other three MTX congeners (MTX2, MTX3 and MTX4) have not been structurally elucidated yet [11-13, 20].

The description of maitotoxin-4 (MTX4, accurate mono-isotopic mass of 3292.4860 Da for the free acid form) is recent and included neuro-2a cytotoxicity data and high resolution mass spectrometry (HRMS) analysis in negative electrospray ionization mode (ESI^-) [13]. Production of MTX4 seems to be specific to *G. excentricus* species [13].

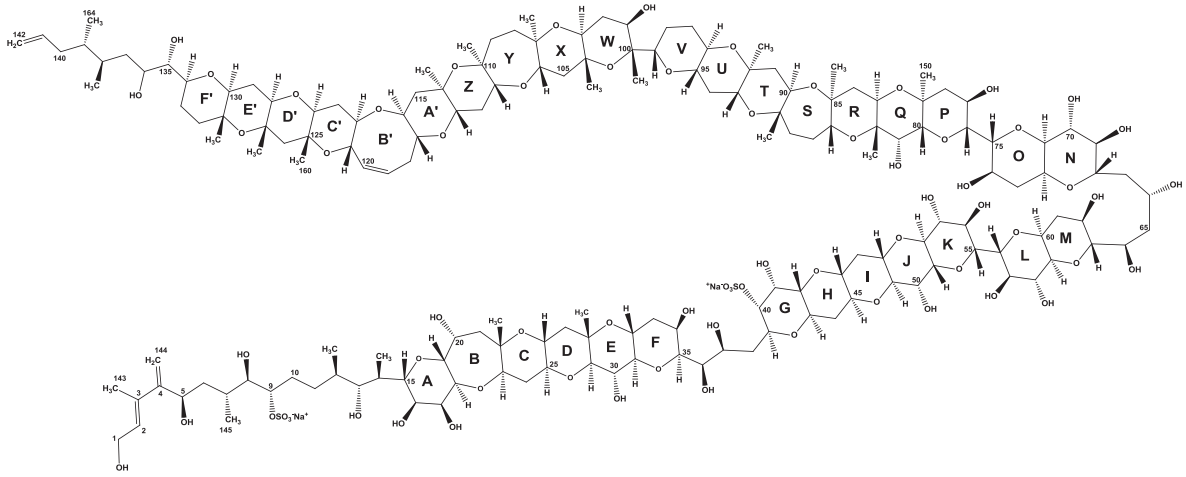

Figure 50. Structure of maitotoxin (MTX) [6] with absolute stereochemistry according to Sasaki, et al. [14] and Nonomura, et al. [15].

The previous HRMS/MS (ESI^-) experiments on MTX and MTX4 were poorly informative, as they only resulted in the loss of sulfate group(s) [3]. The aim of the present study was to make a step further into the chemical characterization of MTX4. In order to achieve this goal, chemical analyses were performed using a UHPLC system coupled to a diode array detector (DAD) and a HRMS Q-Tof 6550 iFunnel (Agilent) mass spectrometer operating in positive electrospray ionization mode (ESI^+). Comparison of UV, HRMS (ESI^+) and HRMS/MS (ESI^+) spectra brought to light new similarities and differences between MTX and MTX4. Data from the present study suggest that MTX4 has two sulfate ester groups and shares the same terminal structure as that of MTX (rings W through F', **Figure 50**).

2.2. **Experimental**

2.2.1. Chemicals and reagents

Maitotoxin (MTX) was purchased from Wako Chemicals USA, Inc. (Richmond, Virginia, USA) and was used as the reference standard for chemical analyses. MTX was dissolved and stored in MeOH:H_2O (1:1, v/v). The stock solution was prepared at a concentration of 20 µg mL^{-1}.

HPLC grade methanol was purchased from Sigma Aldrich (Saint Quentin Fallavier, France). Milli-Q water was supplied by a Milli-Q integral 3 system (Millipore, Saint-Quentin-Yvelines, France). Water (Optima quality), acetonitrile (Optima quality), formic acid (Puriss quality) and ammonium formate (Purity for MS) were used to prepare mobile phases. They were purchased from Sigma Aldrich (Saint Quentin Fallavier, France).

2.2.2. Sample preparation

Toxic fractions of *G. excentricus* extracts containing maitotoxin-4 (MTX4) were obtained in a previous study [13]. Details on strain denomination and origin, culture conditions, extraction and purification steps can be found in Pisapia, et al. [13]. In particular, the most concentrated LH-20 fraction of *G. excentricus* VGO792 was used in this study.

2.2.3. Liquid chromatography-high resolution mass spectrometry (Q-Tof 6550 iFunnel)

Liquid chromatography-high resolution mass spectrometry (LC-HRMS) analyses were performed using a UHPLC system (1290 Infinity II, Agilent Technologies, CA, USA) coupled to a diode array detector (DAD, Agilent Technologies, CA, USA) and to a high resolution time-of-flight mass spectrometer (Q-Tof 6550 iFunnel, Agilent Technologies, CA, USA) equipped with a Dual Jet Stream® electrospray ionization (ESI) interface operating in the positive mode.

Toxins were separated using a reversed-phase C_{18} Kinetex column (100 Å, 2.6 µm, 50 × 2.1 mm, Phenomenex, Le Pecq, France) with water (A) and 95% acetonitrile/water (B), both containing acidic buffer consisting of formic acid (HCOOH), 50 mM, and ammonium formate ($HCOO^-NH_4^+$), 2 mM. The column oven and the sample tray temperatures were set at 40 °C and 4 °C, respectively. The flow rate was set at 0.4 mL min^{-1}, the injection volume was set at 3 µL. Separation was achieved using the following mobile phase gradient: from 10% to 95% B in 10 min, plateau at 95% B for 2 min, return to the initial condition (10% B) in 0.1 min and a re-equilibration period (10% B) for 3.9 minutes. The chromatographic run lasted 16 min per analysis. The MTX standard used for LC-HRMS experiments was at a concentration of 20 µg mL^{-1} MeOH:H_2O (1:1, v/v).

DAD detector (Agilent Technologies, CA, USA) was set to acquire spectra in a range of wavelengths comprised between 200 and 400 nm each 2 nm. The extracted signal for MTX and MTX4 was chosen as follows: 260 ± 30 nm. UV spectra of a blank sample, consisting of MeOH:H_2O (1:1, v/v) injected under the same analytical conditions, were subtracted from MTX and MTX4 spectra.

The conditions of the ESI source were set as follows: source temperature, 200 °C; drying gas, N_2; flow rate, 11 mL min^{-1}; sheath gas

temperature, 350 °C; sheath gas flow rate, 11 mL min^{-1}; nebulizer, 45 psig; capillary voltage, -3.5 kV; nozzle voltage, 500 V. The instrument was mass calibrated in positive ionization mode before each analysis, using the Agilent tuning mix. A mixture solution of reference mass compounds (purine, 2 mL L^{-1}; HP-0921, 1 mL L^{-1}; HP-1221, 1 mL L^{-1}; HP-1821, 2 mL L^{-1}; HP-2421, 2 mL L^{-1}) in MeOH:H_2O (95:5, v/v) was infused with an isocratic pump to a separate ESI sprayer in the dual spray source at a constant flow rate of 1.5 µL min^{-1}. HP-0921 and purine allowed for correction of the measured m/z throughout the batch.

Mass spectrum detection was carried out in full scan and targeted MS/MS mode in positive ion acquisition. The full scan acquisition operated at a mass resolution of 45,000 Full Width at Half Maximum (FWHM) over a mass-to-charge ratio (*m/z*) range from 100 to 3200 with a scan rate of 2 spectra s^{-1}. The targeted MS/MS mode was performed in a Collision Induced Dissociation (CID) cell using a mass resolving power of 45,000 FWHM over the scan range *m/z* from 40 to 3200 with a MS scan rate of 10 spectra s^{-1} and a MS/MS scan rate of 3 spectra s^{-1}. Different collision energies (from 10 to 50 eV) were applied to the precursor ions to obtain a good fragmentation pathway.

All the acquisition and analysis data were controlled by MassHunter software (Agilent Technologies, CA, USA). Raw data were processed using the Molecular Feature Extraction (MFE) algorithm of the Agilent MassHunter Qualitative Analysis software (version B.07.00, service pack 1). The algorithm permitted to find all sample components down to the lowest-level abundance (abundance cut-off set at 500 counts) and to extract all relevant spectral and chromatographic information.

2.3. **Results and discussion**

2.3.1. Retention times

In the present study, the chromatographic conditions were the same as Pisapia, et al. [13], except for the composition of the mobile phases. For the present study in positive ionization mode, mobile phases were buffered with formic acid (HCOOH, 50 mM) and ammonium formate ($HCOO^-NH_4^+$, 2 mM). The acidic buffer improved positive ionization of MTXs as it allowed the formation of intense ammonium adducts.

An increase of retention times (*RT*) of both MTXs was observed compared to the previous study [13]. MTX eluted at 5.82 min (4.09 in Pisapia, et al. [13]), MTX4 eluted at 5.64 min (4.58 min in Pisapia, et al. [13]) (**Table 24**). Such increase can be explained by the presence of the sulfate groups, i.e. anionic functional groups. In aqueous mixtures, sulfated compounds undergo acid-base equilibrium between the acid form (R-OSO_3H) and the deprotonated form (R-OSO_3^-). In acidic conditions, a shift of this equilibrium in favor of the acid form make MTXs less polar, and, consequently, it results in increased retention on a reversed-phase column. Interestingly, increase in *RT* was less pronounced in MTX4 (ΔRT = +1.06 min) than in MTX (ΔRT = +1.73 min).

Table 24. Comparison of retention times (*RT*, min) in UPLC-HRMS analysis of maitotoxin (MTX) and maitotoxin-4 (MTX4) between this study and Pisapia, et al. [13]. The two studies used the same chromatographic conditions except for the composition of the mobile phases.

Ref.	Mobile phases	Retention time (*RT*, min)		Ionizaton mode
	A: H_2O **B: MeCN:H_2O (95:5, *v/v*)**	**MTX**	**MTX4**	
Pisapia, et al. [13]	**Without acidic buffer**	4.09	4.58	NEG
This study	**With acidic buffer ***	5.82	5.64	POS

* formic acid (HCOOH, 50 mM) and ammonium formate ($HCOO^-NH_4^+$, 2 mM)

2.3.2. UV spectra

Maitotoxin (MTX) standard is characterized by a single UV absorbance maximum at a wavelength of 232 nm (**Figure 51a**), slightly higher than what was previously reported by Yokoyama, et al. [21] (i.e. λ_{max} = 230 nm). UV absorption is due to the presence of a diene function at one extremity of the molecule (C_2-C_3-C_4-C_{144}, **Figure 50**).

Maitotoxin-4 (MTX4) has a UV spectrum composed of a UV maximum at a wavelength of 275 nm between two shoulders (**Figure 51b**). The bathochromic effect of +43 nm compared to MTX suggests the presence of more conjugated unsaturations or the presence of an amine (or even amide) substituent on the diene function, plausibly situated at the same extremity of the molecule.

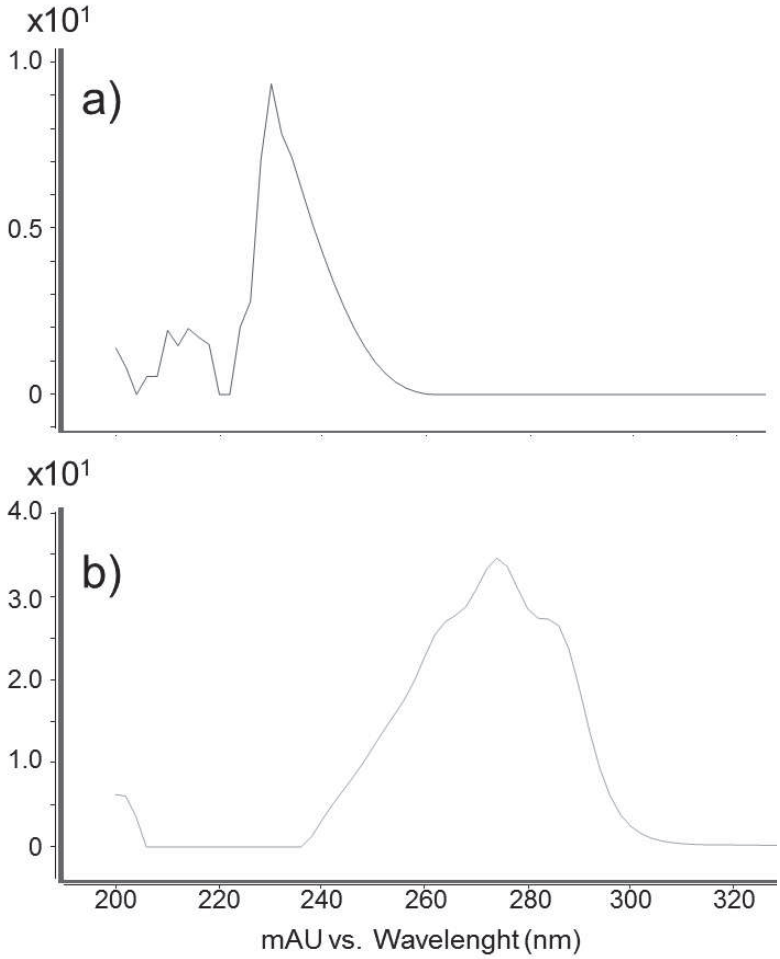

Figure 51. UV spectra of **(a)** maitotoxin (MTX) standard (20 µg mL^{-1}) and **(b)** maitotoxin-4 (MTX4) from *G. excentricus* VGO792. MTX standard is characterized by a single UV absorbance maximum at λ_{max} = 232 nm. MTX4 has a UV maximum peak at λ = 275 nm between two shoulders.

