Harmful Algae 110 (2021) 102135

Contents lists available at ScienceDirect

Harmful Algae

journal homepage: www.elsevier.com/locate/hal

Nucleic acid lateral flow dipstick assay for the duplex detection of *Gambierdiscus australes* and *Gambierdiscus excentricus*

Iris Ginés [a], Greta Gaiani [b], Ankur Ruhela [a], Vasso Skouridou [a], Mònica Campàs [b], Lluis Masip [a,*]

[a] Departament d'Enginyeria Química, Universitat Rovira i Virgili, 26 Països Catalans, 43007 Tarragona, Spain
[b] IRTA, Ctra Poble Nou km 5.5, 43540 Sant Carles de la Ràpita, Spain

ARTICLE INFO

Editor Proof Rem Exec EMAN

Keywords:
Harmful microalgae
Gambierdiscus
Lateral flow dipstick
scCro DNA binding protein
Carbon nanoparticles

ABSTRACT

The proliferation of harmful microalgae endangers aquatic ecosystems and can have serious economic implications on a global level. Harmful microalgae and their associated toxins also pose a threat to human health since they can cause seafood-borne diseases such as ciguatera. Implementation of DNA-based molecular methods together with appropriate detection strategies in monitoring programs can support the efforts for effective prevention of potential outbreaks. A PCR-lateral flow assay (PCR-LFA) in dipstick format was developed in this work for the detection of two *Gambierdiscus* species, *G. australes* and *G. excentricus*, which are known to produce highly potent neurotoxins known as ciguatoxins and have been associated with ciguatera outbreaks. Duplex PCR amplification of genomic DNA from strains of these species utilizing species-specific ssDNA tailed primers and a common primer containing the binding sequence of scCro DNA binding protein resulted in the generation of hybrid ssDNA-dsDNA amplicons. These were captured on the dipsticks via hybridization with complementary probes and detected with a scCro/carbon nanoparticle (scCro/CNPs) conjugate. The two different test zones on the dipsticks allowed the discrimination of the two species and the assay exhibited high sensitivity, 6.3 pg/μL of genomic DNA from both *G. australes* and *G. excentricus*. The specificity of the approach was also demonstrated using genomic DNA from non-target *Gambierdiscus* species and other microalgae genera which did not produce any signals. The possibility to use cells directly for amplification instead of purified genomic DNA suggested the compatibility of the approach with field sample testing. Future work is required to further explore the potential use of the strategy for on-site analysis and its applicability to other toxic species.

1. Introduction

Ciguatera fish poisoning (CFP) is a highly common seafood-borne disease (Lewis, 2001) caused by the potent marine microalgal neurotoxins named ciguatoxins (CTXs) (Yasumoto, 2005). Ciguatera has become a global health concern due to the severe symptoms elicited after intoxication, including cardiovascular, gastrointestinal and neurological alterations which might last a few days but can also persist for longer periods of time (Friedman et al., 2017). Several species of the marine dinoflagellate genus named *Gambierdiscus* have been confirmed to produce CTXs (Caillaud et al., 2011; Litaker et al., 2017; Reverté et al., 2018). Ingestion of CTX-producing microalgae by herbivorous fish and subsequent bioaccumulation of CTXs facilitates their introduction into the food chain, which ultimately may reach humans. This genus of microalgae is epibenthic and endemic of tropical and subtropical waters such as the Pacific and Indian Ocean or the Caribbean Sea (Hamilton et al., 2002; Lewis 2001; Litaker et al., 2017). However, their presence has expanded to non-endemic areas. Recently, Tester et al. (2020) reviewed the global distribution of the genus during the last decade or so (2009 – 2018), underlying zones in which the amount and diversity of *Gambierdiscus* species is higher, such as the French Polynesia, Caribbean coasts and Canary Islands (Spain). In fact, several *Gambierdiscus* species have been reported during the last years in the Canary Islands (Fraga et al., 2011; Rodriguez et al., 2017), the Madeira archipelago (Portugal) (Kaufmann and Bohm-Beck, 2013) and the Mediterranean Sea (Aligizaki and Nikolaidis 2008; Tudó et al., 2018). *Gambierdiscus australes and G. excentricus* strains exhibiting high CTX-like toxicities have been recently found in the Canary Islands and are considered the dominant species associated with ciguatera outbreaks in this area (Rossignoli et al., 2020).

Monitoring the presence of these microalgae and the toxins they produce in the marine environment is essential for implementing

* Corresponding author.
E-mail address: lluis.masip@urv.cat (L. Masip).

https://doi.org/10.1016/j.hal.2021.102135
Received 19 September 2021; Received in revised form 2 November 2021; Accepted 8 November 2021
Available online 17 November 2021
1568-9883/© 2021 The Author(s). Published by Elsevier B.V. This is an open access article under the CC BY-NC-ND license (http://creativecommons.org/licenses/by-nc-nd/4.0/).

appropriate risk prevention strategies. A battery of methods has been developed for the direct detection of CTXs in fish and have been extensively reviewed elsewhere (Pasinzski et al., 2020; Reverté et al., 2014). These include animal and cell-based toxicity assays, biochemical assays employing receptors and antibodies, as well as liquid chromatography coupled with mass spectrometry. However, the complex chemical structures and scarce availability of pure CTXs, the low concentration at which they are encountered, and the complexity of fish matrices make the development of highly sensitive and specific assays for CTXs detection very challenging (Pasinzski et al., 2020; Reverté et al., 2014). Biosensors on the other hand, extensively used for the detection of various foodborne pathogens (Lazcka et al., 2007; Velusamy et al., 2010), can offer the required specificity and sensitivity when combined with highly specific biorecognition molecules. The recent generation of CTX specific monoclonal antibodies using synthetic CTX fragments (Tsumuraya et al., 2014) has indeed allowed the development of sensitive sandwich-type immunoassays and biosensors for CTX detection in fish (Leonardo et al., 2020) and microalgal samples (Gaiani et al., 2020; Tudó et al., 2020).

Detection of harmful microalgae like the CTX-producing *Gambierdiscus* species based on molecular methods has also been introduced over the last years to implement the monitoring and containment efforts of harmful algae blooms (Toldrà et al., 2020). These methods are considered as easier, faster and more specific alternatives to the traditional light microscopy. They rely on the amplification of genomic DNA from the target species using specific primers in combination with colorimetric, fluorescent or electrochemical detection techniques. Even though the gold standard for amplification is PCR, isothermal amplification has also been reported (Toldrà et al., 2020). Nevertheless, few reports can be found in the literature exploiting molecular methods for the identification of *Gambierdiscus* species (Vandersea et al., 2012; Nishimura et al., 2016; Lyu et al., 2017; Lozano-Duque et al., 2018; Pitz et al., 2021; Gaiani et al., 2021).

