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

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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 relatively 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 1426 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.

1. 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).

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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., 1992, 1993) 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, 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 1, Section 2.2). Except two strains (CCMP1653 and the strain from Viet Nam), none

Table 1
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 2.2.1).
[b] strains cultured at the IFREMER laboratory (Nantes, France) (section 2.2.2). (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.