2.3.3. Positive electrospray HRMS spectra

Negative electrospray (ESI^-) HRMS spectra of MTX and MTX4 were already presented in a previous study [13]. Characteristic ions found in MTX and MTX4 negative spectra are: $[M-2H]^{2-}$, $[M+Na-3H]^{2-}$, $[M+2Na-4H]^{2-}$ and $[M-3H]^{3-}$ (**Table 25**).

In the present study, positive ion clusters were initially assigned for MTX as HRMS signals had a better signal-to-noise ratio and molecular mass was well known. In particular, singly, doubly and triply ammoniated adducts ($[M+H+NH_4]^{2+}$, $[M+2NH_4]^{2+}$ and $[M-H+3NH_4]^{2+}$) were easiliy attributable to MTX (**Figure 52a**; **Table 25**). Even though signal-to-noise ratios were close to three for several peaks in pseudo-molecular ion clusters, the triply ammoniated adduct was easily visible and the highest peaks in the molecular and other ammoniated clusters presented signal-to-noise ratios more than three. Comparison of the ion clusters attributed for

MTX4 in positive ionization mode with those of a previous study in negative ionization mode [13] also confirm the correct attribution in the present study.

Positive ESI$^+$ HRMS spectra of MTX and MTX4 are shown in **Figure 52**. Differences in intensity ratios between the ion clusters were observed. The ion species $[M+2NH_4]^{2+}$ was predominant in MTX spectrum (**Figure 52a**). In the case of MTX4, $[M-H+3NH_4]^{2+}$ was the most intense cluster observed (**Figure 52b**).

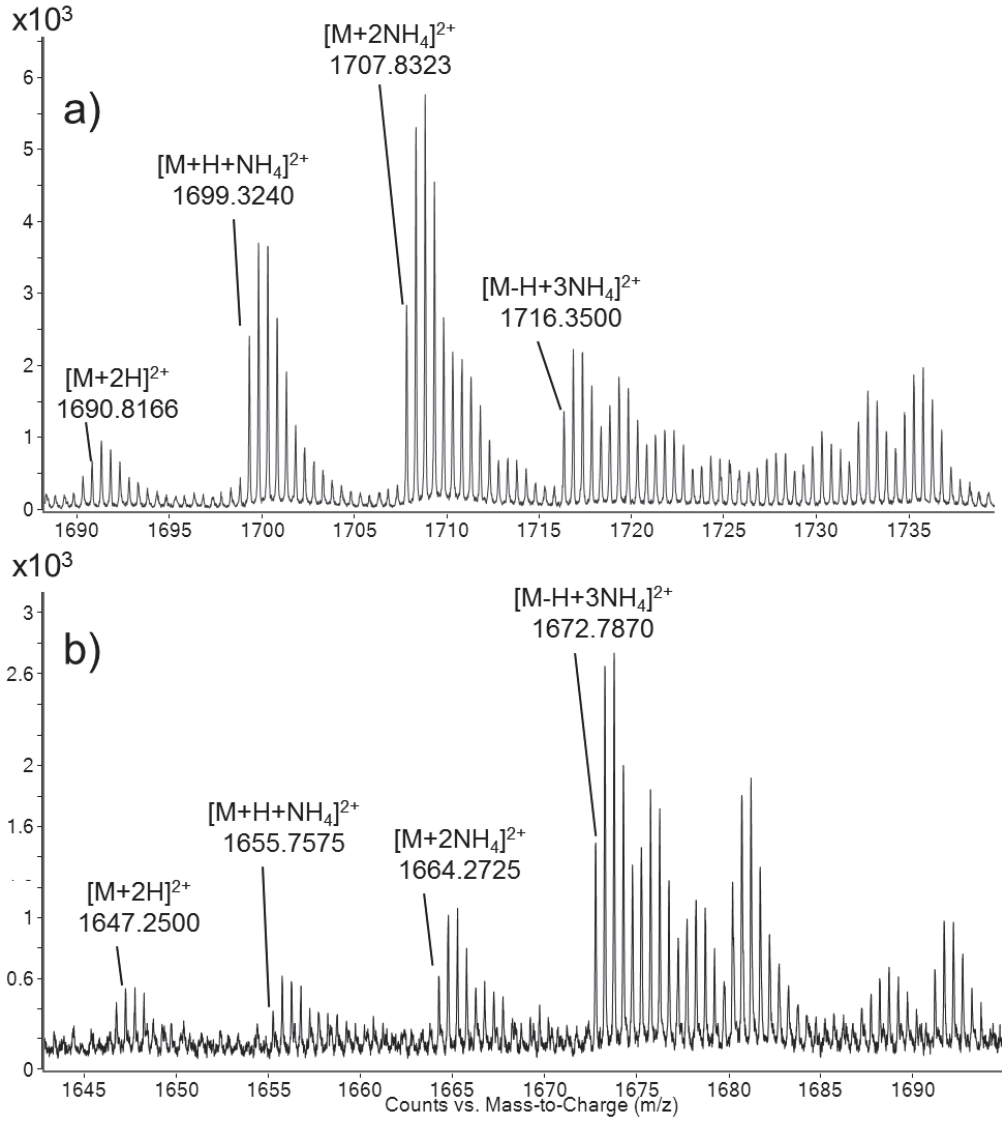

Figure 52. Positive electrospray HRMS spectra of **(a)** maitotoxin (MTX) standard (20 µg mL^{-1}) and **(b)** maitotoxin-4 (MTX4) from *G. excentricus* VGO792.

Table 25. List of the assigned HRMS ion species for MTX and MTX4 in positive and in negative ESI ionization modes. The *m/z* values in the table correspond to the accurate mono-isotopic *m/z*. Δppm for MTX were calculated compared to the exact theoretical mass. Δppm for the bi-charged molecular cation $[M+2H]^{2+}$ of MTX4 was calculated compared to the accurate mass measured in a previous study [13].

		MTX	MTX4	Ref.
Elemental formula (free acid form)		$C_{164}H_{258}O_{68}S_2$	?	
Ion species (accurate mono-isotopic *m/z*)	$[M+2H]^{2+}$	1690.8166 (Δppm: −0.4)	1647.2500 (Δppm: −0.5)	This study
	$[M+H+NH_4]^{2+}$	1699.3240 (Δppm: +3.0)	1655.7575	This study
	$[M+2NH_4]^{2+}$	1707.8323 (Δppm: +5.9)	1664.2725	This study
	$[M-H+3NH_4]^{2+}$	1716.3500 (Δppm: +3.3)	1672.7870	This study
	$[M-2H]^{2-}$	1688.8027 (Δppm: −0.8)	1645.2357	Pisapia et al. [13]
	$[M+Na-3H]^{2-}$	1699.7914 (Δppm: +0.5)	1656.2256	Pisapia et al. [13]
	$[M+2Na-4H]^{2-}$	1710.7814 (Δppm: +1.1)	1667.2075	Pisapia et al. [13]
	$[M-3H]^{3-}$	1125.5334 (Δppm: −1.4)	1096.4889	Pisapia et al. [13]
	$[M-4H]^{4-}$	843.8989 (Δppm: −2.1)	ND	Pisapia et al. [13]

2.3.4. Comparison of fragmentation patterns of MTX and MTX4

Fragmentation of molecular bicharged anions $[M-2H]^{2-}$ of MTX and MTX4 in negative ESI HRMS/MS mode was provided in Pisapia, et al. [13]. In both cases, HRMS/MS spectra were dominated by a single fragment ion peak corresponding to the hydrogenated sulfate anion ($[HOSO_3]^-$).

In positive ion acquisition mode, it was not possible to target the molecular bicharged cation $[M+2H]^{2+}$ (intensity was too weak). Its tri-ammoniated adduct ($[M-H+3NH_4]^{2+}$) was chosen for HRMS/MS experiments in reason of its prominent intensity in the MTX4 spectrum (**Figure 52b**). **Figure 53** represents the HRMS/MS average spectra (CEs from 10 to 50 eV) of MTX and MTX4.

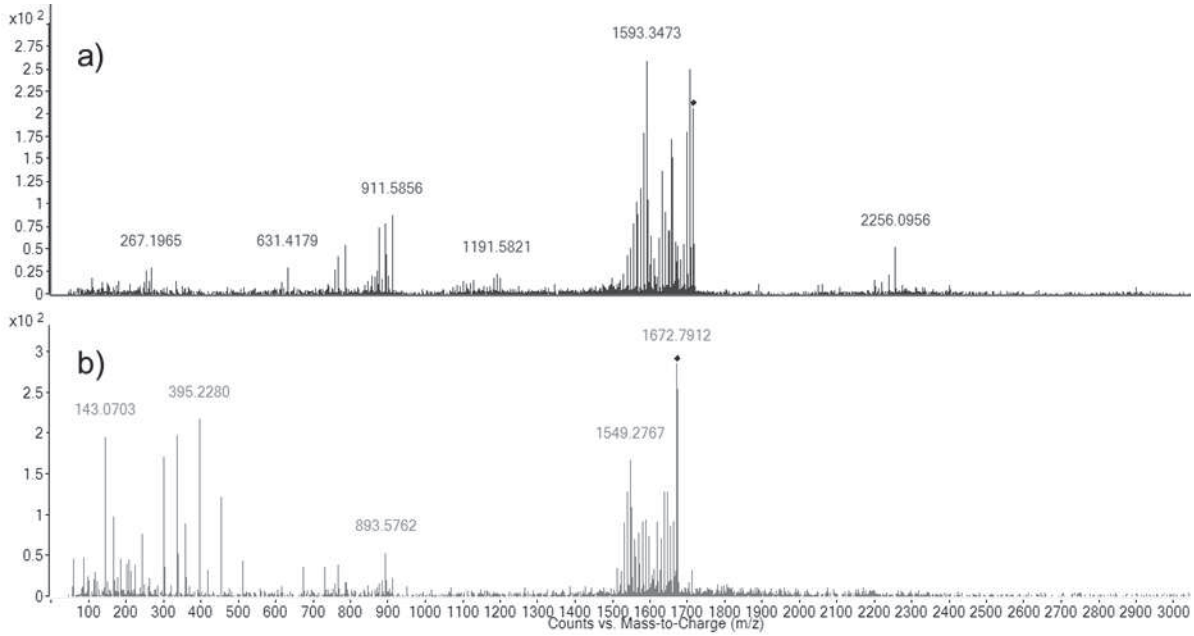

Figure 53. HRMS/MS average spectra (CEs = 10, 20, 30, 40, 50 eV) of the ion speces $[M-H+3NH_4]^{2+}$ of **(a)** maitotoxin (MTX) standard (20 µg mL^{-1}) and **(b)** maitotoxin-4 (MTX4) from *G. excentricus* VGO792. HRMS and MS/MS acquisition operated over a *m/z* range from 40 to 3200.

The comparison of HRMS/MS spectra in the *m/z* region from 1490 to 1720 led to the identification of $[M-2(SO_3)+2H]^{2+}$ and $[M-2(SO_3)+2H-n(H_2O)]^{2+}$ patterns (**Figure 54**). Assigned ion species for MTX and MTX4 are presented in **Table 26**. Pisapia, et al. [13] already proved the presence of sulfate ester group(s) in MTX4 using negative ESI HRMS/MS analysis. HRMS/MS data in positive ESI mode from the present study confirm the previous finding and add important information as they prove that MTX4 presents two sulfate ester groups such as MTX.