In this work, a nucleic acid lateral flow assay (LFA) in a dipstick format was developed for the duplex detection of two toxin-producing *Gambierdiscus* species, *G. australes* and *G. excentricus*. This is the first report of an LFA employed for the identification of *Gambierdiscus* species. It relies on PCR amplification of genomic DNA using specifically modified primers followed by detection with dipsticks. Two reverse primers were designed, each one modified with a distinct single-stranded DNA (ssDNA) tail to allow discrimination of the two species. The amplicons were captured on two separate test lines of the dipsticks via hybridization with capture probes complementary to these ssDNA tails. On the other hand, the common forward primer contained the binding sequence of scCro DNA binding protein to facilitate detection with scCro/carbon nanoparticles (scCro/CNPs) conjugate. Enzyme Linked Oligonucleotide Assay (ELONA) was used to demonstrate correct amplification of both targets before transferring the assay to its final LFA format. The performance of the approach in terms of sensitivity and specificity was evaluated. Genomic DNA from several microalgae genera, different combinations of genomic DNA from five *Gambierdiscus* and one *Fukuyoa* species, and DNA extracted and amplified directly from cells of the two target species, were used to test the potential applicability of the strategy on field samples.

2. Materials and methods

2.1. Materials

Phosphate buffered saline (PBS; 10 mM phosphate, 137 mM NaCl, 2.7 mM KCl, pH 7.4), DreamTaq DNA polymerase, neutravidin and neutravidin-coated microplate strip plates and Tween-20 were obtained from Fisher Scientific (Spain). The BioFX TMB Super Sensitive One Component HRP Microwell Substrate was from Surmodics (USA), the FF170HP nitrocellulose membrane from Cytiva (Spain), the C083 cellulose fiber absorbent pad and Empigen BB from Merck (Spain). The preparation of scCro was based on a previous report (Aktas et al., 2015) with some modifications as described in the Supplementary Data (Fig. S1). HRP-scCro and scCro/CNPs conjugates were prepared as previously described (Aktas et al., 2015; 2019). Primers and DNA probes were purchased from Biomers.net (Germany) and their sequences are shown in Table 1.

2.2. Genomic DNA from microalgae

The microalgae strains used in this work are shown in Table 2. Maintenance of the cultures and genomic DNA extraction from each strain were performed as in previous works (Gaiani et al., 2021). Briefly, cultures were maintained under a photon flux of 100 μmol photons m^{-2} s^{-1} with a 12:12-h light/dark regime and at 24 ± 1 °C. For DNA extraction, cell pellets were re-suspended in lysis buffer (1 M NaCl, 70 mM Tris, 30 mM EDTA, pH 8.6), 1:8 vol of 10% (w/v) DTAB and 1 vol of chloroform. Subsequently, cellular disruption was achieved adding zirconium beads (0.5 mm diameter) to the mixture and using a Bead Beater-8 (BioSpec, USA). Then, DNA was extracted from the aqueous phase using standard phenol/chloroform method. Precipitation of the DNA was achieved by the addition of 2 vol of absolute ethanol and 0.1 vol of 3 M sodium acetate (pH 8.0). Finally, DNA was rinsed with 70% (v/v) ethanol and then dissolved in 1:4 vol of molecular DNAse/RNAse-free water. The concentration and purity of the extracted DNA were evaluated using a NanoDrop 2000 spectrophotometer (Thermo Fisher Scientific, Spain) and it was stored at −20 °C until analysis. Each strain was analyzed individually or in mixtures.

2.3. PCR amplification of genomic DNA

The primers used for amplification of genomic DNA from *G. australes* and *G. excentricus* were designed within the D1-D3 region of the 28 S large subunit (LSU) ribosomal DNA (Gaiani et al., 2021) and are shown in Table 1. The strategy for duplex PCR amplification of the target *Gambierdiscus* species with the modified primers is illustrated in Fig. 1A. Each reverse primer was extended at its 5′ end with a distinct ssDNA tail to allow the discrimination of the two species. The common forward primer for the two species was extended at its 5′ end with the DNA binding site for the scCro DNA binding protein. For simplex PCR, 200 nM of each primer were used whereas duplex PCR was performed using 200 nM of *G. australes* reverse primer, 300 nM of *G. excentricus* and 500 nM of the common forward primer for both *G. australes* and *G. excentricus*. Purified genomic DNA from each species was added to the PCR reactions at final concentrations of 400 pg/μL down to 400 fg/μL. PCR was performed using an initial denaturation step for 3 min at 95 °C, 30 cycles of denaturation for 10 s at 95 °C, annealing for 10 s at 58 °C and extension of 10 s at 72 °C, and a final extension step for 5 min at 72 °C. Strains *G. australes* IRTA-SMM-13-11 and *G. excentricus* IRTA-SMM-17-407 were used in all experiments unless otherwise stated. The compatibility of the primers with other strains of these species was also tested (Fig. S2). The PCR reactions were analyzed by agarose gel (2.6% w/v agarose in Tris/borate/EDTA buffer) electrophoresis.

2.4. Enzyme linked oligonucleotide assay (ELONA) for colorimetric detection of PCR amplicons

Correct incorporation of the ssDNA tails and scCro DNA binding site to the *G. australes* and *G. excentricus* PCR amplicons was verified by a colorimetric ELONA. Several parameters of the assay were optimized as shown in the Supplementary Data (Fig. S3). For the ELONA, biotinylated capture probes (Table 1) specific for each species (50 μL of 50 nM in PBS with 0.05% (v/v) Tween-20 (PBST)) were immobilized on separate wells of neutravidin-coated microplates for 15 min at room temperature. After washing (3 × 300 μL of PBST), 1 μL of PCR reaction was mixed with 49 μL of PBS and added to the wells for a 30-min incubation step. After washing, the HRP-scCro conjugate was added (50 μL of 15 nM in PBS)

Table 1
Oligonucleotides used in this work. The scCro DNA binding site is in bold and the DNA tails are in italics.

Oligonucleotide	Sequence (5′ – 3′)
G. australes & *G. excentricus* Forward primer	**TATCACTTGCGGTGATA**TGCTGCATGYGGAGATTCTTTYYTKG
G. australes Reverse primer	*GTTTTCCCAGTCACGAC*-C3-ATGCATAACTCTTCATTGCCAGTAG
G. excentricus Reverse primer	*TCTACAGGCTCGTATATGTA*-C3-AGCTTGGGTCACAGTGCAACAGAG
G. australes Capture probe	GTCGTGACTGGGAAAACTTTTTTTTTTTTTTT-TEG-biotin
G. excentricus Capture probe	TACATATACGAGCCTGTAGATTTTTTTT TTTTTTT-TEG-biotin
NALFA control line probe	biotin-AGTCCGTGGTAGGGCAGGTTGGGGTGACTTTTTTTTT**TATCACCGCAAGTGATA***TTTT***TATCACTTGCGGTGATA**

Table 2
Microalgae strains used in this work.

