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

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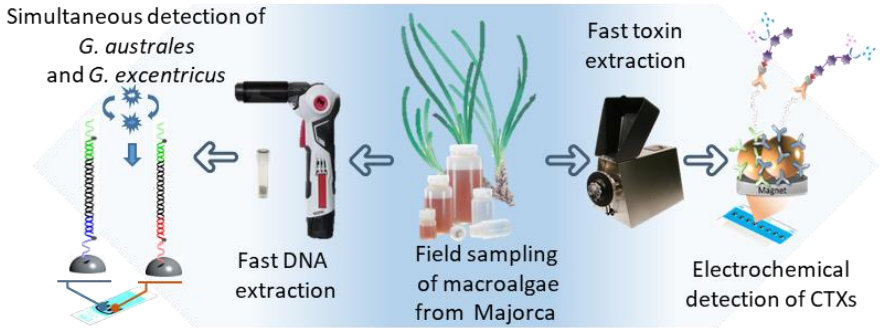
HIGHLIGHTS

- First biosensor for the dual detection of two toxin-producing *Gambierdiscus* species
- Fast DNA extraction technique using a portable bead beater and magnetic beads
- Simultaneous detection of *G. australes* and *G. excentricus* in field samples
- First report of G. *excentricus* in the Balearic Islands waters
- Ciguatoxins detection in field samples using a biosensor

GRAPHICAL ABSTRACT



Abstract

Several genera of marine dinoflagellates are known to produce bioactive compounds that affect human health. Among them, *Gambierdiscus* and *Fukuyoa* stand out for their ability to produce several toxins, including the potent neurotoxic ciguatoxins (CTXs), which accumulate through the food web. Once fishes contaminated with CTXs are ingested by humans, it can result in an intoxication named ciguatera. Within the two genera, only some species are able to produce toxins, and *G. australes* and *G. excentricus* have been highlighted to be the most abundant and toxic. Although the genera *Gambierdiscus* and *Fukuyoa* are endemic to tropical areas, their presence in subtropical and temperate regions has been recently recorded. In this work, the combined use of species-specific PCR primers for *G. australes* and *G. excentricus* modified with short oligonucleotide tails allowed the development of a multiplex detection system for these two toxin-producing species. Simultaneous detection was achieved using capture probes specific for *G. australes* and *G. excentricus* immobilized on maleimide-coated magnetic beads (MBs), separately placed on the working electrodes of a dual electrode array. Additionally, a rapid DNA extraction technique based on a portable bead beater system and MBs was developed, significantly reducing the extraction time (from several hours to 30 min). The developed technique was able to detect as low as 10 cells of both *Gambierdiscus* species and allowed the first detection of *G. excentricus* in the Balearic Islands in 8 out of the 9 samples analyzed. Finally, field samples were screened for CTXs with an immunosensor, successfully reporting 13.35 ± 0.5 pg CTX1B equiv. $cell^{-1}$ in one sample and traces of toxins in 3 out of the 9 samples analyzed. These developments provide rapid and cost-effective strategies for ciguatera risk assessment, with the aim of guaranteeing seafood safety.

Keywords

Ciguatera; *Gambierdiscus*; simultaneous detection; species-specific molecular assay; DNA-based biosensor; field sample analysis

1. Introduction

Marine dinoflagellates are known producers of a wide range of toxins. These toxins may be transferred along the food chain and accumulate in the flesh of seafood. Therefore, they can reach seafood consumers resulting in foodborne diseases. Among all the existing foodborne diseases caused by marine toxins, ciguatera is one of the most common in intertropical and nearby areas (Begier et al., 2006; Larsson et al., 2019; Lewis, 2001; Litaker et al., 2017). Ciguatera is caused by the ingestion of fish contaminated with ciguatoxins (CTXs), potent marine neurotoxins that can accumulate in the food webs, rarely in bivalves, echinoderms and crustaceans, and more frequently in herbivorous, detritivorous and carnivorous fish (Kelly et al., 1992; Ledreux et al., 2014; Roué et al., 2016; Silva et al., 2015). CTXs are produced by dinoflagellates of the genera *Gambierdiscus* and *Fukuyoa,* which also produce other bioactive compounds such as maitotoxins (MTXs) (Holmes and Lewis, 1994; Murata et al., 1993; Pisapia et al., 2017b), gambieric acids (Nagai et al., 1992), gambierol (Satake et al., 1993), gambieroxide (Watanabe et al., 2013) and gambierone (Murray et al., 2019; Rodríguez et al., 2015). However, it is not clear yet if these compounds play a role in ciguatera (Kohli et al., 2015). The *Gambierdiscus* and *Fukuyoa* genera are endemic of subtropical areas, although in the past decade they have been found in temperate areas such as Japan (Nishimura et al., 2014), the coast of North Carolina (Litaker et al., 2009), the Gulf of Mexico (Litaker et al., 2017), Brazil (Gómez et al., 2015), the Canary Islands (Fraga and Rodriguez, 2014; Fraga et al., 2011), Madeira (Kaufmann and Böhm-Beck, 2013) and the Mediterranean Sea, first detected in Greece (Aligizaki and Nikolaidis, 2008; Aligizaki et al., 2009) and then a few years later in the Balearic Islands (Laza-Martínez et al., 2016; Tudó et al., 2018). The intensification of monitoring efforts could give the impression of an increase in the spread of the genera, as it has been demonstrated for the perceived global increase in algal blooms (Hallegraeff et al., 2021). Nevertheless, the global warming trend is supposed to favor the proliferation and expansion of these harmful genera.

Therefore, the probable raise in the occurrence of ciguatera poisoning events in new areas increases the threat to human health.