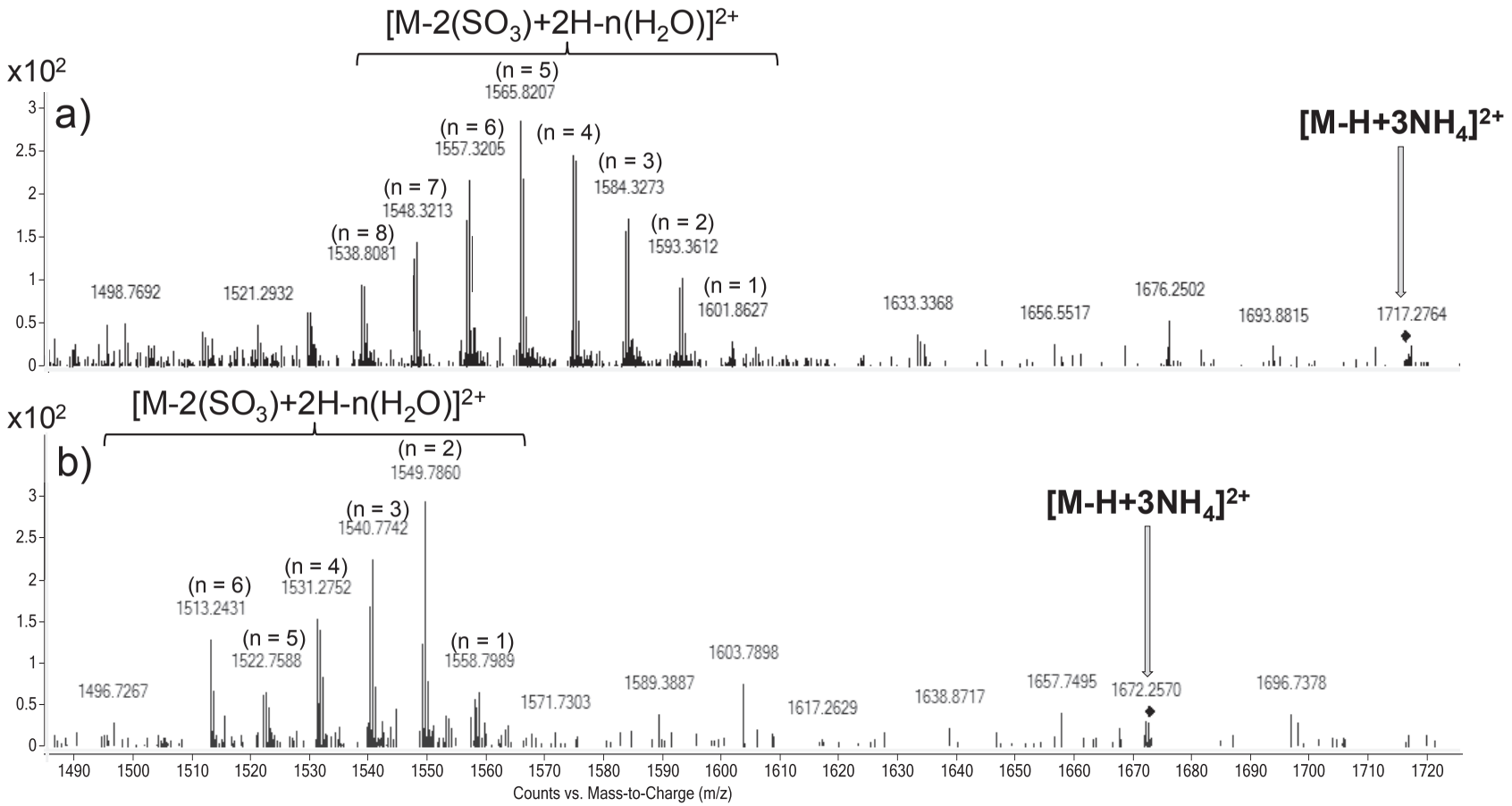

Figure 54. HRMS/MS spectra of **(a)** maitotoxin (MTX) standard (20 µg mL^{-1}) and **(b)** maitotoxin-4 (MTX4) from *G. excentricus* VGO792. Spectra were obtained targeting $[M-H+3NH_4]^{2+}$ with a collision energy (CE) of (a) 50 eV and (b) 40 eV. The figure focuses on a *m/z* range of 1490-1720. Note the clusters $[M-2(SO_3)+2H-n(H_2O)]^{2+}$.

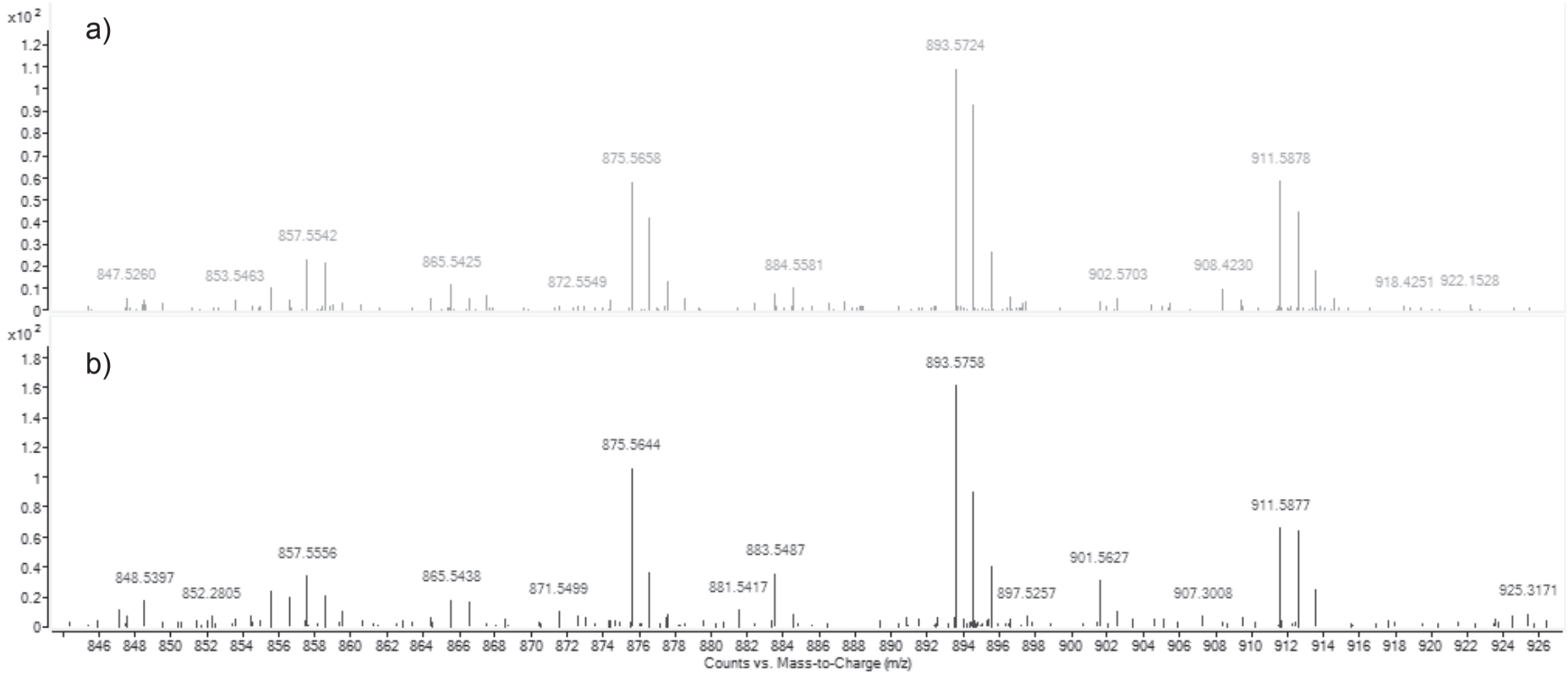

Figure 55. HRMS/MS fragmentation patterns of **(a)** maitotoxin (MTX) standard (20 µg mL^{-1}) and **(b)** maitotoxin-4 (MTX4) from *G. excentricus* VGO792. HRMS/MS spectra were obtained targeting $[M-H+3NH_4]^{2+}$ with a collision energy (CE) of 50 eV. The figure focuses on a *m/z* range of 846-926.

Table 26. List of the assigned HRMS/MS fragment ion species for MTX and MTX4. Accurate mass data were obtained from HRMS/MS spectra of $[M-H+3NH_4]^{2+}$ with a collision energy (CE) of 50 eV for MTX and 40 eV for MTX4, except where indicated otherwise. The *m/z* values in the table correspond to the accurate mono-isotopic *m/z*.

	Ion species	MTX	MTX4
Parent ion	$[M-H+3NH_4]^{2+}$	1716.3637 (Δppm: -4.7)	1672.7870
Fragment ions	$[M-(SO_3)+2H]^{2+}$	1650.8379 (Δppm: -0.2)	
	$[M-(SO_3)+2H-H_2O]^{2+}$	1641.8215 (Δppm: +6.5)	
	$[M-(SO_3)+2H-2(H_2O)]^{2+}$	1632.8171 (Δppm: +6.0)	
	$[M-2(SO_3)+2H]^{2+}$	1610.8975 (Δppm: -23.8) *	1567.3 **
	$[M-2(SO_3)+2H-H_2O]^{2+}$	1601.8404 (Δppm: +8.4)	1558.2819
	$[M-2(SO_3)+2H-2(H_2O)]^{2+}$	1592.8453 (Δppm: +2.0)	1549.2723
	$[M-2(SO_3)+2H-3(H_2O)]^{2+}$	1583.8332 (Δppm: +6.3)	1540.2789
	$[M-2(SO_3)+2H-4(H_2O)]^{2+}$	1574.8350 (Δppm: +1.8)	1531.2603
	$[M-2(SO_3)+2H-5(H_2O)]^{2+}$	1565.8295 (Δppm: +2.0)	1522.2777
	$[M-2(SO_3)+2H-6(H_2O)]^{2+}$	1556.8169 (Δppm: +6.7)	1513.2920
	$[M-2(SO_3)+2H-7(H_2O)]^{2+}$	1547.8326 (Δppm: -6.8)	1504.2389
	$[M-2(SO_3)+2H-8(H_2O)]^{2+}$	1538.8164 (Δppm: +0.3)	1495.2651

* detected only at CE = 30 eV; ** barely detected at CE = 30 eV.

Figure 55 shows another similarity between the positive HRMS/MS spectra of MTX and MTX4 in the *m/z* region of 850-920. For both molecules, the ion peaks observed at *m/z* 911.59, 893.58, 875.57 and 857.56 were respectively assigned to the following ion species: $[C_{53}H_{85}O_{13}\text{-}1(H_2O)]^+$, $[C_{53}H_{85}O_{13}\text{-}2(H_2O)]^+$, $[C_{53}H_{85}O_{13}\text{-}3(H_2O)]^+$ and $[C_{53}H_{85}O_{13}\text{-}4(H_2O)]^+$ (**Table 27**).

Table 27. Assignment of HRMS/MS fragment ion peaks to MTX4 in comparison with the fragment ion peaks assigned to MTX standard. Accurate mass data were obtained from HRMS/MS spectra of $[M-H+3NH_4]^{2+}$ with a collision energy (CE) of 50 eV for both molecules (**Figure 55**). The *m/z* values in the table correspond to the accurate mono-isotopic *m/z*.

Fragment ion species	Exact *m/z*	MTX	Δppm	MTX4	Δppm
$[C_{53}H_{85}O_{13}\text{-}H_2O]^+$	911.5879	911.5878	+0.11	911.5877	+0.22
$[C_{53}H_{85}O_{13}\text{-}2(H_2O)]^+$	893.5773	893.5724	+5.48	893.5758	+1.68
$[C_{53}H_{85}O_{13}\text{-}3(H_2O)]^+$	875.5668	875.5658	+1.14	875.5644	+2.74
$[C_{53}H_{85}O_{13}\text{-}4(H_2O)]^+$	857.5562	857.5542	+2.33	857.5556	+0.70

For MTX, the interpretation of the HRMS/MS fragment ions include: (i) the breaking of the C_{99}-C_{100} bond, with the positive charge attributed to the C_{100} fragment ($C_{53}H_{85}O_{13}^+$, not observed) and (ii) subsequent losses of molecules of water (**Figure 56**). As a consequence, MTX4 shares the same terminal part of MTX (rings W through F', **Figure 56**).