Species	Strain	Origin	GenBank accession
Gambierdiscus australes	IRTA-SMM-13-09	Hierro Island, Spain	KY564322
	IRTA-SMM-13-11	Selvagem Grande Island, Portugal	KY564324
	IRTA-SMM-16-286	Lanzarote Island, Spain	MT119197
Gambierdiscus balechii	VGO920	Manado, Indonesia	KX268469
Gambierdiscus belizeanus	IRTA-SMM-17-421	Hierro Island, Spain	MT379471
Gambierdiscus caribaeus	IRTA-SMM-17-03	Hierro Island, Spain	MT119203
Gambierdiscus excentricus	IRTA-SMM-17-407	Gomera Island, Spain	MT119200
	IRTA-SMM-17-428	Gomera Island, Spain	MT119201
	VGO791	Tenerife, Spain	JF303066; JF303075
Fukuyoa paulensis	VGO1185	Ubatuba, Brazil	KM886379
	IRTA-SMM-17-211	Menorca Island, Spain	MT119205
Coolia monotis	IRTA-SMM-16-285	Formentera Island, Spain	MW328563
Ostreopsis cf. *ovata*	IRTA-SMM-16-133	Catalonia, Spain	MH790463
Prorocentrum lima	IRTA-SMM-17-47	Lanzarote Island, Spain	MW328564

and the plate was incubated for another 30 min. After a final washing step, 50 µL of TMB ELISA substrate were added and signal generation was stopped after 10 min with the addition of equal volume of 1 M H_2SO_4. The absorbance was finally recorded at 450 nm. Target genomic DNA calibration curves were constructed using 1/50 diluted PCR reactions performed with serially two-fold diluted genomic DNA from each species (final concentrations of 8 pg/µL down to 8 fg/µL). The absorbance data was fitted to four-parameter logistic model using the GraphPad Prism software and the limits of detection (LOD) were interpolated from the curves as the blank signals (ntc) plus three times their standard deviation (ntc + 3xSD_{ntc}). Four replicates were prepared for each concentration examined.

2.5. Preparation of the LFA dipsticks

Nitrocellulose FF170HP membrane (height 3 cm) was used to prepare the dipsticks. The control line was constructed at 1.8 cm from the bottom of the membrane and separated from the two test lines by 0.4 cm and 0.8 cm. To facilitate ssDNA immobilization, each biotinylated probe was mixed with neutravidin in PBS at final concentrations of 35 µM and 8.3 µM, respectively. The mixtures were incubated for 15 min at ambient temperature and then dispensed on the membrane using a Lateral Flow Reagent Dispenser (Gentaur, Belgium). *G. australes* and *G. excentricus* capture probes were used for the construction of the two test lines, whereas a hairpin probe forming the scCro dsDNA binding site was used for the control line (see Table 1 for sequences). The membranes were dried at room temperature for at least 2 h and then blocked for 30 min with 2% (w/v) skim milk and 0.1% (v/v) Empigen BB in 10 mM carbonate-bicarbonate buffer pH 9.4. The strips were assembled on backing cards with a 2 cm absorbent pad overlapping the nitrocellulose membrane by 2 mm to ensure correct wicking. Finally, the dipsticks were cut at a width of 4 mm using an Autokun Cutter (Hangzhou Autokun Technology, India), packaged in plastic pouches and stored at 4 °C until use.

2.6. Detection of the PCR amplicons with the LFA dipsticks

The PCR amplicons were detected on the dipsticks using scCro/CNPs conjugate for carbon black signal (Fig. 1B and 1C). The scCro/CNPs conjugate was prepared as previously described (Aktas et al., 2019). The dipsticks were dipped vertically in the wells of a microtiter plate containing 10 µL of PCR reaction, 1 µL of scCro/CNPs conjugate suspension (0.2% w/v) and 39 µL of PBS with 1% (w/v) skim milk and 0.1% (v/v) Tween-20. Black color signals were observed within 15 min and the dipsticks were dried and finally imaged by flatbed scanning. Each experiment was performed in triplicate for the construction of the calibration curves or at least in duplicate for other experiments. The volume of the PCR reaction used for analysis with the dipsticks was optimized beforehand as described in the Supplementary Data (Fig. S4).

2.7. Direct PCR amplification of G. australes and G. excentricus cells and detection with LFA dipsticks

The possibility of detecting the two *Gambierdiscus* species using directly cells instead of purified genomic DNA was also evaluated. Cell suspensions containing 40 cells/µL from each strain (*G. australes* IRTA-SMM-16-286 and *G. excentricus* VGO791) were prepared in milli-Q water and heated for 5 min at 95 °C to promote lysis and release of genomic DNA. Crude cell extracts (2 µL) from each species were then added directly, individually or in a mixture, to PCR reactions containing primers for both strains to a final volume of 20 µL. Purified genomic DNA (100 pg/µL) was used for PCR in parallel as a control. PCR amplification was performed as detailed in Section 2.3 but with an extended initial denaturation step of 10 min at 95° C instead of 5 min. Amplicons were finally detected with the LFA dipsticks as described in Section 2.6.

3. Results

3.1. Specificity of the primers and PCR amplicon detection by ELONA

The specificity of the primers was first evaluated with simplex PCR. DNA from each species was added to a PCR reaction containing only its corresponding primers, and after amplification, the reactions were analyzed by agarose gel electrophoresis. As shown in Fig. 2A, only the specific genomic DNA was amplified with its corresponding primers

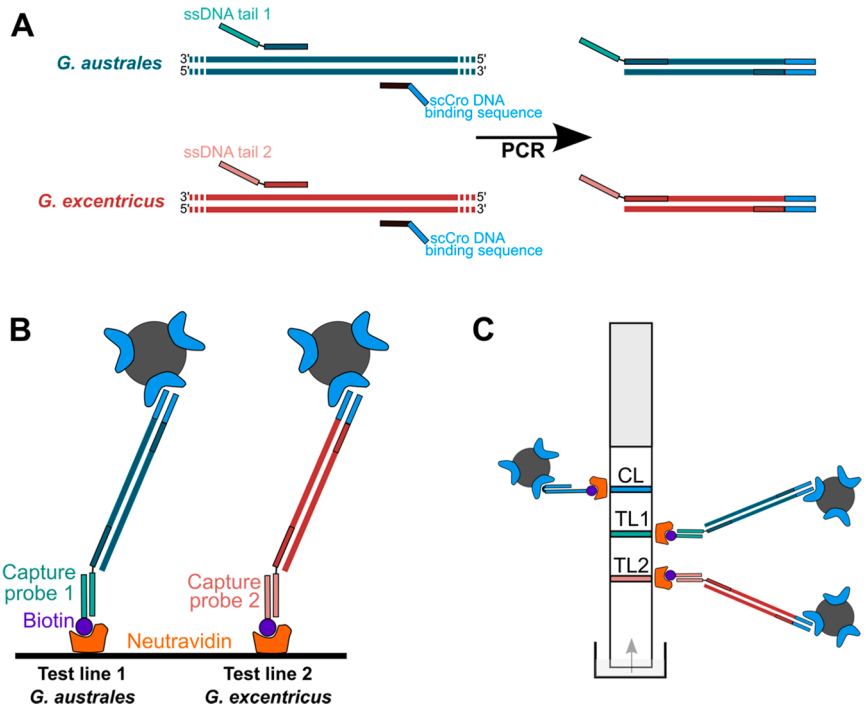

Fig. 1. Strategy for the detection of the target *Gambierdiscus* species with the PCR-LFA dipstick. (A) PCR amplification of *G. australes* and *G. excentricus* genomic DNA using modified primers. (B) PCR amplicon detection by LFA. Capture of the ssDNA-dsDNA hybrid amplicons on the dipsticks by complementary probes and detection with scCro/CNPs conjugate. (C) Design of the dipsticks. CL: control line; TL1: test line 1 for *G. australes*; TL2: test line 2 for *G. excentricus*.