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

In this work, we used species-specific PCR primers for *G. australes* and *G. excentricus* modified with short oligonucleotide tails to create a multiplex detection system for these two toxin-producing species (Figure 1). The species-specific detection was achieved using capture probes of *G. australes* and *G. excentricus* immobilized on maleimide-coated magnetic beads (MBs), and subsequently capturing them separately on the working electrodes of a dual electrode array. One tail of the amplified products binds specifically to the corresponding capture probe and the other to an enzyme-labelled reporter probe. A similar approach was previously used for the detection of other toxic marine dinoflagellates (Toldrà et al., 2019b), and this is the first time that such a strategy is combined with a dual electrochemical biosensor and used for the simultaneous detection of two toxin-producing *Gambierdiscus* species in field samples. In addition, a rapid DNA extraction technique combining a portable bead beater system and MBs was developed, which reduces the extraction time from several hours to a few minutes, which can be considered as a step forward for the extraction of samples directly in field. Moreover, we used the sandwich immunosensor previously developed by our group (Gaiani et al., 2020), to screen CTX contents directly in field samples. This technique involves the use of monoclonal antibodies (mAbs) specific for CTXs. Specifically, two capture antibodies were used, the 3G8 mAb which has affinity for the left wing of CTX1B and 54-deoxyCTX1B (Tsumuraya et al., 2012), and the 10C9 mAb which has affinity for the left wing of CTX3C and 51-hydroxyCTX3C (Oguri et al., 2003). Moreover, a detector antibody, 8H4 mAb was used for the recognition of the right wing of the four congeners (Tsumuraya et al., 2006).

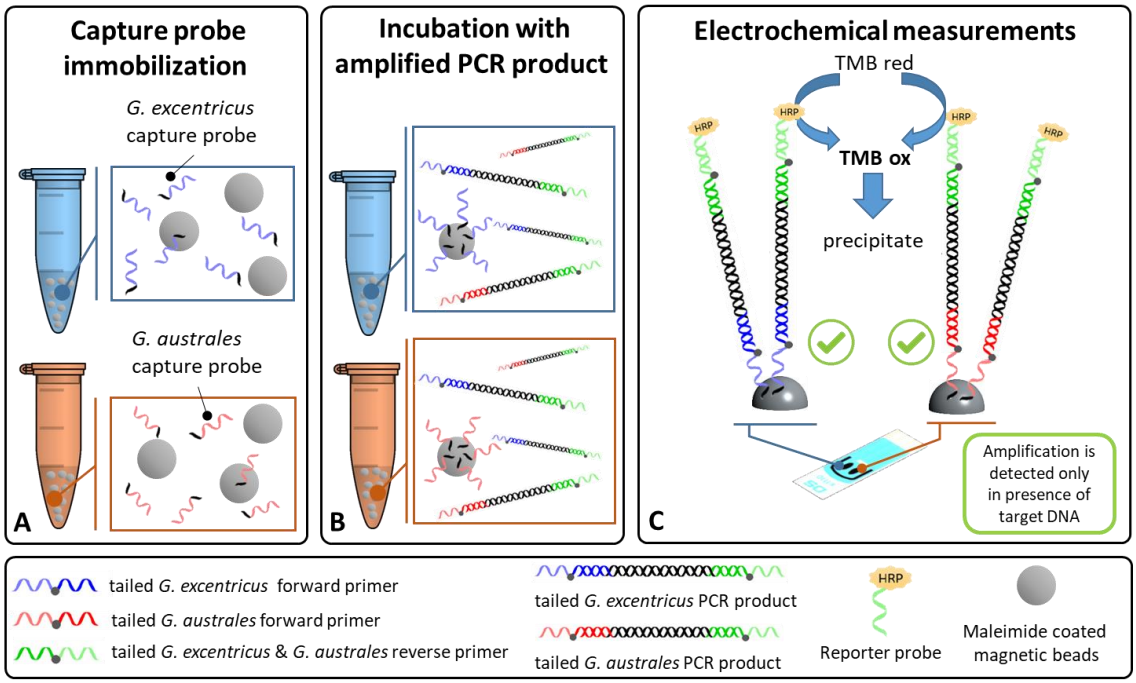


Figure 1. Schematic representation of the strategy developed in this work. (A) Species-specific capture probes were immobilized separately on maleimide-coated MBs and then (B) exposed to PCR products. (C) Detection of tailed *G. australes* and *G. excentricus* PCR products was achieved on each working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

2. Materials and methods

2.1. Microalgal cultures and field samples

One strain of *G. australes* obtained from the IRTA collection (IRTA-SMM-16_286) and one of *G. excentricus* from the Culture Collection of Microalgae of the Instituto Español de Oceanografía (CCVIEO) in Vigo, Spain (VGO791) were used in this work. Monoclonal cultures were grown in polystyrene flasks containing 500 mL of modified ES medium (Provasoli, 1968) prepared with filtered and autoclaved seawater from L'Ametlla de Mar, Spain (salinity adjusted to 36 psu). Cultures were maintained at 24 ± 1 °C under a photon flux rate of 100 µmol m^{-2} s^{-1} with a 12:12h light:dark regime. Culture aliquots were fixed with 3% *v/v* Lugol's iodine and counted using a Kolkwitz chamber (Hydro-Bios, Altenholz, Germany) under an inverted light microscope (Leica DMIL, Spain), following the Sedgwick-Rafter method (Greeson, 1977) every second day. Once the cultures reached the early exponential phase (*ca.* 21 days), microalgal pellets of 10^4 cells were prepared splitting accordingly the entire culture volume in 50 mL tubes.

The tubes were then centrifuged at 2,500 rpm for 25 min (Allegra X-15R, Beckman Coulter, Brea, USA). Supernatants were discarded and tubes were stored at -20 °C until DNA extraction. A total of 12 samples were collected in Majorca during October 2020 (Table 1). For the sampling, 100-200 g fresh weight of microalgae substrate were mixed with 250 mL of seawater, vigorously shaken and filtered through a 200-µm mesh. Once the 250-mL bottles reached the laboratory, the entire volume was fixed with 3% *v/v* Lugol's iodine solution and 10 mL of the fixed samples were stained with Calcofluor white M2R (Sigma Aldrich, Spain) for identification and counted under UV light using an epifluorescence microscope (LEICA DMLB) with the Utermöhl method (Utermöhl, 1958). Cell abundances were expressed as cell L^{-1}. Fifty millilitre aliquots from each field sample were centrifuged at 2,500 rpm for 25 min. Supernatants were discarded and tubes were stored at -20 °C until DNA extraction.