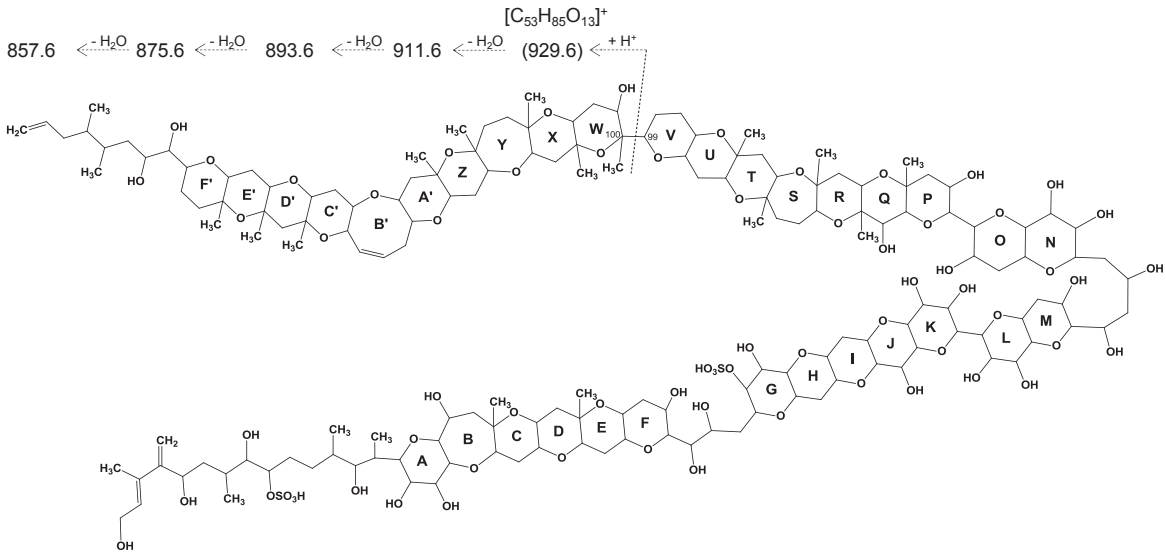

Figure 56. Proposed fragmentation of maitotoxin (MTX) in positive ESI HRMS/MS analysis.

HRMS/MS spectra of MTX and MTX4 in the *m/z* range from 80 to 800 unveiled considerable differences in the molecular structure of these two molecules (**Figure 57**). The ion peaks in this region of the MS/MS spectrum were found to be more informative (more numerous and more intense) for MTX4 (**Figure 57b**) compared to MTX (**Figure 57a**). Interpretation of the fragmentation observed is currently being investigated. Interestingly, the MS/MS spectrum of MTX4 (**Figure 57b**) showed a recurrent neutral loss of 58.04 Da (usually followed by the loss of two molecules of H_2O), probably corresponding to a primary allylic alcool (CH_2=$CHCH_2OH$). Further in-depth studies on this *m/z* region will aid structure elucidation of MTX4.

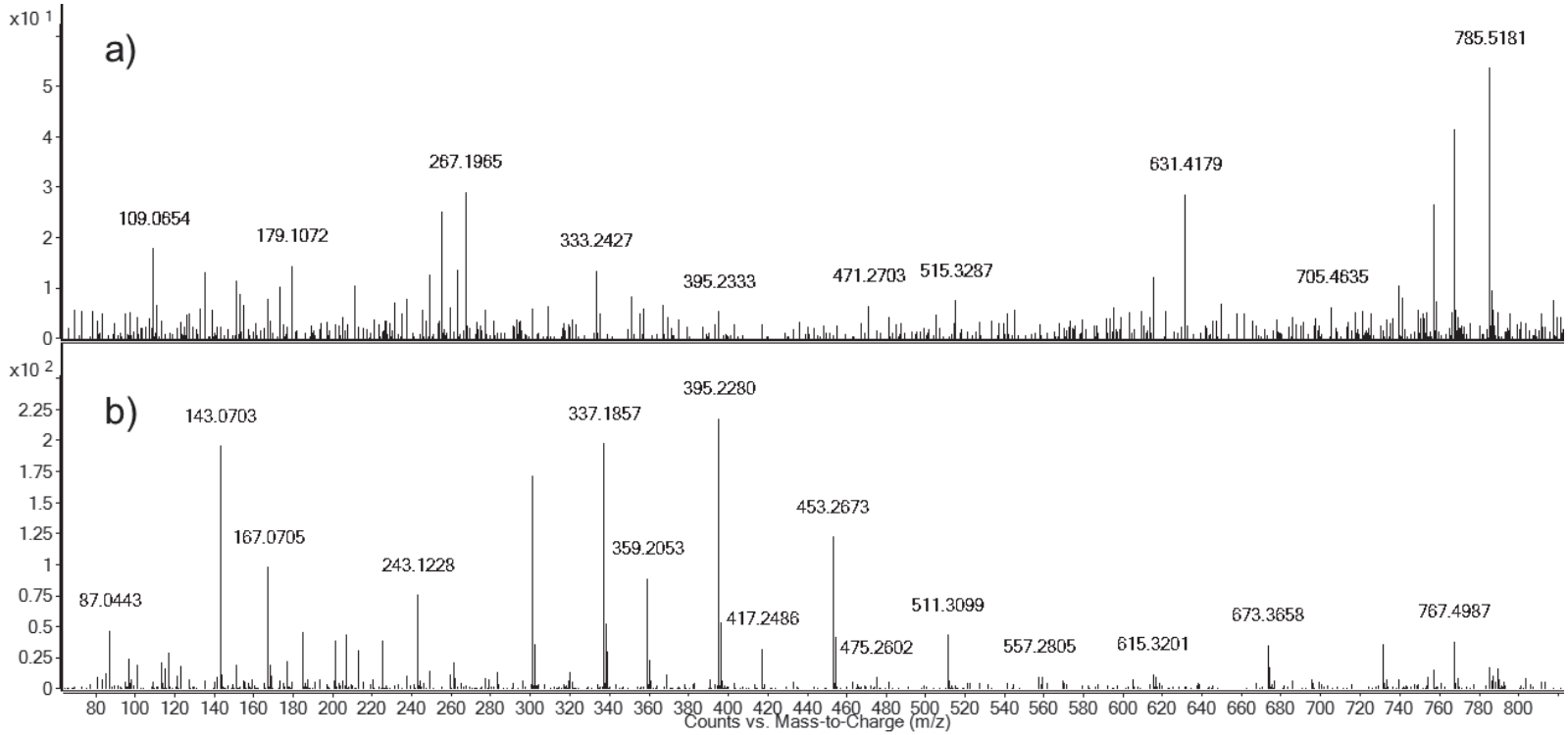

Figure 57. HRMS/MS spectra were obtained targeting $[M-H+3NH_4]^{2+}$and represent an average of five collision energies (CEs = 10, 20, 30, 40, 50 eV). The figure focuses on a *m/z* range of 80-800. HRMS/MS spectra of MTX and MTX4 present considerable differences in this *m/z* region. Note that MTX4 fragments more intensely than MTX, with a recurrent neutral loss of 58.04 Da and subsequent loss of two molecules of water.

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3. In search of CTXs produced by *Gambierdiscus excentricus*

Article in preparation: Hess, P.; **Pisapia, F.**; Clausing, R.; Sibat, M.; Dechraoui-Bottein M.-Y.; Herrenknecht, C. (201X). "In search of CTXs produced by *Gambierdiscus excentricus*". (title and author-list to be finalized prior to submission)

3.1. Introduction

Recent studies using functional bioassays showed that the species *Gambierdiscus excentricus* exhibits particularly high ciguatoxicity, in the picogram or sub-picogram range of CTX3C equivalents (eq) per cell, depending on the strain considered (Fraga et al., 2011; Litaker et al., 2017; Pisapia et al., 2017a). Toxicity of *G. excentricus* falls into the same order of magnitude as *G. polynesiensis* from the South Pacific, the most ciguatoxic species known to date (Chinain et al., 2010). The toxin profile of *G. polynesiensis* has been described by Chinain et al. (2010) using LC-LRMS/MS analysis and consists of several Pacific ciguatoxins (CTXs) previously described and other minor congeners tentatively assigned. Even though *G. excentricus* is, to date, the most ciguatoxic species in the Atlantic region, no information is available on the CTX congeners produced by this species. Therefore, this species needs to be examined in detail to identify the algal precursor(s) of CTXs involved in CFP in the Atlantic Ocean.

Preliminary results of a bioguided fractionation of *G. excentricus* VGO791 followed by $RBA_{(R)}$ assay and LC-LRMS/MS analysis are presented as follows.

3.2. Materials and Methods

3.2.1. Reference toxins and chemicals

Pacific ciguatoxin-3C (CTX3C) standard was purchased from Wako (Wako-Pure Chemical, Osaka, Japan). A standard solution mix of CTX1B, 49-*Epi*CTX3C, CTX4A, CTX4B, M-*Seco*CTX3C and 51-HydroxyCTX3C were kindly provided by Louis Malardé Institute (ILM, Tahiti, French Polynesia). Brevetoxin-3 (BTX3) was obtained from Latoxan (Rosam,

France). [^{3}H]BTX3 was purchased from American Radiolabelled Chemicals, Inc.

3.2.2. Sample preparation

Strains of *Gambierdiscus excentricus* were cultured, harvested and extracted as described in Pisapia et al. (2017b). Strain names and number of cells harvested are detailed as follows: Pulley Ridge Gam2 (1,630,000 cells), UNR-07 (1,013,833 cells), UNR-08 (441,490 cells), VGO1035 (242,050 cells), VGO790 (171,711 cells), VGO791 (2,200,160 cells) and VGO792 (2,159,997 cells). Larger biomass of *G. excentricus* VGO791 was available as this strain had shown high CTX-toxicity in a previous study (Pisapia et al., 2017a). Thus, the following procedure focuses on this sample.

An aliquot of crude extract of *G. excentricus* VGO791 was used for LC-MS analysis, the remnant was partitioned between dichloromethane (CH_2Cl_2) and an aqueous methanol mixture (MeOH:H_2O, 3:2, v/v) (Pisapia et al., 2017b). An aliquot of the CH_2Cl_2-soluble fraction (DSF) of *G. excentricus* VGO791 (0.745 mL, 1.082 million cells, 35.0 mg DSF residue) was processed using size-exclusion chromatography (SEC, LH-20). Prior to use, Sephadex™ LH-20 powder (10 g) was soaked in methanol (MeOH) overnight, then gently packed in an open glass column (internal diameter: 1 cm) with one continuous motion. The column was then rinsed with MeOH. Bed height was 52.7 cm, giving a bed volume of 41.4 mL. After sample loading, compounds were eluted with MeOH under atmospheric pressure (flow rate: 0.4 mL min^{-1}). Fractions were collected as follows: 1st fraction of 10 mL, 45 fractions of 1 mL and two last fractions of 30 mL.

3.2.3. $RBA_{(R)}$ analysis

Samples were analysed for CTX content using the radioactive receptor binding assay ($RBA_{(R)}$) at the International Atomic Energy Agency (IAEA), Environment Laboratories (Monaco). Analysis was carried out in a microplate format (Millipore 96-well filter plate) following the IAEA TecDoc No. 1729 (IAEA, 2013) with some modification. Reagents included phosphate buffered saline buffer (PBST- Tween®) with bovine serum albumin (1mg mL^{-1}), [^{3}H]BTX3 solution (1 nM), and a porcine brain membrane preparation (0.8 mg protein mL^{-1}). The plate was incubated in

the dark with liquid scintillant (Optiphase, PerkinElmer) for 2 h before beta emissions quantification in a beta counter with normalisation (MicroBeta Plate Counter, PerkinElmer). Data were analysed using GraphPad Prism software (version 6.0; San Diego, USA).

Samples were assayed three or four times (three or four separate RBAs, one well per sample each time) at concentrations ranging from 25,000 to 150,000 cell eq mL^{-1} in assay. Results were analysed against a standard curve of CTX3C (780 nM to 0.08 nM). Binding competition was assessed against a standard curve of brevetoxin (6 uM to 0.6 nM). Samples were considered positive for the presence of CTXs if activity fell between 25 and 75% total binding. Potential quenching effects were assessed using Spectral Quench Parameters provided by the MicroBeta instrument software.

3.2.4. LC-LRMS/MS analysis

Samples were analysed using an LC-MS/MS method adapted from Yogi et al (2011). The instrument used was an LC system (UFLC XR Nexera, Shimadzu, Japan) coupled to a hybrid triple quadrupole-linear ion trap mass spectrometer (API-4000 QTrap, AB Sciex, CA, USA) equipped with a turboV® ion spray interface. A 1.8 µm C_{18} Zorbax Eclipse plus column (50*2.1 mm, Agilent Technologies, CA, USA) was employed at 40 °C and eluted at 0.4 mL min^{-1} with a linear gradient. Eluent A was water and eluent B was methanol, both eluents containing 2 mM ammonium formiate and 50 mM formic acid. The elution gradient ran from 78 to 88% over 10 min and was held for 4 min before a re-equilibration period of 5 min. Sample injector was set at 5 µL.