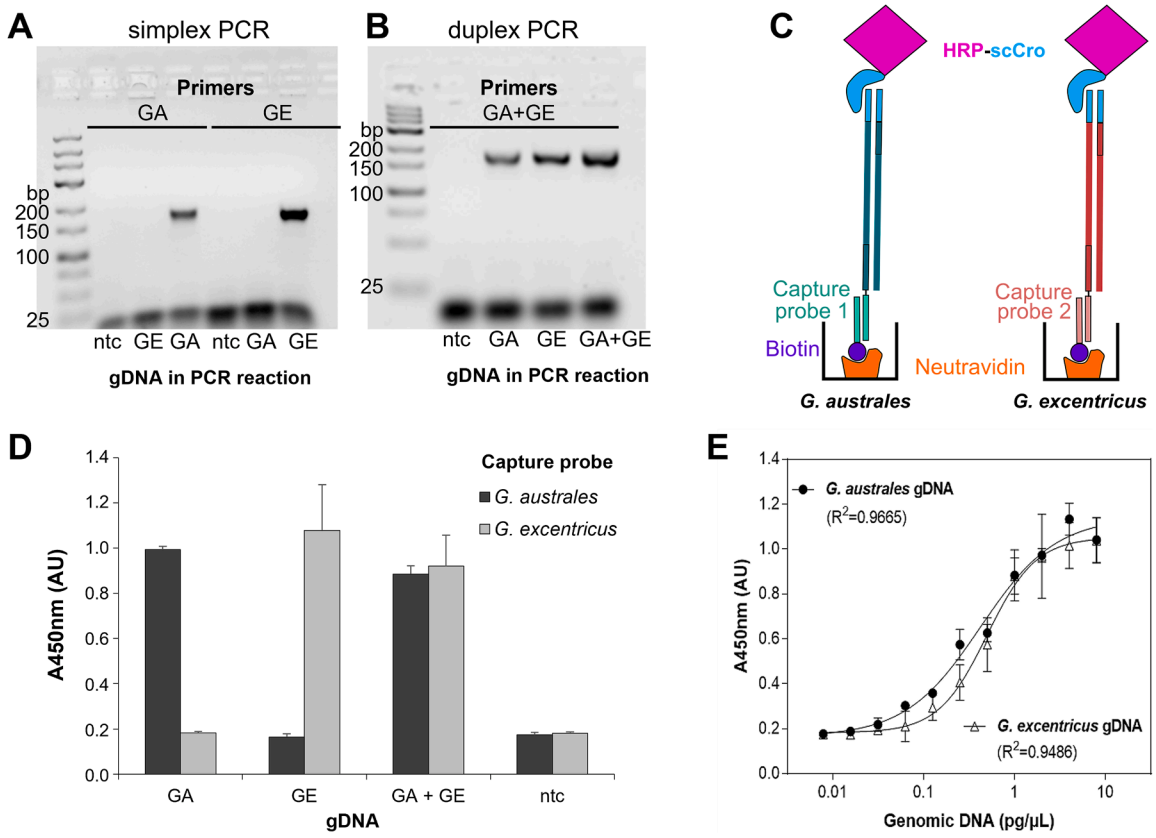

Fig. 2. PCR amplification of *G. australes* (GA) and *G. excentricus* (GE) genomic DNA (gDNA) and detection by ELONA. (A) Simplex PCR using primers for each species and (B) duplex PCR using primers for both strains. (C) Format of the ELONA for the detection of the two *Gambierdiscus* species. (D) Specificity of the duplex PCR-ELONA for the detection of the amplicons. (E) Calibration curves for the detection of genomic DNA from *G. australes* and *G. excentricus* by ELONA. ntc: no template control.

whereas no amplification was observed for the other one. Duplex PCR was then performed using a mixture of the two reverse primers and the common forward primer. Again, successful amplification for each strain was observed when only one of the targets was present (Fig. 2B). Since the expected size of the two amplicons is very similar (approximately 150 bp), it is not possible to differentiate the two amplicons in the duplex PCR reaction solely by gel electrophoresis. Therefore, ELONA was performed using capture probes specific to each target, which were complementary to the ssDNA tails at one end of the generated PCR amplicons for each species. For colorimetric detection, the HRP-scCro conjugate was used to bind the dsDNA binding site of scCro formed at the other end of the amplicons after incorporation of the specific sequence in the common forward primer of the two species (Fig. 2C). Correct amplification of each target genomic DNA in the duplex PCR reaction was verified when added individually or simultaneously whereas no signal was observed when the non-specific capture probe was used (Fig. 2D). The sensitivity of the ELONA was finally assessed using optimized conditions for PCR amplification and ELONA detection as described in the Supplementary Data (Fig. S3). Genomic DNA from each species was used for PCR amplification in a master mix containing primers for both species and representative agarose gels are shown in Fig. S5. The calibration curves are shown in Fig. 2E and the LODs were calculated to be 22.8 fg/μL and 52.3 fg/μL of genomic DNA for *G. australes* and *G. excentricus*, respectively. These correspond to 1.1 pg of genomic DNA for *G. australes* and 2.6 pg for *G. excentricus*, considering the 50 μL sample volume used for analysis.

3.2. Detection of G. australes and G. excentricus PCR amplicons by dipstick LFA

The approach used for the detection of the PCR amplicons by LFA and the design of the dipsticks, based on two test lines, one for each of the *Gambierdiscus* species, are illustrated in Fig. 1B and 1C, respectively. Duplex PCR reactions were performed using 100 pg/μL of genomic DNA from each species, separately or in a mixture, and the amplicons were detected with the dipsticks as described in Section 2.6. As it can be seen in Fig. 3, successful detection was achieved when genomic DNA from each strain was added separately or simultaneously, while no signal was observed in the absence of both targets.

3.3. Sensitivity of the dipsticks for G. australes and G. excentricus

To assess the sensitivity of the LFA dipsticks, PCR reactions were performed using a series of genomic DNA concentrations from each species (1.6 – 400 pg/μL) and a mixture of the primers for the two species. After amplification, the PCR reactions, previously optimized as shown in Fig. S4, were analyzed by agarose gel electrophoresis (Fig. S5). Finally, each PCR reaction was mixed with the scCro/CNPs conjugate and analyzed with the dipsticks. The visual LODs were determined as the minimum amounts of genomic DNA used for PCR amplification which resulted in visible test lines on the strips. As it can be seen in Fig. 4, the visual LODs were 6.3 pg/μL of genomic DNA for both *G. australes* and *G. excentricus*, corresponding to 63 pg of genomic DNA for each species considering that 10 μL of PCR reactions were analyzed with the dipsticks.