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

Sampling point	Sample code	Macrophyte substrate	Biosensor			Light microscopy
			G. australes **(cell L^{-1})**	***G. excentricus*** **(cell L^{-1})**	**Total (cell L^{-1})**	***Gambierdiscus*** **spp. (cell L^{-1})**
Cala Gat	2020-ME-886*	*Posidonia oceanica*	ND	ND	ND	ND
Platja Canyamel	2020-ME-906*	*Posidonia oceanica*	ND	ND	ND	ND
Portocolom	2020-ME-946*	*Posidonia oceanica*	ND	ND	ND	ND
Platja Canyamel	2020-ME-914	*Corallina elongata* *Digenea simplex*	484	176	660	5,800
Cala Anguila	2020-ME-930	*Digenea simplex* *Corallina elongata* *Jania adhaerents*	349	109	458	7,800
Cala Anguila	2020-ME-934	*Corallina elongata*	ND	280	280	700
Cala Llombards	2020-ME-966	*Cladostephus spongiosus*	515	108	623	2,700
Cala Llombards	2020-ME-970	*Halopteris scoparia* *Jania adhaerents*	1,181	58	1,239	36,100
Cala Galiota	2020-ME-986	*Halopithys incurva*	428	ND	428	3,200
Cala Galiota	2020-ME-990	*Halopithys incurva*	4,824	1,883	6,707	1,000
Cala Galiota	2020-ME-994	*Posidonia oceanica*	536	163	699	100
Cala Mosques	2020-ME-1034	*Posidonia oceanica* (rizoma)	2,536	273	2,809	2,300

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

2.2. DNA extraction methods

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

Additionally, DNA was extracted from cell culture pellets using the Biomeme Sample Prep Kit for DNA (Biomeme Inc., Philadelphia, USA) using the protocol optimized by Toldrà et al. (2018b), with some minor modifications. Briefly, cell pellets were re-suspended in 250 µL of lysis buffer and moved to 2-mL screw-cap cryotubes containing *ca.* 20 µg of 0.5-mm diameter zirconium glass beads and cell disruption was performed as described above. Homogenized cell pellets were added to tubes containing 500 µL of Biomeme Lysis Buffer and pumped through a syringe with an ion exchange cartridge attached (5 pumps). Subsequently, samples were washed with 500 µL of Biomeme Protein Wash and 500 µL of Biomeme Salt Wash. Each wash step consisted

of a single pumping. Samples were then dried by pumping only air through the columns (*ca.* 50 pumps), and finally the samples were eluted in 250 µL of Biomeme Elution Buffer (5 pumps).

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

The genomic DNA obtained with the different techniques was quantified and checked for purity by measuring the absorbance at 260/280 using a NanoDrop 2000 spectrophotometer (Thermo Fisher Scientific, Spain), and subsequently stored at -20 °C until analysis.

DNA from field samples was extracted from one of the 50 mL tubes, by resuspending the pellets in 1 mL of seawater, and then taking 500 µL to be processed with the chosen technique (Terralyzer and MBs). The remaining 500 µL were used for CTX extraction.

2.3. DNA amplification

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

DNA was amplified using the Invitrogen Taq DNA kit (Thermo Fisher Scientific, Madrid, Spain). In the amplification of just one target DNA with its corresponding pair of primers (single PCR reactions), each reaction mixture contained 0.5 µL of 0.2 µM of each primer, 3 µL of 600 µM dNTP, 5 µL of PCR Buffer 1X (-Mg), 2 µL of 2 mM $MgCl_2$, 0.2 µL of 1 U of Taq polymerase, 2 µL of template DNA, and DNAse/RNAse-free water up to 50 µL. The amplification reactions in presence of the three primers (multiplex PCR reaction) contained 0.5 µL of 0.2 µM of each reverse primer and 1 µL of 0.4 µM of the forward primer, all the other reagents were kept at the same concentrations, and DNAse/RNAse-free water was added up to 50 µL. Non-target controls (NTCs, only DNAse/RNAse-free water) were included in the experimental design. To optimize the system, 1 µL of DNA (1 ng μL^{-1}) of each target species was used. After optimization, the amplification protocol was as follows: 95 °C for 5 min, 40 cycles of 95 °C for 30 s, 59 °C for 30s

and 72 °C for 30s, terminated by a final elongation at 72 °C for 5 min. Amplifications were carried out in a Nexus Gradient Thermal Cycler (Eppendorf Iberica, Madrid, Spain). PCR products were then purified using a GeneJet PCR purification kit (Thermo Fisher Scientific, Madrid, Spain) following the manufacturer´s instructions, resulting in 50 µL of DNA in TE (Tris-acetate-EDTA) buffer following the final elution step. The size of the products from the PCR reactions were checked with agarose (2% *w/v*) gel electrophoresis.

2.4. Colorimetric assay

Thiolated capture probes were prepared in 100 mM phosphate, 150 mM NaCl, pH 7.4, at a concentration of 500 nM and 50 µL were incubated in each well of a maleimide-coated plate (Pierce maleimide-activated microtitre plates from Thermo Fisher Scientific, Madrid, Spain). A first blocking of the non-functionalised maleimide groups was performed via the addition of 200 µL of a 100 µM 6-mercapto-1-hexanol solution dissolved in Milli-Q water. A secondary blocking was executed with 200 µL of 5% *w*/*v* skimmed milk in PBS, to avoid non-specific adsorption. Subsequently, 45 µL of PCR product was exposed to the immobilized capture probes, followed by addition of 50 µL of 10 nM HRP-conjugated reporter probe in washing buffer (100 mM phosphate, 150 mM NaCl, 0.05% *v*/*v* Tween-20, pH 7.4). Three washing steps were performed between each step. Capture probe immobilization was performed overnight at 4 °C, whereas all the other incubations were performed at room temperature for 30 min. For all the incubation steps, a microplate shaker was used, to obtain a constant gentle agitation. Finally, 100 µL of TMB (3,3',5,5'-tetramethylbenzidine) Liquid Substrate System for ELISA (Sigma-Aldrich, Tres Cantos, Spain) were added and after 10 min, the absorbance was measured at 620 nm using a Microplate Reader KC4 (BIO-TEK Instruments, Winooski, USA). Gene 5 software was used to collect and evaluate the data. Colorimetric measurements were performed in duplicate.