Mass spectrometry detection was operated in positive mode using Multiple Reaction Monitoring (MRM). The selected *m/z* transitions are shown in **Table 28**. The MRM experiments were established using the following source settings: curtain gas set at 25 psi, ion spray at 5500 V, a turbogas temperature of 300 °C, gas 1 and 2 set, respectively, at 40 and 60 psi, an entrance potential of 10 V.

Data processing and analysis were achieved with Analyst software (Sciex, CA, USA). To verify the presence of known toxins, a standard solution of CTX3C (Wako Chemicals, Ltd.) and a mixture of purified CTX1B, 49-*Epi*CTX3C, CTX4A, CTX4B, M-*Seco*CTX3C and 51-

HydroxyCTX3C (ILM, Tahiti, French Polynesia) were also injected. The limit of detection (LOD) for CTX3C was 2 ng mL^{-1}.

Table 28. List of the MRM transitions (*m/z*) for each of the ciguatoxin congeners examined in this study (LC-LRMS/MS, API 4000 QTrap).

Compound	Precursor ion (Q1) *m/z*	Product ion (Q2) *m/z*	DP (eV)	CE (eV)	CXP (eV)
CTX1B, 52-*Epi*CTX, 54-*Epi*CTX & 52-54-*Diepi*CTX	1128.6 $[M+NH_4]^+$	1093.6 $[M-H_2O+H]^+$	105	20	12
		1075.6 $[M-2H_2O+H]^+$	105	30	12
		95.1	105	90	20
52-*Epi*-54-deoxyCTX (CTX2) & 54-DeoxyCTX (CTX3)	1112.6 $[M+NH_4]^+$	1077.6 $[M-H_2O+H]^+$	105	20	12
		1059.6 $[M-2H_2O+H]^+$	105	30	12
		95.1	105	90	20
CTX4B & CTX4A (52-*Epi*CTX4B)	1078.6 $[M+NH_4]^+$	1043.6 $[M-H_2O+H]^+$	105	30	12
	1061.6 $[M+H]^+$	1043.6 $[M-H_2O+H]^+$	105	20	12
		125.1	105	50	18
CTX3C & 49-*Epi*CTX3C (CTX3B)	1040.6 $[M+NH_4]^+$	1005.6 $[M-H_2O+H]^+$	105	30	12
	1023.6 $[M+H]^+$	1005.6 $[M-H_2O+H]^+$	105	20	12
		125.1	105	50	18
2,3-Dihydro-2,3-dihydroxyCTX3C	1074.6 $[M+NH_4]^+$	1039.6 $[M-H_2O+H]^+$	105	30	12
	1057.6 $[M+H]^+$	1039.6 $[M-H_2O+H]^+$	105	20	12
		125.1	105	50	18
51-HydroxyCTX3C	1056.6 $[M+NH_4]^+$	1021.6 $[M-H_2O+H]^+$	105	30	12
	1039.6 $[M+H]^+$	1021.6 $[M-H_2O+H]^+$	105	20	12
		1003.6 $[M-2H_2O+H]^+$	105	20	12
M-*Seco*CTX3C (or 3B)	1041.6 $[M+H]^+$	1023.6 $[M-H_2O+H]^+$	105	30	12
		1005.6 $[M-2H_2O+H]^+$	105	20	12
		125.1	105	50	18
2,3-Dihydro-2-hydroxyCTX3C & 2,3-Dihydro-3-hydroxyCTX3C	1058.6 $[M+NH_4]^+$	1023.6 $[M-H_2O+H]^+$	105	30	12
		1005.6 $[M-2H_2O+H]^+$	105	20	12
		125.1	105	50	18
C-CTX1, C-CTX2, I-CTX1 & I-CTX2	1141.4 $[M+NH_4]^+$	1108.6 $[M-H_2O+H]^+$	105	30	12
	1123.4 $[M+H]^+$	1108.6 $[M-H_2O+H]^+$	105	30	12
		1087.6 $[M-2H_2O+H]^+$	105	30	12

3.3. **Preliminary results and discussion**

LC-LRMS/MS analysis did not confirm the presence of any CTXs examined (**Table 28**), in crude extracts of any of *G. excentricus* strains available.

The presence of CTXs in LH-20 fractions of *G. excentricus* VGO791 was monitored using the $RBA_{(R)}$ assay. The sensitivity of the assay with algal cell matrix was particularly poor (LOD: 0.3 pg $cell^{-1}$) due to the presence of pigments (chlorophylls and carotenoids) which induced a quenching effect on the liquid scintillation counting (i.e. reduced counting efficiency). Significant ciguatoxin-like binding activity was nonetheless detectable in five LH-20 fractions, although signals were below the limit of quantification. CTX-activity was detected for two separate peaks of the LH-20 chromatography, one consisting of four fractions eluting from 35 to 39 mL and the other one of one fraction eluting at a V_e = 46–47 mL (**Figure**

58). Due to the low amount of toxin in the sample, quantification by RBA was not possible in these fractions.

The LH-20 fractions of the DSF of *G. excentricus* VGO791 which showed CTX activity were also screened for the presence of known CTX analogs using LC-LRMS/MS analysis (**section 3.2.4**). None of them was confirmed in any of the fractions examined. Therefore, these fractions will need to be examined for unknown analogs using LC-HRMS analysis.

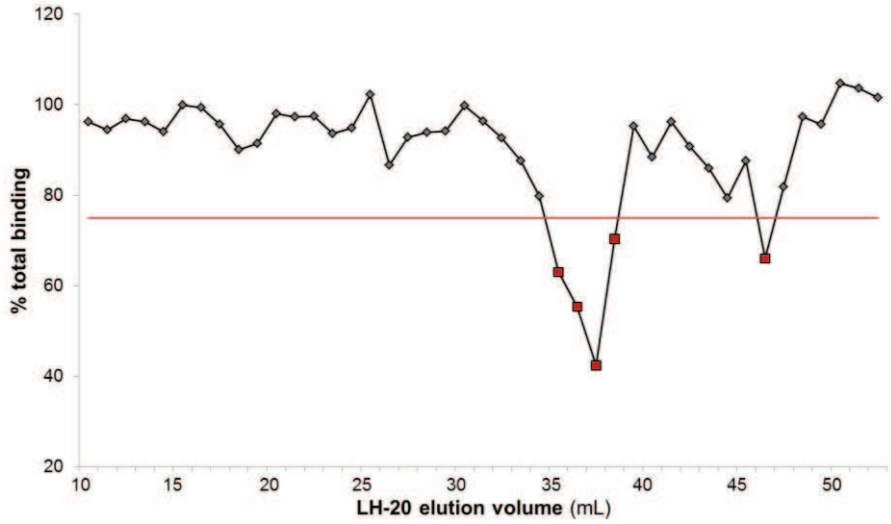

Figure 58. CTX-activity exhibited by LH-20 fractions of DSF of *G. excentricus* VGO791 as shown by the $RBA_{(R)}$ assay. The figure depicts one representative run of four replicate $RBA_{(R)}$ assays. X axis represents elution volume of the LH-20 chromatography. Y axis represents the binding of the radiolabeled [^{3}H]BTX3 expressed as % total binding. 100% total binding represents binding of [^{3}H]BTX3 in the absence of unlabeled toxin. Fractions reducing binding of [^{3}H]BTX3 by more than 25% (less than 75% total binding) were considered active (red squares), i.e. V_e = 35–39 mL and V_e = 46–47 mL. LH-20 fractions with V_e < 10 mL and V_e > 53 mL were omitted because they exhibited no CTX-activity.

Our past work showed that maitotoxin-4 (MTX4, accurate mono-isotopic mass of 3292.4860 Da for the free acid form) mostly eluted in a V_e range of 12.5–20 mL using the same LH-20 chromatographic conditions (Pisapia et al., 2017b). $RBA_{(R)}$ results are consistent with the presence of CTXs in a V_e range of 35–39 mL, since known CTXs have a lower molecular weight (MW = 1000-1200 Da) than MTX4. The bioactive fraction eluting at V_e = 46–47 mL may contain one (or more) smaller CTX congener(s). The identification of such bioactive compounds may help to trace the biosynthetic pathways of cyclic polyethers in *Gambierdiscus* and *Fukuyoa*. To date, only few studies specifically addressed biosynthetic pathways of these dinoflagellates (Kohli et al., 2017a; Kohli et al., 2015). The discovery of novel bioactive compounds would provide additional information about the structure-activity relationship of such intriguing natural products.

3.4. References

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Conclusions and perspectives

Ciguatera Fish Poisoning (CFP), the most common non-bacterial food-borne intoxication worldwide, is caused by toxins produced by dinoflagellates of the genera *Gambierdiscus* and *Fukuyoa*. The principal toxins responsible for CFP are ciguatoxins (CTXs), which mainly bioaccumulate in fish flesh through the marine food chain. Maitotoxins (MTXs) may potentially have a role in CFP as well, especially if fish viscera are consumed. In addition, these dinoflagellates produce other cyclic polyether compounds (e.g. gambierol, gambieroxide, gambieric acids and gambierone) and their role in CFP has not yet been evaluated. Over the last decades, the increased incidence of CFP in the Pacific Ocean and in the Caribbean Sea (Friedman et al., 2017), as well as the emergence of CFP in temperate areas (e.g. Canary Islands) (Rodríguez et al., 2017), necessitates additional efforts in the study of CFP worldwide and are at the origin of this thesis.

In recent years, molecular biology techniques have been developed for the identification of almost all species and phylotypes of *Gambierdiscus* and *Fukuyoa* described to date (Lyu et al., 2017; Nishimura et al., 2016; Vandersea et al., 2012). On the other hand, little is known about the degree to which toxicity varies among dinoflagellate species/phylotypes or strains, and even less is known about the chemical diversity of the toxins produced. Therefore, the objectives of this study were: (i) to assess the relative CTX- and MTX-toxicity of *Gambierdiscus* and *Fukuyoa* species and (ii) to pinpoint CTX and MTX candidates in the most toxic strains.

Relative toxicity of different species of *Gambierdiscus* and *Fukuyoa*

Only a few recent studies have assessed the relative toxicity between species or phylotypes of the dinoflagellate genera *Gambierdiscus* and *Fukuyoa* (Chinain et al., 2010a; Holland et al., 2013; Lewis et al., 2016; Munday et al., 2017). One reason for this lack of information is that the taxonomy of those organisms have undergone several updates in the last decade (reviewed in **chapter 1**, **section 2.2**). To date, the most ciguatoxic strain belongs to the species *G. polynesiensis* (TB-92, from the South Pacific) (Chinain et al., 2010a), while the most maitotoxic strain belongs to the phylotype *Gambierdiscus* sp. ribotype 2 (CCMP1655, from the Caribbean) (Holland et al., 2013). Knowledge of the toxin-producing potential of a given species or phylotype is critical for the development of effective strategies for cell-based risk evaluation of CFP.

In the present PhD study, the evaluation of the relative toxicity between different species or phylotypes of *Gambierdiscus* and *Fukuyoa* was carried out using functional bioassays (i.e. neuro-2a and erythrocyte lysis assays) (**chapter 2**). Substantial differences in toxicity among species or phylotypes were highlighted, as was expected from the existing literature. Even though functional assays cannot distinguish between production of large amounts of low toxicity CTX or MTX congeners as compared to lower production of high toxicity congeners, the data provided by this study on the overall toxicity per cell corroborated the previous findings by Fraga et al. (2011) that show *G. excentricus* as the most CTX-producing species in the Atlantic area. This is particularly relevant since two different approaches were used: suppression of MTX activity by SK&F 96365 in crude extracts (Fraga et al., 2011) and liquid-liquid partitioning in this study (Litaker et al., 2017; Pisapia et al., 2017a).

Gambierdiscus excentricus exhibited a CTX-type toxicity at sub-picogram or picogram levels of CTX3C eq per cell, depending on the strain, which is in the same order of magnitude as *G. polynesiensis* from the South Pacific (Chinain et al., 2010a). All other species or phylotypes tested had relatively low CTX-toxicity ranging from non-detectable to 10-50 femtograms of CTX3C eq per cell. All strains also displayed MTX-toxicity in the picogram range (MTX eq per cell), with *G. excentricus* also being the most maitotoxic. Even though *G. excentricus* cells divide more slowly than

all other species or phylotypes examined, the toxicity data provided in this study suggest that *G. excentricus* has a high probability to be a major contributor to the flux of ciguatera toxins through the food webs in the Atlantic region. Nevertheless, methodological limitations should be highlighted. Growth rates and toxin production in the laboratory may not reflect what occurs in the natural environment, and only a limited number of strains per species have been examined to date.