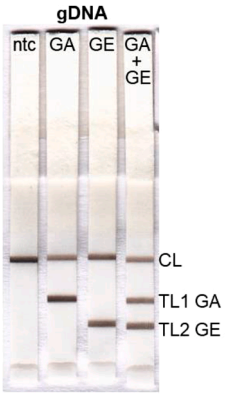

Fig. 3. LFA dipsticks for the detection of genomic DNA (gDNA) from *G. australes* (GA) and *G. excentricus* (GA) after duplex PCR amplification of genomic DNA (100 pg/μL) from each species. CL: control line; TL1 GA: test line 1 for *G. australes*; TL2 GE: test line 2 for *G. excentricus*.

3.4. Specificity of the PCR-LFA

The dipsticks were tested with PCR reactions performed with genomic DNA from different non-target microalgae species to evaluate the specificity of the developed approach. Each PCR reaction contained the three primers for *G. australes* and *G. excentricus* and 100 pg/μL of genomic DNA from each species. Only the PCR reactions containing genomic DNA from *G. australes* and *G. excentricus* resulted in amplification as seen after agarose gel electrophoresis (Fig. S6) and positive test lines signals on the dipsticks (Fig. 5). The genomic DNA from the other microalgae did not cross-react with the primers, the capture probes or the reporter scCro/CNPs conjugate used for detection, thus demonstrating the specificity of the approach.

3.5. Detection of genomic DNA from G. australes and G. excentricus in the presence of non-target Gambierdiscus or Fukuyoa species

The effect of various non-target *Gambierdiscus* and a *Fukuyoa* species on the detection of genomic DNA from *G. australes* and *G. excentricus* was evaluated next. Mixtures containing equal concentrations of genomic DNA from each of the *Gambierdiscus* and *Fukuyoa* species (Fig. 6) were thus prepared and used for PCR amplification with the *G. australes* and *G. excentricus* primers. Analysis of the PCR reactions by agarose gel electrophoresis revealed successful amplification only in mixtures containing genomic DNA either from *G. australes* or *G. excentricus* or both (Fig. S7). Likewise, amplicons were detected as black lines on the dipsticks when either one or both target *Gambierdiscus* species were present (Fig. 6). The presence of the non-target *Gambierdiscus* or the *Fukuyoa* species included in this study did not interfere with the detection of the two target species since the intensity of the test lines was similar in all cases regardless of the composition of the DNA mixtures.

3.6. Detection of G. australes and G. excentricus cells

The possibility to detect the two target *Gambierdiscus* species with the PCR-LFA approach developed in this work using directly cells was finally evaluated. To this end, cell suspensions from each species were heated briefly and the crude cell lysates were used directly for PCR amplification instead of purified genomic DNA. PCR amplicons were detected by agarose gel electrophoresis for both species when used individually or in a mixture (Fig. S8). Similar amplification efficiency of the duplex reaction was observed when using cells or 100 pg/μL of purified genomic DNA from each species. The amplicons were finally detected with the LFA dipsticks (Fig. 7). However, the intensity of the test lines of the duplex reactions using crude cell extracts was slightly lower compared to when purified genomic DNA from each strain was used for amplification.

4. Discussion

The main objective of this work was to detect two toxin-producing *Gambierdiscus* species, *G. australes* and *G. excentricus*, associated with ciguatera outbreaks using PCR combined with lateral flow dipsticks. The spread of ciguatoxin-producing microalgae of the *Gambierdiscus* and *Fukuyoa* genera in non-endemic areas has increased the demand for fast and reliable detection methods to monitor these microalgae and prevent

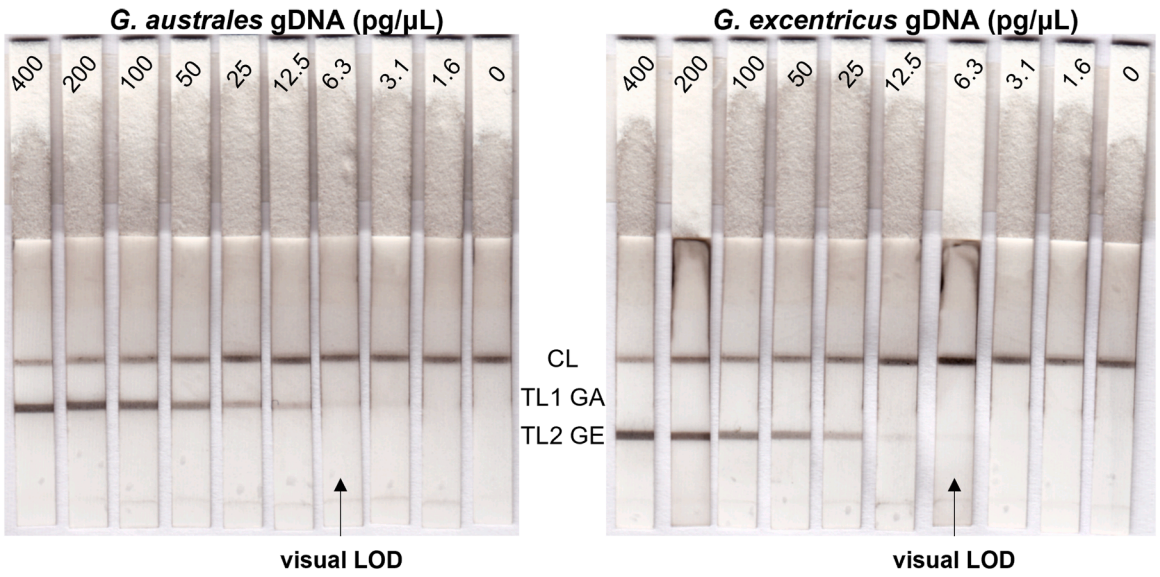

Fig. 4. Sensitivity of the PCR-LFA for each *Gambierdiscus* species. Serially two-fold diluted genomic DNA (gDNA) from each strain (400 to 1.6 pg/µL) was used in each PCR reaction containing primers for both species and added to the dipsticks for visual detection. CL: control line; TL1 GA: test line 1 for *G. australes*; TL2 GE: test line 2 for *G. excentricus*.