2.5. Electrochemical biosensor

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

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

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

Single cells from field samples were isolated as described in our previous work (Gaiani et al., 2021). Extraction of genomic DNA from these single microalgal cells was performed using an Arcturus PicoPure DNA Extraction Kit (Thermo Fisher Scientific, Spain) following the

manufacturer's instructions. Briefly, 155 µL of reconstitution buffer were added to one of the provided vials with lyophilized proteinase K and mixed. Once dissolved, 10 µL of the solution were added to each tube containing single cells isolated from field samples and identified as *Gambierdiscus* with light microscopy. DNA extraction was then achieved with a Nexus Gradient Thermal Cycler (Eppendorf, Spain) by incubating at 65 °C for 3 h ending with a step at 95 °C for 10 min. Extracted DNA was stored at -20 °C until analysis. The D1-D3 domain of the 28 S rDNA gene was amplified using *G. excentricus* primers (Table S1) in the single PCR mode as described in Section 2.3. The PCR reactions of single cell DNA preparations were executed in a total volume of 25 µL containing 600 µM dNTP, 2 mM $MgCl_2$, 0.2 µM of each primer, 1 U of Taq polymerase, 5% *v/v* DMSO, and 2 µL of the DNA extracted from single cells. Amplifications were performed in a Nexus Gradient Thermal Cycler (Eppendorf, Spain) and included 45 cycles of amplification following a three-step protocol (95 °C for 30 s, 60 °C for 45 s and 72 °C for 30 s). Each PCR reaction was checked by agarose (2% *w/v*) gel electrophoresis. PCR products of 150 bp were purified with QIAquick PCR Purification Kit and bidirectionally sequenced (Sistemas Genomicos, LLC, Valencia, Spain). Forward and reverse sequence reads were edited using BioEdit v7.0.5.2 (Hall, 1999), and the consensus sequences obtained were checked for similarities with the NCBI BLAST function.

2.7. Ciguatoxin extraction and detection

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

Analyses of extracts were performed as described in our previous work (Gaiani et al., 2020). Briefly, Dynabeads M-270 Carboxylic Acid MBs (Invitrogen, Life Technologies S.A., Alcobendas, Spain) were first activated with an EDC and NHS solution and then incubated with the capture mAb 3G8 (left wing of CTX1B and 54-deoxyCTX1B) or 10C9 (left wing of CTX3C and 51-hydroxyCTX3C) (Tsumuraya and Hirama, 2019). After incubation, the mAb-MB conjugates were washed, and an equal volume of both was placed into new tubes, exposed to microalgal extract (previously evaporated and suspended in PBS-Tween) or CTX1B standard (for the construction of the calibration curve). A blocking step was then performed in PBS-Tween-BSA. The conjugates were then incubated with a biotinylated 8H4 mAb, which binds to the right wing of CTX1B and 54-deoxyCTX1B and has cross-reactivity with the right wing of CTX3C and 51-hydroxyCTX3C. Finally, immunocomplexes were incubated with polyHRP-streptavidin, washed, and re-suspended in PBS-Tween. Electrochemical measurements were performed on the working electrodes of an 8-electrode array, following addition of TMB and H_2O_2, and measuring the reduction current using amperometry (-0.2 V *vs.* Ag for 5 s). Measurements were performed in triplicate.

3. Results and discussion

3.1. Optimization of PCR and DNA extraction methods

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

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

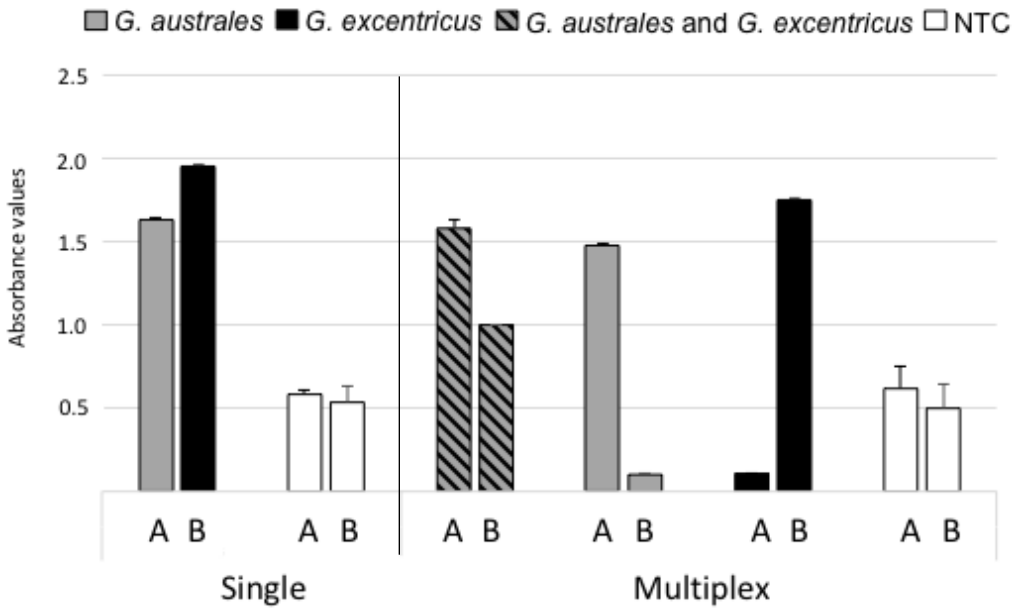


Figure 2. Absorbance values of the PCR-SHA on microtiter plates using single and multiplex PCR. Measurements were performed in triplicate and bars indicate standard deviations. Black line separates results obtained with the single and multiplex strategies. **A**: *G. australes* capture probe; **B**: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

Using the optimized PCR, the different DNA extraction protocols (described in Section 2.2) were evaluated. For this test, 1,000 cells from *G. australes* IRTA-SMM-16_286 and 1,000 cells from *G. excentricus* VGO791 were extracted with each technique. This concentration of cells was chosen as a compromise between a low number of cells and the possibility to obtain sufficient

344 good-quality DNA for the amplification. In this experiment, 1 µL of each extracted DNA was
345 amplified with the multiplex PCR protocol, and the amplified product was then exposed to the
346 *G. australes* and *G. excentricus* capture probes. For all protocols and for both species, higher
347 absorbance values were observed in the presence of the amplified product obtained from a
348 target DNA with its corresponding capture probe (i.e., *G. australes* target DNA with *G. australes*
349 capture probe, and *G. excentricus* target DNA with *G. excentricus* capture probe) than in the
350 presence of the non-corresponding capture probe (Figure 3). Comparing the extraction
351 techniques, the use of the TerraLyzer with MBs was the only one that provided absorbance
352 values close to the ones obtained with the phenol/chloroform/isoamyl alcohol method for both
353 species, which in this experiment is considered as the standard method. This method is also
354 advantageous because the bead beating step with the TerraLyzer can be performed in the field,
355 since it is a portable device with a compact charging system, and also because the use of MBs
356 significantly reduces the DNA extraction time (from several hours to 30 min).