In search of bioactive compounds produced by *Gambierdiscus excentricus*

The recognized biodiversity of *Gambierdiscus* and *Fukuyoa* is increasing rapidly, with several new species described in the last decade. Nonetheless, knowledge of the MTX and CTX congeners produced by these species has not grown at the same pace, mainly due to methodological constraints (lack of reference compounds and strains) and the costs associated with the production of the high biomasses required for isolation and characterization of unknowns.

Once *G. excentricus* was identified as the most toxic species of our culture collection, the subsequent objective was to describe its toxin profile. For this purpose, a simple clean-up strategy consisting of liquid-liquid partitioning and size-exclusion chromatography was employed to obtain pre-purified fractions that were subsequently assayed for toxicity against neuro-2a cells or using a receptor-binding assay ($RBA_{(R)}$) (**chapter 3**). Toxicity screening of such fractions revealed the presence of bioactive compounds exhibiting CTX and MTX activities that need to be isolated in order to determine their molecular structure. As MTXs were detected in higher concentrations in this dinoflagellate, the subsequent studies focused on the isolation of the MTX congeners.

The structure of maitotoxin (MTX) was elucidated by FAB and NMR spectroscopy. Nevertheless, although the interpretation of NMR and FAB spectra is well documented, this is not the case for electrospray (ESI) coupled with high resolution mass spectrometry (HRMS). Thus, the development of an LC-ESI-MS method was undertaken at the Phycotoxins Laboratory, using MTX reference material. The method was developed in high resolution mass spectrometry (HRMS), both in negative and positive full scan acquisition modes. In addition, HRMS/MS fragmentation spectra of MTX were produced and a fragmentation pathway has been proposed. The

assignment of fragment ions will help the structural elucidation of hitherto undescribed MTX congeners.

The method developed for detection of MTX congeners and structural elucidation was applied to the analysis of crude extracts and purified fractions. The main finding of these studies was the discovery of a novel MTX congener, named maitotoxin-4 (MTX4) (Pisapia et al., 2017b). This discovery was possible by comparison with spectra of MTX reference material. Complete purification of MTX4 was not possible due to the scarcity of *G. excentricus* cells and its structure has not been fully elucidated. Still, the following evidence indicates that MTX4 is a MTX analog. High cytotoxicity and Ca^{2+}-influx activity were positively correlated with MTX4 content, after pre-purification with liquid-liquid partitioning and size-exclusion chromatography. HRMS analysis of MTX4, conducted in negative ionization mode, revealed ion cluster similarity with MTX, with the assignment of molecular bi-charged anions and their sodiated and di-sodiated ion clusters (Pisapia et al., 2017b). In positive ionization mode, molecular bi-charged cations and their ammoniated ion clusters (n=1 to 3) were also assigned (Pisapia et al., *in preparation*). Targeted HRMS/MS analyses revealed the presence of two sulfate ester groups and the same terminal structure as MTX. The fragment ions that have been interpreted include the hydrogenated sulfate anion ($[HOSO_3]^-$), the bi-charged cation clusters after loss of two sulfonic acid groups (SO_3) with subsequent losses of several molecules of water and the mono-charged cation clusters corresponding to the terminal region ($C_{53}H_{85}O_{13}^+$) with subsequent losses of molecules of water (Pisapia et al., 2017b; Pisapia et al., *in preparation*).

Another aim of this thesis was to assess the diversity of previously reported MTX congeners in several species or phylotypes of *Gambierdiscus* and *Fukuyoa* using LC-MS analyses (Pisapia et al., 2017b). The main finding was that the production of certain MTX analogs is likely to be species-specific. Indeed, MTX was detected in *G. australes* only, in accordance with previous and simultaneous studies (Munday et al., 2017; Rhodes et al., 2014; Rhodes et al., 2016; Rhodes et al., 2017c; Rhodes et al., 2017d). MTX4 was detected in *G. excentricus* only, independently of the geographical origin of the strains examined (Canary Islands, Brazil, Caribbean Sea) (Pisapia et al., 2017b).

Perspectives

Evidence provided in this study suggested that the recently described species *Gambierdiscus excentricus* is a major contributor to CFP in the Atlantic region. As a conclusion of this study, the distribution and abundance of *G. excentricus* should be monitored in the field for risk assessment purposes. Field studies can be used to determine species distribution in specific areas, but toxin analysis of algal field samples remains difficult due to sensitivity problems and lack of knowledge on the toxin congeners produced. Among the bioactive compounds produced by *G. excentricus*, this study highlighted the presence of CTX- and MTX-related compounds, as demonstrated using functional bioassays. Nonetheless, it cannot be excluded that compounds other than CTXs or MTXs could have interfered with functional assays. Hence, all the bioactive compounds produced by this species need to be examined in detail. Chemical characterization of these compounds is required to better assess CFP risk linked to *G. excentricus* proliferation. For this purpose, large-scale culturing of *G. excentricus* strains needs to be undertaken.

In this study, small-scale experiments on *G. excentricus* extracts suggested that liquid-liquid partitioning and size-exclusion chromatography are efficient clean-up steps for CTX and MTX isolation. Once sufficient biomass is available, such an approach might unveil the compounds responsible for the CTX-activity observed. Chemical analysis (e.g. full scan HRMS) of the LH-20 fractions active on the $RBA_{(R)}$ will need to be carried out to pinpoint CTX congeners produced by *G. excentricus*. Then, the link between the algal congeners and CFP events in the Atlantic area needs to be established. The examination of the stomach content of ciguatoxic fish from the Atlantic area may represent a promising approach for the discovery of the CTX congeners involved in CFP events in this region. A recent study conducted by Diogène et al. (2017) on a ciguateric shark from the Indian Ocean demonstrated that it was possible to detect CTXs in the stomach content using LC-HRMS, whereas they were not detected in extracts of either muscle or fin of the same animal.

Untargeted LC-HRMS analysis conducted in this study permitted the discovery of a novel MTX congener, maitotoxin-4 (MTX4), in strains of the species *G. excentricus* (Pisapia et al., 2017b). Since all other species or phylotypes examined did not produce MTX4, this compound could serve as a biomarker for the highly toxic *G. excentricus* species, at least until the

actual CTXs produced by *G. excentricus* are described. Detection of MTX4 in field samples would indirectly reveal the presence of *G. excentricus* and help identification of CFP hotspots. A sufficiently large biomass of *G. excentricus* cells is needed to complete the isolation of MTX4 and to fully examine the extract for other minor congeners. Further HRMS/MS and NMR studies are also needed to complete structural characterization of MTX4. In addition, *in vivo* and *in vitro* toxicological studies need to be undertaken to better characterize the biological activity of MTX4 and, ultimately, its molecular target. Bioaccumulation studies in fish and in other seafood have to be conducted in order to assess the potential risk of MTX4 to humans. The elucidation of the molecular structure and the discovery of the molecular target would also permit to clarify the structure-activity relationship of MTXs. Furthermore, chemical synthesis of truncated MTX4 analogs may provide useful applications in biomedical sciences, e.g. cancer therapy, as demonstrated for a MTX truncated analog (Nicolaou et al., 2014).

The role of the other bioactive metabolites (gambierol, gambierone, gambieric acids and potentially gambieroxide) produced by *Gambierdiscus* and *Fukuyoa* in CFP needs to be assessed as well. Taking into account the biological activity described in the literature, determination of their content in field samples is required, in addition to CTXs and MTXs, for a comprehensive risk assessment. The identification of all the ladder-shaped polyethers produced by these dinoflagellates could potentially contribute to the understanding of their biosynthetic pathways. One of the most promising approaches to pinpoint these compounds could be the establishment of molecular networking as a dereplication strategy (Brito et al., 2015; Yang et al., 2013).

Scientific publications and communications

Published articles

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

- **Pisapia, F.**; Sibat, M.; Herrenknecht, C.; Lhaute, K.; Gaiani, G.; Ferron, P.-J.; Fessard, V.; Fraga, S.; Nascimento, S. M.; Litaker, R. W.; Holland, W. C.; Roullier, C.; Hess, P. (2017). "Maitotoxin-4, a novel MTX analog produced by *Gambierdiscus excentricus*". Mar Drugs **15**(7): 220.

- Litaker, R. W.; Holland, W. C.; Hardison, D. R.; **Pisapia, F.**; Hess, P.; Kibler, S. R.; Tester, P. A. (2017). "Ciguatoxicity of *Gambierdiscus* and *Fukuyoa* species from the Caribbean and Gulf of Mexico". PLoS One **12**(10): e0185776.

Articles in preparation

- **Pisapia, F.**; Watanabe, R.; Sibat, M.; Roullier, C.; Suzuki, T.; Hess, P.; Herrenknecht, C. (201X). "Towards the characterization of maitotoxin-4 (MTX4)". Rapid Communication in Mass Spectrometry. (title and author-list to be finalized prior to submission)

- Hess, P.; **Pisapia, F.**; Clausing, R.; Sibat, M.; Dechraoui-Bottein M.-Y.; Herrenknecht, C. (201X). "In search of CTXs produced by *Gambierdiscus excentricus*". (title and author-list to be finalized prior to submission)

Oral communications

- 2016, March 14th – 16th: **Pisapia, F.**; Holland, W. C.; Hardison, D. R.; Litaker, R. W.; Fraga, S.; Nishimura, T.; Adachi, M.; Nguyen-Ngoc, L.; Séchet, V.; Amzil, Z.; Herrenknecht, C.; Hess, P. "Toxicity screening of *Gambierdiscus* strains using neuro-2a and erythrocyte lysis bioassays" at the national conference GdR PHYCOTOX 2016 in Villefranche-sur-Mer (France).

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- 2016, November 3rd – 4th: **Pisapia, F.**; Gaiani, G.; Lhaute, K.; Holland, W. C.; Hardison, D. R.; Litaker, R. W.; Ferron, P.-J.; Fessard, V.; Sibat, M.; Séchet, V.; Amzil, Z.; Herrenknecht, C.; Hess, P. "In search of the elusive bioactive compounds of *Gambierdiscus* spp." at the conference Journées scientifiques de l'École Doctorale VENAM 2016 in Nantes (France). Prize: "best oral presentation".

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Posters with ignite talks

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- 2016, October 9th – 14th: **Pisapia, F.**; Holland, W. C.; Hardison, D. R.; Litaker, R. W.; Fraga, S.; Nishimura, T.; Adachi, M.; Nguyen-Ngoc, L.; Séchet, V.; Amzil, Z.; Herrenknecht, C.; Hess, P. "Assessment of 13 *Gambierdiscus* strains using neuro-2a and erythrocyte lysis assays" at the international conference ICHA 2016 in Florianópolis (Brazil) (**Figure 60**, **annexes**).

Posters

- 2015, March 31st – April 2nd: **Pisapia, F.**; Séchet, V.; Sibat, M.; Raimbault, V.; Herrenknecht, C.; Amzil, Z.; Hess, P. "Culture of *Gambierdiscus* strains for the evaluation of extraction efficiency and inter-and intra-specific variability of growth as function of nutrition" at the national conference GdR PHYCOTOX & GIS CYANO 2015 in Brest (France) (**Figure 59**, **annexes**).

- 2015, June 14th – 17th : Pisapia, F.; Séchet, V.; Sibat, M.; Raimbault, V.; Herrenknecht, C.; Amzil, Z.; **Hess, P.** "Culture of *Gambierdiscus* strains for the evaluation of extraction efficiency and inter-and intra-specific variability of growth as function of nutrition" at the Marine and Freshwater Toxins Analysis: Joint Symposium and Task Force Meeting (5th Edition) in Baiona (Spain) (**Figure 59**, **annexes**)

- 2016, November 8th – 9th: **Pisapia, F.**; Gaiani, G.; Lhaute, K.; Roullier, C.; Ferron, P.-J.; Bertrand, S.; Sibat, M.; Amzil, Z.; Herrenknecht, C.; Hess, P. "Bioguided fractionation of *Gambierdiscus* extracts" at the seminar Journées ODE in Logonna-Daoulas (France) (**Figure 61**, **annexes**).

Annexes

Culture of *Gambierdiscus* strains for the evaluation of extraction efficiency and inter- and intra-specific variability of growth as function of nutrition

Francesco Pisapia[1], Véronique Séchet[1], Manoëlla Sibat[1], Virginie Raimbault[1], Christine Herrenknecht[2], Zouher Amzil[1], Philipp Hess[1]

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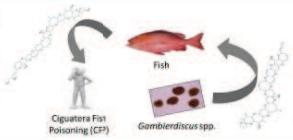
Background

- **Ciguatera Fish Poisoning (CFP)** is a food-borne illness caused by consumption of fish contaminated with polyether toxins known as ciguatoxins (CTXs).
- Originally known as a tropical disease, CFP has recently been reported from areas previously not considered endemic (e.g. Canary Islands).
- The benthic dinoflagellate *Gambierdiscus* is considered to be the primary producer of CTXs. An increasing number of species has recently been discovered in this genus, even in areas where it had previously not been observed (e.g. Mediterranean Sea). This genus also produces other types of potent toxins, namely maitotoxins.
- CTXs are bio-accumulated and transformed in herbivorous and carnivorous fish along the marine trophic chain.