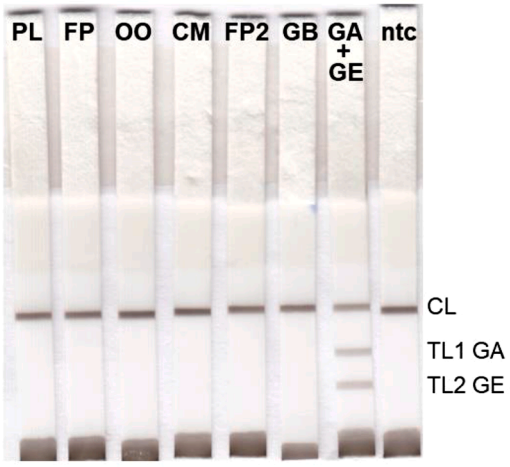

Fig. 5. Specificity of the PCR-LFA in the presence of various microalgae genera. PCR reactions containing the *G. australes* and *G. excentricus* primers were performed in the presence of 100 pg/µL of genomic DNA from each strain. PL: *P. lima* (IRTA-SMM-17-47); FP: *F. paulensis* (IRTA-SMM-17-211); OO: *O.* c.f. *ovata* (IRTA-SMM-16-133); CM: *C. monotis* (IRTA-SMM-16-285); FP2: *F. paulensis* (VGO1185); GB: *G. balechii* (VGO920); GA: *G. australes* (IRTA-SMM-13-11); GE: *G. excentricus* (IRTA-SMM-17-407); ntc: no template control; CL: control line; TL1 GA: test line 1 for *G. australes*; TL2 GE: test line 2 for *G. excentricus*.

future outbreaks. Hence, a lot of effort has been invested over the last years into moving from laboratory-based techniques, which are inherently slow and equipment-dependent, to decentralized, user-friendly platforms such as biosensors which are suitable for on-site detection (McPartlin et al., 2017). The sensitivity and specificity provided by nucleic acid amplification tests has allowed the implementation of molecular methods as promising tools for the detection of microalgae (Ebenezer et al., 2012; Medlin and Orozco, 2017). There is a plethora of reports on the detection of microalgae nucleic acids using both PCR and isothermal amplification (Toldrà et al., 2020).

Studies on the detection of ciguatoxin-producing microalgae based on molecular methods are scarce though. The first one reported the development of a qPCR assay for the simultaneous detection of several *Gambierdiscus* species with an LOD of 10 cells (Vandersea et al., 2012). In another qPCR-based study, identification and enumeration of four *Gambierdiscus* species was demonstrated with LODs of 10 gene copies (Nishimura et al., 2016). Alternatively, restriction fragment length polymorphism (RFLP) was used to identify *Gambierdiscus* and *Fukuyoa* species in field samples (Lyu et al., 2017; Lozano-Duque et al., 2018). In addition to these methods, species-specific fluorescence in situ hybridization (FISH) probes were also designed for the multiplex detection of several *Gambierdiscus* species in another report (Pitz et al., 2021). Finally, isothermal recombinase polymerase amplification in combination with a sandwich hybridization assay was recently developed for the detection of single cells from *Gambierdiscus* and *Fukuyoa* strains (Gaiani et al., 2021). Most of these studies targeted the detection of *G. australes* strains (Nishimura et al., 2012; Lyu et al., 2017, Lozano-Duque et al., 2018; Pitz et al., 2021, Gaiani et al., 2021), among other species, however only two of them focused also on *G. excentricus* (Lyu et al., 2017; Gaiani et al., 2021).

The combination of molecular methods for target DNA amplification with a sensitive detection strategy that is also simple, cost-effective and compatible with field testing would be ideal for monitoring the presence of toxic algae species in the marine environment. LFAs can potentially serve this purpose. The low cost and easy operation of these simple devices have encouraged their use for the detection of a wide variety of target analytes such as proteins, nucleic acids, toxins, drugs and others even in complex samples, and are especially suitable for on-site testing (Bahadir and Sezgintürk, 2016). For nucleic acid targets, detection of DNA amplicons generated by PCR or isothermal amplification is generally achieved using different labels (Bahadir and Sezgintürk, 2016; Zheng et al., 2021). Gold nanoparticles have been the mainstay in nucleic acid LFAs (Aveyard et al., 2007; Jauset-Rubio et al., 2016), mainly for qualitative or semiquantitative detection. Other labels have also been reported, such as CNPs (Noguera et al., 2011; Aktas et al., 2019; El-Tholoth et al., 2019), fluorescent nanoparticles (Takalkar et al., 2017), colored latex beads (Mao et al., 2013), enzymes (Aktas et al., 2019) or enzymes combined with nanoparticles (He et al., 2011; Aktas et al., 2019). Label materials such as quantum dots, upconversion nanoparticles, NIR dye-doped beads, SERS tags and semiconductor polymer dots were further developed to improve sensitivity and achieve quantitative analysis when combined with specific reader devices (Gui et al., 2014; Liu et al., 2021). Magnetic nanoparticles are also extremely

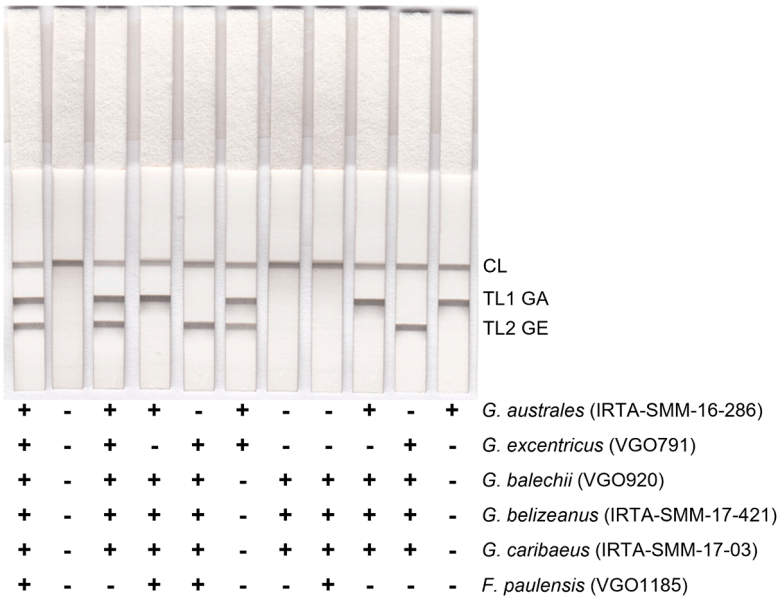

Fig. 6. Specificity of the PCR-LFA in the presence of non-target *Gambierdiscus* and *Fukuyoa* species. Each combination used for PCR amplification contained 400 pg/µL genomic DNA from each strain. CL: control line; TL1 GA: test line 1 for *G. australes*; TL2 GE: test line 2 for *G. excentricus*.

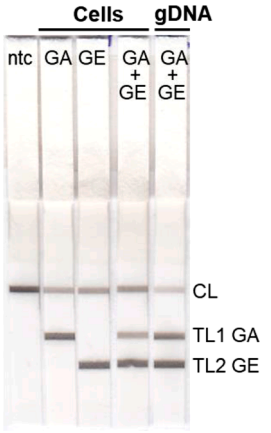

Fig. 7. Detection of *G. australes* (GA) and *G. excentricus* (GE) cells. Each PCR reaction contained 4 cells/µL while 100 pg/µL of purified gDNA (gDNA) from each species was used in parallel as a control. ntc: no template control; CL: control line; TL1 GA: test line 1 for *G. australes*; TL2 GE: test line 2 for *G. excentricus*.

useful as labels because of their dual magnetic and optical properties (Liu et al., 2021; Yan et al., 2019). On the other hand, CNPs are particularly attractive because of their low cost, high stability, easy modification and high signal-to-noise ratio (Posthuma-Trumpie et al., 2012; Zheng et al., 2021). CNPs were previously shown to provide enhanced sensitivity in LFAs when compared to gold nanoparticles, silver-enhanced gold nanoparticles and blue latex beads (Linares et al., 2012). Generic lateral flow immunoassay strips based on CNPs are in fact commercially available by Abingdon Health (UK) for the detection of double-labeled (biotin/fluorophore) DNA amplicons. CNPs were chosen as the colorimetric label for this work as well. Even though CNPs are not typically used for quantitative analysis, the main advantage they provide compared to the more sophisticated labels mentioned above is the simplicity of use and facile interpretation of the visual results without the need of a specific reader when a yes/no response is appropriate for the target analyte.