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

365 In fact, these results are in agreement with those previously obtained with the recombinase
366 polymerase amplification (RPA), which showed different absorbance values between several
367 *G. australes* and *G. excentricus* strains at the same DNA template concentration (Gaiani et al.,
368 2021). The chosen rapid DNA extraction technique (Terralyzer and MBs) enables the

procurement of DNA quality and quantity equivalent to that obtained using the reference method, moving towards the realization of an *in situ* DNA extraction method.

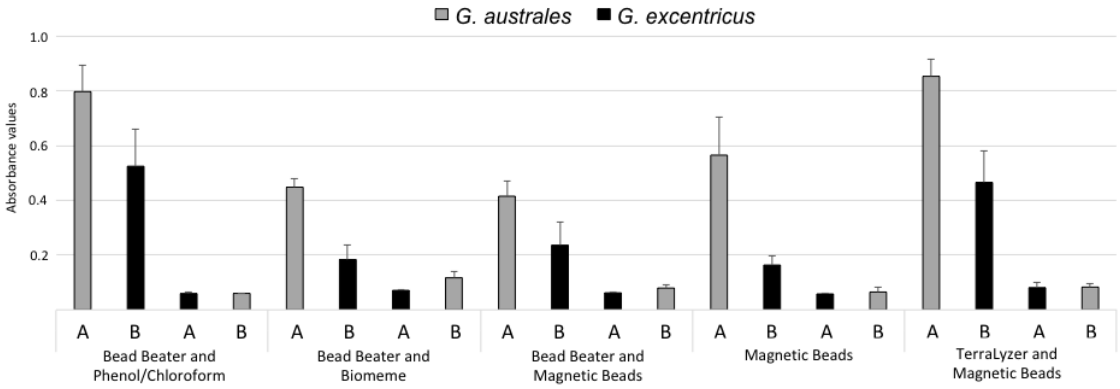


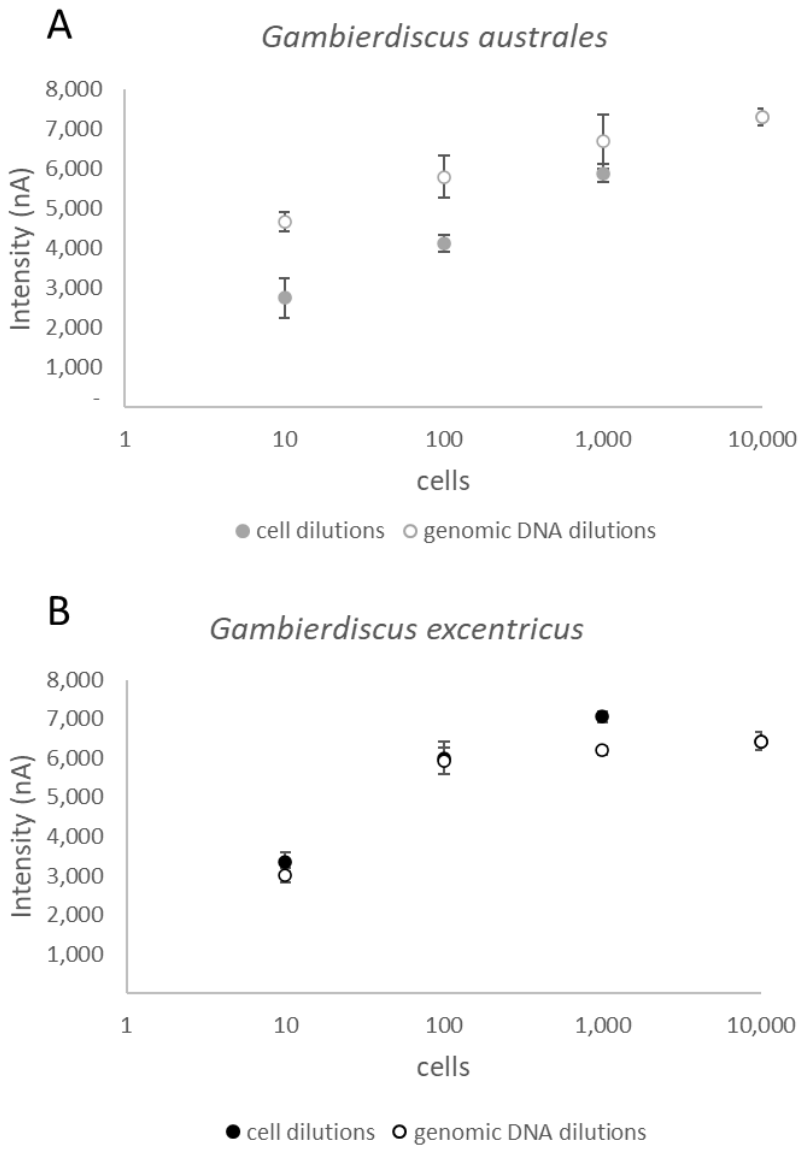
Figure 3. Absorbance values of the PCR-SHA (single) on microtiter plates using 10^3 cells and different DNA extraction methods. Measurements were performed in triplicate and bars indicate standard deviations. **A**: *G. australes* capture probe; **B**: *G. excentricus* capture probe.

3.2. Electrochemical biosensor for DNA detection

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

Results showed higher reduction current intensity with increasing concentrations of DNA for both DNA extracted from cells as well as dilutions of genomic DNA, as expected. The analysis of dilutions of *G. australes* genomic DNA resulted in higher absorbance values in comparison to the

391 corresponding extracted cells (Figure 4A). It is important to take into account that in the
392 calibration curve obtained from the cell dilutions, there is an extraction step for each point of
393 the curve. The efficiency of this extraction step may be compromised by the number of cells,
394 and may be lower when cells are more dispersed in the lysis buffer. However, on the other hand,
395 the analysis of dilutions of *G. excentricus* genomic DNA and extracted cells resulted in closer
396 absorbance values between equivalent concentrations (Figure 4B). We postulate that the cells
397 of this strain and culture may be easier to disrupt in comparison to the *G. australes* ones, and
398 thus the effect of the efficiency of the extraction is less notable. For both *G. australes* and
399 *G. excentricus*, the strategy facilitated successful extraction of DNA, allowing detection of a small
400 number of cells (10).