Materials & Methods

Experiment 1

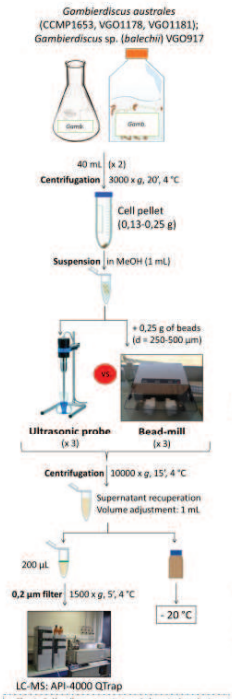


Fig. 1: Cell pellet extraction and chemical analysis.

Cell pellets of four *Gambierdiscus* strains (*G. australes* CCMP1653 from Hawaii, VGO1178 and VGO1181 from Canary Islands and *G.* sp. (*balechii*) VGO917 from Indonesia) were collected by centrifugation.
Pellets were extracted in MeOH (1 mL x 3) using two different disruption methods: an ultrasonic probe and a mixer mill with glass beads in polypropylen Eppendorf vials.
Sonication was conducted at 0 °C for 30 minutes in pulse mode (10 s ON, 5 s OFF). Power was fixed at 30% of total power (500 W). Grinding with the bead-mill was conducted for 30 min at a vibration frequency of 30 Hz (**Fig. 1**).
CTXs analysis was carried out using a LC system (Nexera, Shimadzu) coupled to a hybrid triple quadrupole/ion trap mass spectrometer (API-4000 QTrap, AB SCIEX) equipped with a turbo spray® interface. A 2.6 µm C18 Kinetex column (50*2.1 mm, Phenomenex) was employed at 40 °C and eluted at 400 µL/min with a linear gradient. Eluent A was water and eluent B was 95% acetonitrile/water, both eluents containing 2 mM ammonium formiate and 50 mM formic acid. Mass spectrometry detection was operated in positive mode and using Multiple Reaction Monitoring (MRM) (**Fig. 1**).

Experiment 2

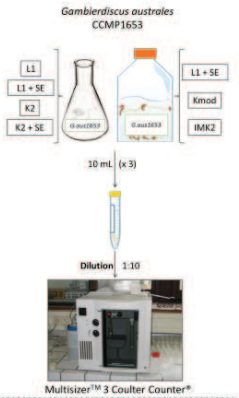


Fig. 2: Cell counting and cell volume estimation.

Gambierdiscus australes CCMP1653 (a Hawaiian strain) was grown in filtered natural Mediterranean seawater (33 psu)

- in Erlenmeyer glass flasks, enriched with the following culture media: L1, L1 + soil extract, K2, K2 + soil extract;
- in Corning Cellbind flasks: L1 + soil extract, K modified and IMK2.

Cultures were maintained at 25 °C, with a photon flux density at 200 µmol m^{-2} s^{-1} and a photoperiod of 16 D / 8 N. Cell volumes and concentrations (cells/mL) were estimated at late stationary growth phase using a Multisizer™ 3 Coulter Counter® (**Fig. 2**).

Results & Discussion

Experiment 1: Evaluation of extraction efficiency

As the CTXs in these algal strains are not yet known, extraction efficiency was evaluated with compounds of similar mass and RT as known (Pacific) CTXs. Three compounds in the molecular size range of CTXs eluting respectively at the beginning (RT ~ 2 min), at the middle (RT ~ 4 min) and at the end (RT ~ 6 min) of the chromatographic run were chosen and peak areas were measured (**Fig. 3**). Average recovery yields between the four strains were calculated for each step of the disruption (**Fig. 4**).

- Both ultrasonic probe and bead-mill disruption techniques had very similar extraction efficiency.
- Regardless the strain and the number of cells extracted (from ~ 70000 to ~ 400000 in this experiment), duplicate extraction of pellets (2 x 1 mL) was sufficient for retrieval of more than 95% of the metabolites.

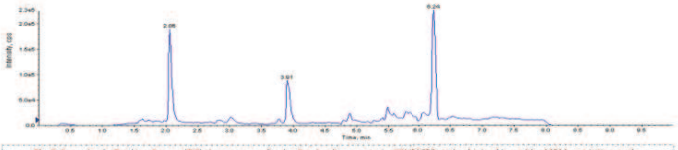


Fig. 3: Example of a Total Ion Current (TIC) chromatogram for the *Gamberdiscus australes* CCMP1653 strain obtained in positive MRM acquisition mode.

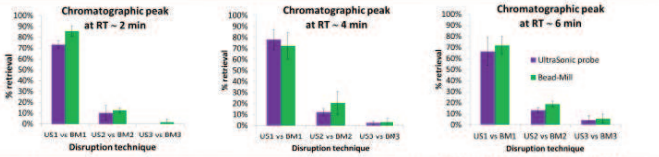


Fig. 4: Evaluation of extraction efficiency using "pseudo-"CTX peaks for the following *Gambierdiscus* strains: CCMP1653, VGO1178, VGO1181 and VGO917.

Experiment 2: Cell growth as function of nutrition

In general terms, laboratory cultures of *Gambierdiscus* spp. showed that cell concentration was inversely proportional to cell volume for any given bottom surface. For the *G. australes* CCMP1653 strain, this relationship seemed more evident when using Erlenmeyer glass flasks than Corning Cellbind flasks (**Fig. 5**).
In this experiment, L1 medium gave the best results in terms of cell concentration, reaching 11520 cells/mL. The presence of soil extract (SE, 1 mL/L) seemed to inhibit cell division and to increase cell size.
Estimated spherical volumes of this particular strain at the late stationary growth phase varied between 1,00*10^5 µm^3/cell (L1 medium) and 1,72*10^5 µm^3/cell (K2 + SE medium) (**Fig. 5**).
The culture medium seemed to have a great influence on cell development. Further studies on toxin production as function of nutrition will help optimize our laboratory conditions.

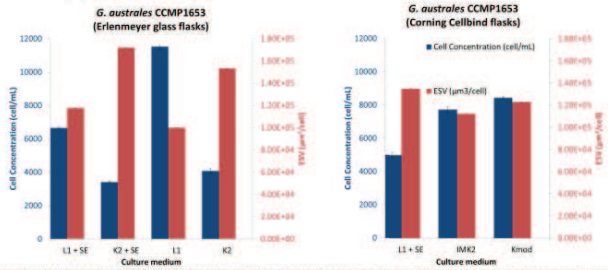


Fig. 5: Cell concentration and volume as function of culture medium for the *Gambierdiscus australes* CCMP1653 strain at late stationary growth phase.

Conclusions & Perspectives

- Both ultrasonic probe and bead-mill disruption techniques have very similar extraction efficiency.
- Duplicate extraction of pellets is sufficient for retrieval of more than 95% of the metabolites in the molecular size range of CTXs.
- The strain *Gambierdiscus australes* CCMP1653 shows some differences both in cell volume and concentration depending on growth medium.

Still to come...

- Optimisation of laboratory cultures: studies on growth rates and toxin production as function of culture medium, salinity, temperature...
- Optimisation of the extraction procedure: solvent (nature, volume), amount of beads, grinding time...
- Comparative studies on cell growth and toxin production kinetics of the 19 strains currently maintained in our laboratory.
- Bioguided fractionation of toxic strains in combination with high resolution mass spectrometry to pinpoint previously undescribed compounds.

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GdR PHYCOTOX: 2015, March 31st – April 2nd, Brest

Figure 59. Culture of *Gambierdiscus* strains for the evaluation of extraction efficiency and inter- and intra-specific variability of growth as function of nutrition.

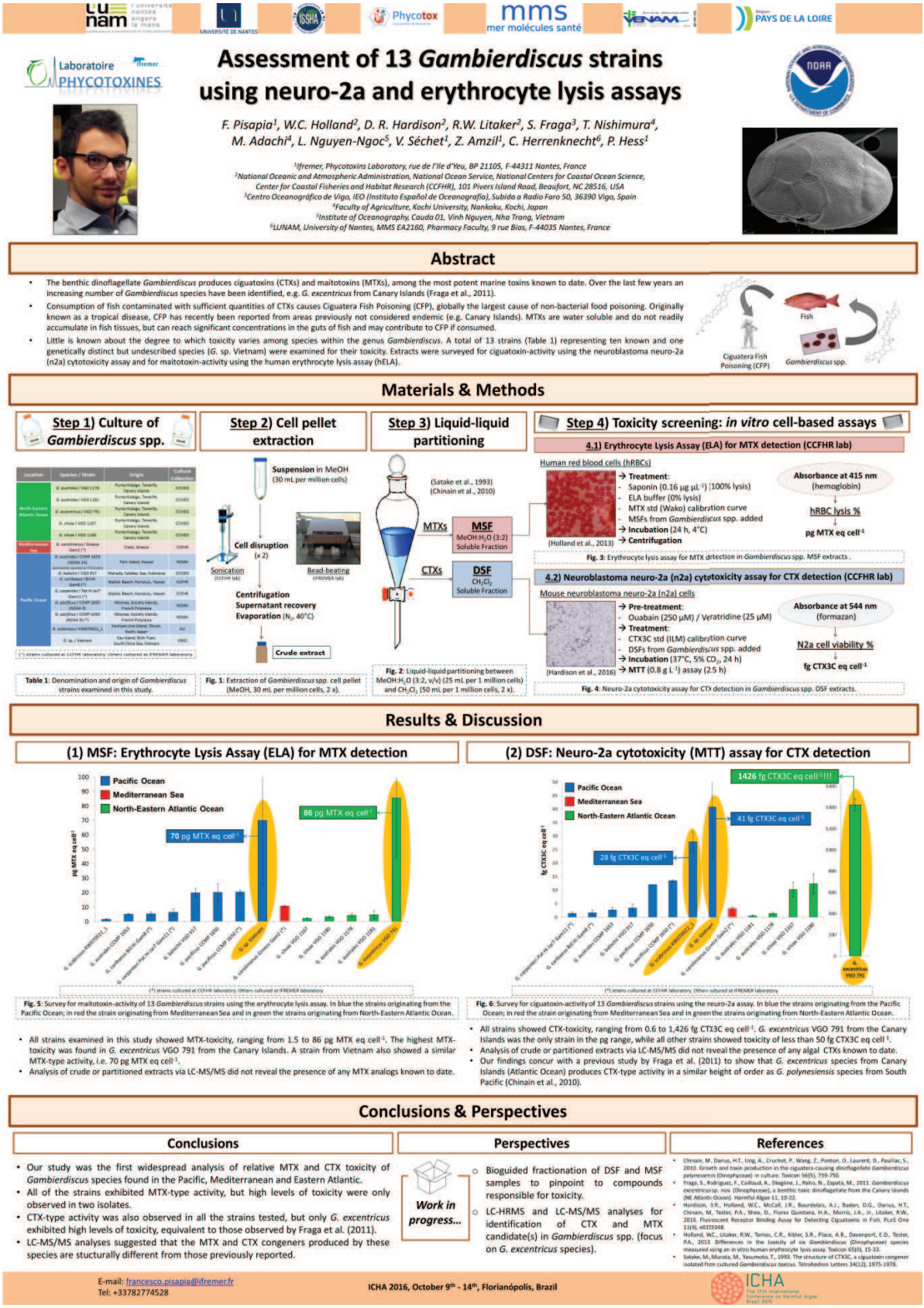

Figure 60. Assessment of 13 *Gambierdiscus* strains using neuro-2a and erythrocyte lysis assays.

Bioguided fractionation of *Gambierdiscus* extracts

Francesco Pisapia[1], Greta Gaiani[2], Korian Lhaute[1], Catherine Roullier[3], Pierre-Jean Ferron[4]
Samuel Bertrand[3], Manoëlla Sibat[1], Zouher Amzil[1], Christine Herrenknecht[3], Philipp Hess[1]

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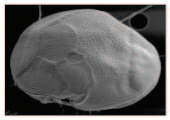

Background

- Species in the benthic dinoflagellate genus *Gambierdiscus* produce ciguatoxins (CTXs) and maitotoxins (MTXs), among the most potent marine toxins isolated to date. Over the last few years an increasing number of *Gambierdiscus* species have been identified, e.g. *G. excentricus* from Canary Islands (Fraga et al., 2011).
- Consumption of fish tainted with sufficient quantities of CTXs causes Ciguatera Fish Poisoning (CFP), globally the largest cause of non-bacterial food poisoning. Originally known as a tropical disease, CFP has recently been reported from areas previously not considered endemic (e.g. Canary Islands).
- MTXs are water soluble and do not readily accumulate in fish tissues, but can reach significant concentrations in the guts of fish and may contribute to CFP if consumed. Three MTX analogs are known to date. The structural elucidation by NMR technique has been obtained only for MTX-1.
- 13 *Gambierdiscus* strains have previously been screened for their toxicity using two *in vitro* assays (N2a and hemolytic assays). The most toxic ones are shown in **Table 1**.