The primers used for PCR amplification of DNA from the two target toxic microalgal *Gambierdiscus* species were based on a previous work (Gaiani et al., 2021). They were designed within the large subunit of ribosomal DNA (rDNA) which provides the necessary sequence variability to allow specific amplification of the target strain and high copy number for increased sensitivity. The same region of rDNA was also targeted in previous studies with regards to the detection of several *Gambierdiscus* species including *G. australes* and *G. excentricus* (Lyu et al., 2017; Lozano-Duque et al., 2018; Pitz et al., 2021). To facilitate capture of the amplicons on the dipsticks, each reverse primer was modified at its 5′ end with a ssDNA sequence (tail) separated from the rest of the sequence with a carbon-based spacer. For detection, the common forward primer for both species was extended with the specific DNA sequence recognized by scCro DNA binding protein. In this way, the generated hybrid ssDNA-dsDNA amplicons were tagged at one end with distinct ssDNA tails to allow discrimination of the two species on the two individual test lines comprised of complementary ssDNA probes. The other end of the amplicons contained the dsDNA binding site for scCro DNA binding protein to facilitate detection with the scCro/CNPs conjugate. The performance of HRP-scCro, scCro/CNPs and HRP-scCro/CNPs conjugates regarding the detection of *Escherichia coli* bacterial DNA by PCR-LFA was compared in a previous report (Aktas et al., 2019). Even though all three approaches exhibited very similar sensitivity, the use of scCro/CNPs provided the fastest (less than 20 min) and more reliable detection with no false positives, and this conjugate was chosen for this work as well. The use of tailed primers for amplification combined with LFAs for detection via hybridization with complementary probes has also been reported previously (Jauset-Rubio et al., 2016, 2018). Isothermal recombinase polymerase amplification (RPA) was used in these reports for amplification of target DNA from the bacterial biowarfare agents *Yersinia pestis* and *Francisella tularensis*, whereas gold nanoparticles served as the colorimetric reporter achieving high sensitivity (< 1 pg of genomic DNA). However, purified amplicons were used in these studies for detection, resulting in extended time-to-result and requirement of additional material or infrastructure. In the current work, the assay was simplified by solely diluting the PCR reactions prior to analysis. Since scCro specifically binds only to dsDNA, no interference from unreacted primers was expected thus eliminating the need for amplicon purification.

For the initial validation of the primer design and to ensure correct incorporation of the tags at the two ends of the generated amplicons (ssDNA tail and dsDNA binding site for scCro), an ELONA was developed. Since the expected length of the two amplicons was similar, it was not possible to discriminate them by agarose gel electrophoresis. Therefore, the same capture probes later used for the preparation of the LFA dipsticks were employed for the ELONA which allowed the validation of the specificity of the primers and of the overall strategy for amplification and detection. Similar enzyme-based DNA hybridization assays have been developed before for other toxic algae like *Karlodinium* (Toldrà et al., 2018) and *Ostreopsis* (Toldrà et al., 2019). In these studies, ssDNA tails were used for capturing the amplicons with complementary ssDNA probes whereas a ssDNA-HRP conjugate was utilized for detection. The LODs reported for example for *Ostreopsis* (50 – 70 pg of gDNA) were more than 10-fold higher compared to the ones achieved in this work for the two *Gambierdiscus* species (1.1 – 2.6 pg). However, it is important to note that these LODs are expressed in genomic DNA amount. In *Gambierdiscus* species, the rDNA copy number per cell has been reported to be as high as 4560–21,500 (Vandersea et al., 2012) or even up to 3197,000 (Nishimura et al., 2016), probably due to the large cell size and high amount of genomic DNA. This means that the genomic DNA amount detected in this work for *Gambierdiscus* species may have a higher number of copies that of *Ostreopsis* species. Additionally, the rDNA copy number can vary between genus, species, strains, geographic origins, and even cell growth phases and thus sample harvesting times (Gaiani et al., 2021). Also, in this work, detection of the amplicons was achieved using an HRP-scCro conjugate. Because of the dimeric nature of scCro and the imperfect dyad symmetry of its specific DNA binding sequence, stoichiometries of scCro/DNA binding sites can range from 1:1 to 2:1, potentially allowing more than one HRP-scCro conjugate to associate with each amplicon and resulting in increased sensitivity. This target-independent HRP-scCro conjugate can be very useful for colorimetric assays and it could be potentially used in any assay provided that the dsDNA binding site for scCro is incorporated in the target amplicon.

As mentioned earlier, there are very few reports in the literature on the detection of harmful algae using DNA amplification combined with visual LFAs and they are summarized in Table S1. In these reports, amplification of the target microalgae species was performed with PCR, loop-mediated isothermal amplification (LAMP), RPA and rolling circle amplification (RCA), exploiting one or two labeled primers. Commercially available generic dipsticks were used for detection of the generated amplicons via hybridization or by sandwich formation with capture/reporter probes in almost all the reports. In the case of hybridization, only one labeled (biotinylated) primer was required for amplification, and the biotinylated amplicon was hybridized with a ssDNA probe labeled with a fluorophore, followed by capture on the test line using a biotin-binding biomolecule and detection with an AuNPs/anti-fluorophore IgG conjugate. On the other hand, for sandwich detection, two labeled primers were used and the biotin-fluorophore double-labeled amplicon was captured on the test line and detected again with an AuNPs/anti-fluorophore IgG conjugate. The generic LFA dipsticks used were obtained from two different companies (Ustar Biotech Ltd, China and Milenia Biotec GmbH, Germany) and were used directly for the detection of the amplicons. The reported LODs were in the range of 0.34 pg/μL – 10 ng/μL, with few exceptions reporting $\leq$ 1 fg/μL when variations of RCA were employed for the amplification step. The sensitivity achieved in this work for the two *Gambierdiscus* species with the PCR-LFA dipsticks (6.3 pg/μL) was in line with the previous reports on the other microalgal species where an isothermal amplification method was used. Two reports were found in the literature showing the combination of PCR with LFAs for the detection of toxic microalgae. In these reports, ssDNA tagged primers were used for PCR amplification and the detection of the ssDNA double-tagged DNA amplicons was performed with LFA dipsticks via hybridization with complementary DNA probes (Nagai et al., 2016; Chen et al., 2020). In one of these works, several *Alexandrium* species were detected using commercially available universal dipsticks from Kaneka Co. (Japan) and detection limits of $<$ 0.1 – 10 pg were achieved (Nagai et al., 2016). In the second work, homemade dipsticks were prepared for the detection of *Karlodinium veneficum* exhibiting a sensitivity of 91.3 pg/μL (Chen et al., 2020) in comparison to the 63 pg (6.3 pg/μL) of genomic DNA from the two *Gambierdiscus* species demonstrated in this work also exploiting homemade LFA dipsticks.