401

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

405 Subsequently, to demonstrate the ability of the dual biosensor to simultaneously detect both
406 species, nine mixtures of cells (Table 2) were prepared and DNA was extracted with the
407 TerraLyzer and MBs, amplified with PCR (multiplex), and analyzed with the biosensor. The results
408 obtained demonstrate that reduction currents above the background are observed when the
409 target amplified products are exposed to the corresponding capture probes. The system allows
410 discrimination between amplified products belonging to *G. australes* and *G. excentricus* species.
411 Negligible signals were observed in the absence of both targets, i.e. mix 9 (Figure 5). As observed

412 in previous experiments, the presence of the non-target amplified product affects the detection
413 of the target ones. Indeed, the analysis of the mixes in which only one of the two target species
414 is present (mixes 1 and 4 for *G. australes* and mixes 7 and 8 for *G. excentricus*) gave higher
415 current intensity values in comparison with the mixes with the same amount of target cells, but
416 in the presence of different concentrations of the other species (mixes 2, 3, 5 and 6). The
417 observed differences could be attributed to a better affinity of the primers for the target region
418 of *G. australes,* since its presence seems to hinder more the detection of *G. excentricus* than the
419 contrary even though, when only *G. excentricus* is present (mixes 7 and 8), current intensities
420 are higher than for *G. australes* (mixes 1 and 4). Again, as observed in the previous experiment
421 (Figure 4), it seems that *G. excentricus* cells are easier to lyse than those of *G. australes*.
422 Therefore, a better extraction efficiency of *G. excentricus* cells can also be the explanation for
423 the differences observed. However, as mentioned above, the rDNA copy number cannot be
424 excluded as one of the reasons contributing to these differences. Moreover, the presence of
425 *G. australes* cells has a higher effect on the detection of *G. excentricus* than the contrary (mixes
426 2 and 6). Nevertheless, at an equal concentration of cells (mixes 3 and 5), the intensity values
427 are similar, indicating that, even if the detection is to some extent influenced by the non-target
428 species, the system recognizes both. In summary, since the specificity of the primers allows them
429 to amplify target DNA even in the presence of non-target species belonging to the same genus
430 (as demonstrated in this work and in Gaiani et al., 2021), the system is suitable for the screening
431 of field samples.

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434

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

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

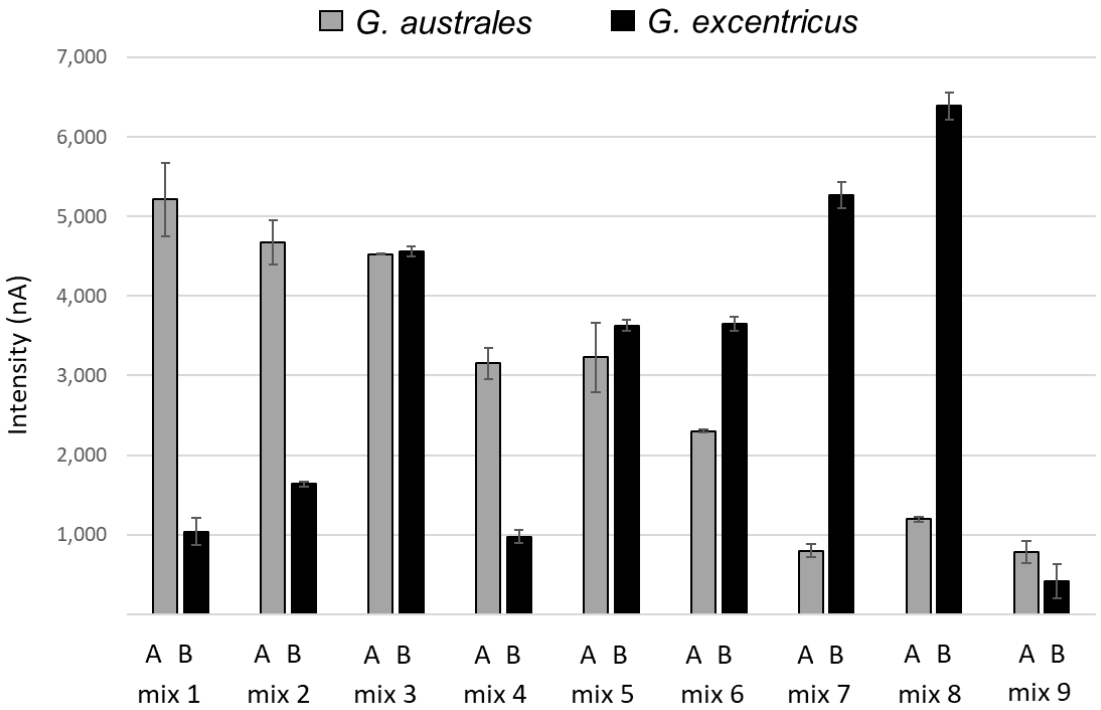

Figure 5. Current intensity values of the PCR-SHA (multiplex) on the dual electrode using mixes with different amounts of *G. australes and G. excentricus* cells. Measurements were performed in triplicate and bars indicate standard deviations. **A**: *G. australes* capture probe; **B**: *G. excentricus*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

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

To evaluate the applicability of the TerraLyzer and MBs protocol combined with the dual biosensor for the analysis of field samples, a preliminary experiment was performed using several dilutions (pure, 1:10, 1:100, 1:1,000) of DNA extracted from samples in which no *Gambierdiscus* sp. had previously been detected with light microscopy (2020-ME-886, 2020-ME-906, 2020-ME-946 in Table 1), but other microalgae were present (Table S2). Results

448 demonstrate that the presence of other genera of microalgae did not give current intensity
449 values higher than the limit of detection (LOD). The amount of *Gambierdiscus* spp. cells, if any,
450 was below the LOD for both *G. australes* and *G. excentricus*. Subsequently, 10^2, 10^3 and 10^4
451 *G. australes* cells were spiked into those field samples. Samples spiked with *G. excen*tricus cells
452 were prepared in a similar manner. DNA was again extracted with the TerraLyzer and MBs
453 protocol and PCR amplification was performed with several dilutions of the extracted DNA (pure,
454 1:10, 1:100, 1:1,000). Results were very similar to those obtained in the construction of the
455 calibration curves, but at 1:1,000 DNA dilutions, indicating that the field sample matrix affects
456 the detection of the target species, in agreement with that observed by Nishimura et al. (2016).