Table 1: The most toxic *Gambierdiscus* strains cultured at Phycotoxins laboratory (Ifremer, Nantes).

Strain	fg CTX3C eq cell^{-1}	pg MTX eq cell^{-1}
G. excentricus VGO 791	1,426	86
G. sp. Vietnam	41	70
G. scabrosus KW070922_1	28	1.5

Materials & Methods

Step 1: Harvesting of *Gambierdiscus* cells

Fig. 1: Laboratory cultures of *Gambierdiscus* and cell harvesting.

Step 2: Cell pellet extraction and liquid-liquid partitioning

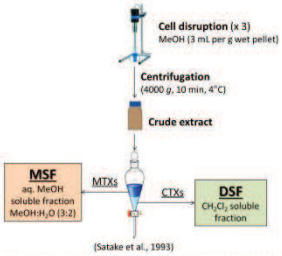

Fig. 2: Sonication of *Gambierdiscus* (MeOH, 3x) and liquid-liquid partitioning between MeOH:H_2O (3:2, v/v) and CH_2Cl_2.

Step 3: Size-exclusion chromatography

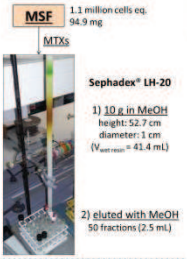

Fig. 3: Example of fractionation on a Sephadex® LH-20 column.

Step 4: Toxicity screening on neuro-2a (N2a) cells

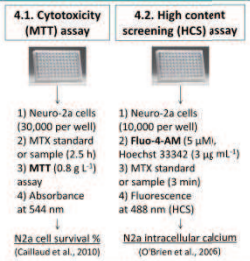

Fig. 4: Toxicity screening on neuro-2a cells using (1) MTT to determine cell survival % or (2) Fluo-4-AM to label iCa^{2+}.

Step 5: Acquisition of data using LC-HRMS

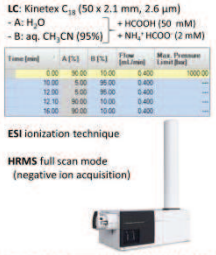

Fig. 5: LC method and HRMS Q-Tof 6550 iFunnel (Agilent) for MTX detection.

Preliminary results & Discussion

N2a cytotoxicity (MTT) assay

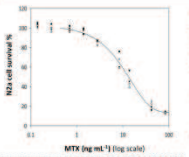

Fig. 6: Sigmoidal dose-response curve (SigmaPlot® 12) of MTX standard on the N2a cytotoxicity (MTT) assay after 2.5 h exposure.

Mitochondrial activity was determined using the quantitative colorimetric MTT assay (Tecan Infinite® M200 plate reader; Tecan, Austria, GmbH) as an indicator of cell viability.

- MTX standard produces mortality in neuro-2a cells in a dose-dependent manner (**Fig. 6**).
- Crude extract and aq. MeOH soluble fraction (MSF) of *G. excentricus* VGO 791 caused more than 90% N2a cells to die (**Fig. 7**).
- Among the 50 LH-20 fractions collected, only those from #06 to #09 (V_{MeOH} = 12.5-22.5 mL) showed to be neurotoxic (**Fig. 7**).
- The early elution of the toxic compound(s) suggests a high molecular weight.

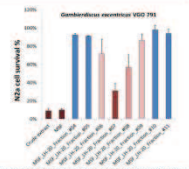

Fig. 7: N2a cell survival % after 2.5 h exposure with *G. excentricus* VGO 791.

N2a high content screening (HCS) assay

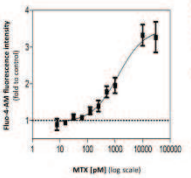

Fig. 8: Sigmoidal dose-response curve (GraphPad Prism 6.0) of MTX standard on the N2a HCS assay after 3 min exposure.

N2a cell nuclei were detected using Hoechst 33342 staining. Fluo-4-AM fluorescence was measured using an ArrayScan VTI HCS Reader (Thermo Scientific, Waltham, MA, USA) and expressed as a fold of intensity compared to vehicle control condition.

- MTX standard increased intracellular calcium in a dose-dependent manner (**Fig. 8**).
- Crude extract and MSF of *G. excentricus* VGO 791 increased Ca^{2+} levels in N2a cells (**Fig. 9**), indicating that the toxic compounds exhibit a similar effect on cells as that of MTX.
- LH-20 fractions of MSF from #06 to #10 (V_{MeOH} = 12.5-25 mL) showed an increase in intracellular calcium levels between 1.5 and 1.8 fold to control (**Fig. 9**).
- Hence, cytotoxicity and changes in intracellular calcium coincided well in early eluting fractions.

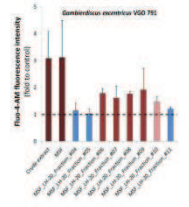

Fig. 9: Fluo-4-AM fluorescence intensity after 3 min exposure with *G. excentricus* VGO 791.

Data-mining of HRMS results

Data-mining via LC-HRMS was carried out to manage data complexity and to correlate MS data to toxicity. Raw data were retreated using MassHunter Qual software (v B.07) using an abundance cut-off of 500 counts. MassHunter algorithms recognize isotope clusters and simple adducts such as water losses. Compound identification was carried out using an in-house developed database. The identification algorithm uses both exact mass and isotope abundance of clusters for assignment to a molecular formula.

- No MTX was found in any of the extracts (crude or purified) suggesting that a hitherto undescribed compound (or more than one) is responsible for the activity found in the cellular assays.
- The crude extract contained 1,312 compounds, while the MSF contained 725 compounds (**Fig. 10**). Thus, the liquid-liquid partitioning step resulted approx. in a purification of a factor 1.8.
- Toxic LH-20 fractions (from #06 to #10) contained between 25 and 51 compounds, with the most toxic fraction (#07) containing only 40 compounds (**Fig. 10**). Comparison of toxic with non-toxic fractions is underway to pinpoint to potential candidates as causative agents for the cytotoxicity observed.
- Size-exclusion chromatography is an efficient clean-up step for high molecular weight compounds as it allows for a purification to a stage where individual compounds may be evaluated as candidates.

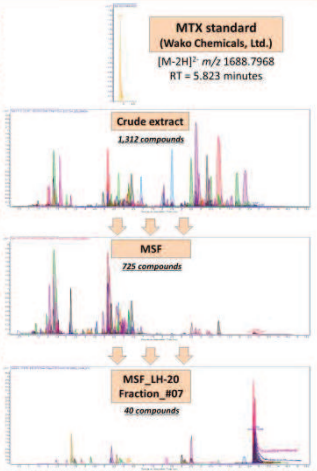

Fig. 10: MS spectra (negative ion acquisition) obtained in full scan mode. -EIC of MTX standard ($[M-2H]^{2-}$ at m/z 1688.7968, RT = 5.823 min). -ECC of *G. excentricus* VGO 791 samples: crude extract, MSF, LH-20 fraction #07.

Conclusions & Perspectives

Conclusions

- *G. excentricus* VGO 791 produces hydrophilic (aq. methanol-soluble) compound(s) that are lethal to neuro-2a cells, causing a rapid increase of intracellular calcium such as MTX.
- Fractionation by size-exclusion chromatography suggests that the toxic components elute early (from 12.5 to 22.5-25 mL) consistent with high molecular weight compounds.
- LC-HRMS data (Q-Tof 6550) in full scan mode (negative acquisition) showed the efficacy of liquid-liquid partitioning and size-exclusion chromatography as purification steps.
- LC-HRMS is a useful approach to identify hitherto undescribed toxic compound(s).

Perspectives

Still to come...

- Preparative isolation for identification of the MTX candidate(s) using a bioguided fractionation approach.
- Screening for toxicity and LC-HRMS analysis of the CH_2Cl_2 soluble fraction (DSF) to pinpoint to lipophilic toxins (CTXs).
- What about the other *Gambierdiscus* strains?

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OCEANEXT, 2016 June 8th - 10th, Nantes

Figure 61. Bioguided fractionation of *Gambierdiscus* extracts.

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UNIVERSITE BRETAGNE LOIRE

UNIVERSITÉ DE NANTES

Thèse de Doctorat

Francesco PISAPIA

Criblage bioguidé et analyse CL-SMHR des toxines et d'autres métabolites d'intérêt chez les dinoflagellés *Gambierdiscus* et *Fukuyoa*

Bioguided screening and LC-HRMS for identification of toxins and other metabolites of interest produced by the dinoflagellates *Gambierdiscus* and *Fukuyoa*

Résumé

Les dinoflagellés epi-benthiques *Gambierdiscus* et *Fukuyoa* produisent les ciguatoxines (CTXs) et les maïtotoxines (MTXs), qui sont parmi les toxines marines les plus puissantes connues. Les CTXs sont bio-accumulées et bio-transformées le long de la chaîne alimentaire marine et provoquent la ciguatéra, l'intoxication alimentaire non bactérienne la plus commune au monde. Récemment, la ciguatéra a été rapporté dans des zones non endémiques, notamment les îles Canaries, et un grand nombre d'espèces a été découvert au sein de ces genres. Peu de données sont disponibles sur la toxicité relative entre les différentes espèces et sur les congénères de toxines produits par ces dinoflagellés, en particulier par des souches en dehors de l'océan Pacifique. Plusieurs souches de *Gambierdiscus* et *Fukuyoa* ont été examinées pour leur cigua- et maïto-toxicité via les tests neuro-2a et hémolytique, respectivement. *G. excentricus* a montré une forte toxicité et deux souches des îles Canaries ont été sélectionnées pour la purification des toxines via une approche de fractionnement bioguidé. L'analyse non-ciblée de spectrométrie de masse (SM) à haute résolution a permis la découverte d'un nouveau congénère de MTX, la maïtotoxine-4 (MTX4). La SM à basse résolution de plusieurs souches de *Gambierdiscus* et *Fukuyoa* n'a révélé la présence de MTX4 que dans *G. excentricus*, y compris des souches provenant des îles Canaries, du Brésil et du Golfe du Mexique. La MTX4 pourrait donc servir de biomarqueur pour l'espèce hautement toxique *G. excentricus*. Les travaux futurs porteront sur l'isolement et l'élucidation de la structure de la MTX4 et l'identification des congénères de CTX produits par *G. excentricus*.

Mots clés

Ciguatéra, *Gambierdiscus*, *Fukuyoa*, ciguatoxines, maïtotoxines, test neuro-2a, test hémolytique, spectrométrie de masse.

Abstract

The epi-benthic dinoflagellates *Gambierdiscus* and *Fukuyoa* produce ciguatoxins (CTXs) and maitotoxins (MTXs), which are among the most potent marine toxins known. CTXs are bio-accumulated and bio-transformed along the marine food chain and cause Ciguatera Fish Poisoning (CFP), the most common non-bacterial food-borne intoxication worldwide. Recently, CFP has been reported from areas previously not considered endemic, namely the Canary Islands, and an increasing number of species has been discovered in these genera. Little is known about the relative toxicity between different species and the toxin congeners produced by these dinoflagellates, especially by strains outside the Pacific Ocean. Several strains of *Gambierdiscus* and *Fukuyoa* were screened for their cigua- and maito-toxicity using neuro-2a and hemolytic assays, respectively. *G. excentricus* showed particularly high toxicity and two strains from Canary Islands were selected for toxin purification using a bioguided fractionation approach. Non-targeted high resolution mass spectrometry (MS) analysis permitted the discovery of a novel MTX congener, maitotoxin-4 (MTX4). Targeted low resolution MS analysis of more than 40 strains of *Gambierdiscus* and *Fukuyoa* revealed the presence of MTX4 in *G. excentricus* only, including strains from the Canaries, the South Western Atlantic (Brazil) and the North Western Atlantic (Gulf of Mexico). As MTX4 was not detected in any other species examined, MTX4 may serve as a biomarker for the highly toxic *G. excentricus*. Future work will include isolation and structural elucidation of MTX4 and identification of CTX congeners produced by *G. excentricus*.

Key Words

Ciguatera Fish Poisoning, Gambierdiscus, Fukuyoa, ciguatoxins, maitotoxins, neuro-2a assay, hemolytic assay, mass spectrometry