457 Subsequently, DNA was extracted from 9 field samples from Majorca in which *Gambierdiscus*
458 spp. had previously been detected with light microscopy, using the TerraLyzer and MBs protocol
459 (Table 1). DNA was diluted 1:1,000, multiplex PCR was performed, and the amplified products
460 were analyzed with the dual biosensor. Cell abundancies were estimated using the calibration
461 curves of cell dilutions. Results showed an overall higher estimated abundance of *G. australes*
462 cells rather than *G. excentricus* (Table 1), with the exception of sample 2020-ME-934 in which
463 *G. australes* was not detected. Furthermore, *G. excentricus* was not detected in sample 2020-
464 ME-986. In general (6 out of 9 samples), the cell abundances estimated with our strategy are
465 lower than the ones obtained with light microscopy (apart from sample 2020-ME-990, 2020-ME-
466 994 and 2020-ME-1034, in which the difference in cell abundance estimation does not go over
467 one order of magnitude), similar to that reported by Vandersea et al. (2012). This may be
468 attributable to the rDNA copy number of the field samples cells differing with that obtained in
469 cultured cells, and this can result in an over/under estimation of the real cell abundances in a
470 sample (Galluzzi et al., 2010; Andree et al. 2011). Additionally, it should be taken into
471 consideration that other species may also be present in the samples and their identification
472 based on morphological features by light microscopy is almost impossible due to the similarities
473 among species of this genus (Litaker et al., 2009). Recently, another biosensor for the detection

474 of different toxin-producing microalgae, including *G. australes* and *G. excentricus,* was
475 developed (Medlin et al., 2020), where they used an approach similar to our system with
476 synthetic DNA or RNA of *G. australes* and *G. excentricus.* The LOD achieved by Medlin and
477 coworkers was close to 1 pM of RNA, but they did not provide a corresponding quantification of
478 cell abundance. Nevertheless, for the other dinoflagellates targeted in their study, the number
479 of cells corresponding to 1 pM of RNA ranged from 10 to 444 cultivated cells, so it is probable
480 that the LOD for *Gambierdiscus* species is in that range, and thus, similar to our results. While
481 the technique presented by Medlin and coworkers is faster, as there is no PCR step, the analysis
482 of genomic DNA/RNA or the screening of field samples was not demonstrated. Additionally, RNA
483 has a highly labile nature, thus detecting it from fixed field samples cells can be problematic
484 (Loukas, et al., 2017). Therefore, despite its limitations, the strategy developed by our group can
485 be considered as a successful step towards practical application in the field, with the developed
486 biosensor allowing the simultaneous discrimination between *G. australes* and *G. excentricus,*
487 both of which are known toxin-producing microalgae species, making the tool suitable for
488 monitoring and research programs. Moreover, *Gambierdiscus* cell abundances in field samples
489 can reach more than 1,000,000 cells per g wet weight algae (Chinain et al., 1999; Litaker et al.,
490 2010; Vandersea et al., 2012), but CTX production has also been detected at very low cell
491 abundances (80.4 ± 56.9 cells per g *Dictyota*, Liefer et al. (2021)). Therefore, the ability to detect
492 low *Gambierdiscus* cell abundances in field samples is of utmost importance to provide timely
493 warnings of possible ciguatera outbreaks, thus enabling informed management decisions.

494 **3.4. First report of *Gambierdiscus excentricus* in Balearic Islands**

495 To date only one species of *Gambierdiscus*, *G. australes*, has been described in the Balearic
496 archipelago (Tudó et al., 2018, Tudó et al., 2020a). Our results obtained from the screening of
497 field samples with the developed biosensor revealed the presence of DNA belonging to
498 *G. excentricus*. Therefore, to have a further confirmation, several single cells were isolated from

499 field samples, and the DNA was extracted and sequenced. Results showed that, among the
500 analyzed cells, 5 belonged to *G. excentricus* species (2 from Cala Galiota, 2 from Platja Canyamel
501 and 1 from Camp de Mar, see Figure 6). As significant as this discovery might seem, it is not
502 entirely surprising since *Gambierdiscus* species have been found in cohabitation in several
503 studies of other locations (Nishimura et al., 2016; Vandersea et al., 2012; Tester et al., 2020;
504 Tudó et al., 2020b). Particularly, in the Canary Islands *G. excentricus* is usually accompanied by
505 the presence of *G. australes* (Tudó et al., 2020b). Nevertheless, *G. excentricus* was the first
506 species identified and described in the Canary Islands (Fraga et al., 2011), and only some years
507 later, *G. australes* was identified in field samples together with the description of another new
508 species, *G. silvae* (Fraga and Rodriguez, 2014). Something similar happened in the
509 Mediterranean, where the presence of *Gambierdiscus* was recorded for the first time in Crete
510 (Aligizaki and Nicolaidis, 2008) (the species was not assigned then, even if the authors stated
511 that it was a "*G. toxicus* type"). Later on, G*. carolinianus* (Holland et al., 2013), *G.* cf. *belizeanus*
512 and *G. silvae* (Aligizaki et al., 2018) were found and identified again in Crete waters. In 2018,
513 Tudó and coworkers reported the presence of *G. australes* in the Balearic Islands (Tudó et al,
514 2018; Tudó et al., 2020a), and the detection of another species in Majorcan waters was
515 somewhat expected. The reason behind the delay in detecting different species is still unclear.
516 It can either be due to an increase in the monitoring effort, which can cause a perceived increase
517 in the reports of *Gambierdiscus* spp. outside of their endemic area (as Hallegraeff et al. (2021)
518 demonstrate for the perceived global increase in algal blooms), or to the true recent
519 introduction of *Gambierdiscus* spp. In this last scenario, coastal ship traffic could play an active
520 role as a vector of introduction, as this has already been found to be responsible for the
521 transport of various genera of harmful dinoflagellates (Butron et al., 2011; Roy et al. 2012).
522 Another hypothesis could involve the transport through plastics or other types of substrates.

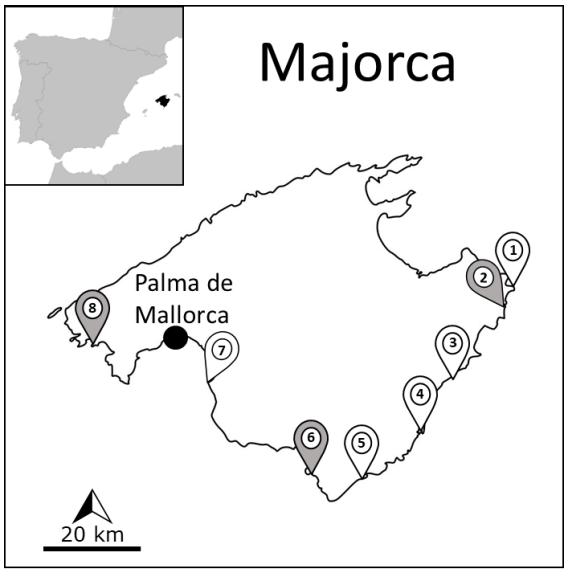

Figure 6. Sampling points of Majorca. (1) Cala Gat, (2) Platja Canyamel, (3) Cala Anguila, (4) Portocolom, (5) Cala Llombards, (6) Cala Galiota, (7) Cala Mosques and (8) Camp de Mar. Grey color indicates the points from which *G. excentricus* single cells have been isolated and identified.

3.5. Ciguatoxin detection in field samples

Analysis with our previously developed immunosensor with the combined 3G8 and 10C9 capture antibodies revealed the presence of quantifiable CTX1B equivalents in 1 (2020-ME-970 from Cala Llombards) out of the 9 analyzed samples. Three samples (2020-ME-990 and 2020-ME-994 from Cala Galiota and 2020-ME-1034 from Cala Mosques) showed very low CTX1B equivalents, which were not quantifiable, since the values were above the LOD but below the LOQ. Recently, Liefer and coworkers (2021) suggested considering a cell toxin quota (pg CTX equiv. $cell^{-1}$) rather than cell abundances to investigate the presence of CTX, since in their studies CTX detection mostly occurred in the presence of low abundances of *Gambierdiscus* cells. Liefer et al. (2021) converted the mouse units (one mouse unit = 18 ng of CTX3C for Pacific samples and 72 ng of C-CTX-1 for Caribbean samples) of the mouse bioassay (MBA) data obtained in previous works to CTX toxin quotas (whose ranges were 0.03-1 (Bagnis et al., 1980), 0.05-1.35 (Bagnis et al., 1990), 0.09-3.60 (Chinain et al., 1999), 1.14-5.14 (McMillan et al., 1986), 0.23 (Holmes et al., 1994), 0.96-1.42 (Yasumoto et al., 1979) and 24 pg CTX equiv. $cell^{-1}$ (Withers, 1983)), in order to make the comparison among different studies easier to interpret. In recent years, due to the ethical controversy and the lack of specificity of the MBA, other tests have been used to detect CTX in

543 field samples such as the Radioligand Receptor Binding Assay (RBA) (Chinain et al., 2020; Darius
544 et al., 2007) (which results ranged respectively from 0.5-13.5 and 0.85-3.90 pg of CTX1B equiv.
545 $cell^{-1}$) and the *in vitro* neuroblastoma cell-based assay (Neuro2a) (Liefer et al., 2021; Pawlowiez
546 et al., 2013) (which results ranged respectively from 0-12.62 and 0.03 ± 0.004 pg of CTX1B equiv.
547 $cell^{-1}$). In our study, sample 2020-ME-970 showed 13.35 ± 0.5 pg CTX1B equiv. $cell^{-1}$, a value that
548 is comparable to the results obtained in the studies mentioned above, indicating that our rapid
549 and reliable strategy is suitable for the analysis of field samples. The CTX contents obtained in
550 the analysis of laboratory cultures are usually much lower than those obtained from field
551 samples. In fact, CTX contents in laboratory cultures of *G. australes* isolates from Majorca
552 analyzed with the Neuro2a assay ranged from 1.38 to 381 fg CTX1B equiv. $cell^{-1}$ (Tudó et al.,
553 2020a). Regarding *G. excentricus*, to date there are no studies regarding the toxicity of strains
554 from Majorca (or the Balearic Islands in general) since it has not yet been isolated and cultured.
555 However, the data available for cultured strains of this species from other regions presented a
556 CTX production comparable to that obtained in our study (0.47 pg CTX3C equiv. $cell^{-1}$ (Litaker et
557 al., 2017) and 1.43 pg CTX3C equiv. $cell^{-1}$ (Pisapia et al., 2017a)). Undoubtedly, laboratory
558 studies of cultured *Gambierdiscus* are essential to better understand the ecotoxicological
559 behavior of this toxin-producing genus, but these artificial systems cannot completely mimic the
560 complex interactions that occur in a natural system. It must be underlined that, even if the cell
561 toxin quota value obtained from the analysis of Balearic Island samples is comparable to the
562 ones obtained in the Great Caribbean region, it has to be considered as a preliminary result, and
563 further studies are definitely needed to better investigate the risk of a future ciguatera outbreak.

564 **4. Conclusion**

565 In this study, the development of the first dual biosensor for the simultaneous detection of
566 *G. australes* and *G. excentricus* in field samples is presented. Additionally, a protocol for the
567 rapid extraction of DNA, based on a portable bead beater and MBs, is developed and successfully

tested on microalgal cultures and field samples. Using the strategy presented in this work, it has been possible to detect *G. excentricus* in the Balearic Islands, the presence of which had not yet been reported. Therefore, the developed strategy could be implemented in monitoring systems to identify new areas of expansion of these two toxin-producing species, preventing the occurrence of a ciguatera intoxication event.

Furthermore, for the first time, an electrochemical immunosensor is exploited for the detection of CTXs in a field sample extract, and the results obtained are similar to those previously observed in the Great Caribbean Region. This result underlines the need for rapid and easy-to-use tools to monitor the Mediterranean Sea for CTXs in order to correctly manage potential ciguatera outbreaks.

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Supplementary material

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

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

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

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

ND: not detected