The PCR-LFA strategy developed in this work was not only sensitive but also very specific. DNA from other microalgae genera potentially present in the same habitat as the two target *Gambierdiscus* species like *Prorocentrum lima, Ostreopsis* cf. *ovata* and *Coolia monotis* as well as non-target *Gambierdiscus* (*G. balechii, G. belizeanus, G. caribaeus*) or *Fukuyoa* (*F. paulensis*) species did not produce positive signals on the dipsticks. This is not only due to the high specificity of the primers but also the specific DNA binding properties of the scCro reporter protein. The analysis of mixtures containing equal concentrations of genomic DNA from non-target *Gambierdiscus* species further demonstrated the specificity of the approach since positive signals on the dipsticks were obtained only when the target species were present. These samples were analyzed in an effort to mimic field samples potentially containing more than one microalgae species. These findings further highlight the importance of the strategy shown herein which is highly specific and does not produce any false positive signals. Research efforts should focus on the validation of the PCR-LFA system for the analysis of field samples and compared to light microscopy. Nevertheless, the results obtained in this work demonstrate that the strategy should be implementable in situ monitoring and research activities, since portable PCR devices can be brought to the field, whereas LFA is easy and fast to perform, and results are easy to interpret by simple visual inspection.

5. Conclusions

In this work, a PCR-LFA dipstick was developed for the simultaneous detection of the toxin-producing *G. australes* and *G. excentricus* species. Duplex PCR amplification of genomic DNA from these species with specifically modified primers allowed facile visual detection with the dipsticks employing a scCro/CNPs conjugate generating black colored line signals. The strategy was highly sensitive whereas the presence of non-target *Gambierdiscus* species or other microalgae genera potentially co-habiting in the same waters did not interfere with the assay. The approach combines the specificity and sensitivity provided by PCR amplification with the simplicity, low cost and on-site testing compatibility of LFAs. Preliminary results demonstrating DNA amplification directly from cells instead of purified genomic DNA and subsequent detection with the LFA dipsticks suggests that the approach could be applied to field sample testing, further highlighting its potential to be implemented in monitoring programs.

Declaration of Competing Interest

The authors declare that there is no conflict of interest.

Acknowledgments

This work was supported financially by the CIGUASENSING (BIO2017-87946-C2-1-R and BIO2017-87946-C2-2-R) project funded by the Ministerio de Ciencia e Innovación (MICINN), the Agencia Estatal de Investigación (AEI) and the Fondo Europeo de Desarrollo Regional (FEDER). The authors thank Dr. Aart van Amerongen (Wageningen University and Research, The Netherlands) for the carbon nanoparticles.

Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.hal.2021.102135.

References

Aktas, G.B., Skouridou, V., Masip, L., 2015. Novel signal amplification approach for HRP-based colorimetric genosensors using DNA binding protein tags. Biosens. Bioelectron. 74, 1005–1010.

Aktas, G.B., Wichers, J.H., Skouridou, V., van Amerongen, A., Masip, L., 2019. Nucleic acid lateral flow assays using a conjugate of a DNA binding protein and carbon nanoparticles. Microchim. Acta 186, 426.

Aligizaki, K., Nikolaidis, G., 2008. Morphological identification of two tropical dinoflagellates of the genera *Gambierdiscus* and *Sinophysis* in the Mediterranean Sea. J. Biol. Res.-Thessalon. 9, 75–82.

Aveyard, J., Mehrabi, M., Cossins, A., Braven, H., Wilson, R., 2007. One step visual detection of PCR products with gold nanoparticles and a nucleic acid lateral flow (NALF) device. Chem. Commun. 4251–4253.

Bahadir, E.B., Sezgintürk, M.K., 2016. Lateral flow assays: principles, designs and labels. Trac. Trends Anal. Chem. 82, 286–306.

Caillaud, A., de la Iglesia, P., Barber, E., Eixarch, H., Mohammad-Noor, N., Yasumoto, T., Diogéne, J., 2011. Monitoring of dissolved ciguatoxin and maitotoxin using solid-phase adsorption toxin tracking devices: application to *Gambierdiscus pacificus* in culture. Harmful Algae 10, 433–446.

Chen, Q., Zhang, C., Liu, F., Ma, H., Wang, Y., Chen, G., 2020. Easy detection of *Karlodinium veneficum* using PCR-based dot chromatography strip. Harmful Algae 99, 101908.

Ebenezer, V., Medlin, L.K., Ki, J.S., 2012. Molecular detection, quantification, and diversity evaluation of microalgae. Mar. Biotechnol. 14, 129–142.

El-Tholoth, M., Branavan, M., Naveenathayalan, A., Balachandran, W., 2019. Recombinase polymerase amplification-nucleic acid lateral flow immunoassays for Newcastle disease virus and infectious bronchitis virus detection. Mol. Biol. Rep. 46, 6391–6397.

Fraga, S., Rodríguez, F., Caillaud, A., Diogène, J., Raho, N., Zapata, M., 2011. *Gambierdiscus excentricus* sp. nov. (Dinophyceae), a benthic toxic dinoflagellate from the Canary Islands (NE Atlantic Ocean). Harmful Algae 11, 10–22.

Friedman, M., Fernandez, M., Backer, L., Dickey, R., Bernstein, J., Schrank, K., Kibler, S., Stephan, W., Gribble, M.O., Bienfang, P., Bowen, R.E., Degrasse, S., Quintana, H.A. F., Loeffler, C.R., Weisman, R., Blythe, D., Berdalet, E., Ayyar, R., Clarkson-Townsend, D., Swajian, K., Benner, R., Brewer, T., Flemming, L.E., 2017. An updated Review of Ciguatera Fish Poisoning: clinical, Epidemiological, Environmental, and Public Health Management. Mar. Drugs 15, 72.

Gaiani, G., Leonardo, S., Tudó, A., Toldrà, A., Rey, M., Andree, K.B., Tsumuraya, T., Hirama, M., Diogéne, J., O'Sullivan, C.K., Campàs, M., 2020. Rapid detection of ciguatoxins in *Gambierdiscus* and *Fukuyoa* with immunosensing tools. Ecotoxicol. Environ. Saf. 204, 111004.

Gaiani, G., Toldrà, A., Andree, K.B., Rey, M., Diogéne, J., Alcaraz, C., O'Sullivan, C.K., Campàs, M., 2021. Detection of *Gambierdiscus* and *Fukuyoa* single cells using recombinase polymerase amplification combined with a sandwich hybridization assay. J. Appl. Phycol. 33, 2273–2282.

Gui, C., Wang, K., Li, C., Dai, X., Cui, D., 2014. A CCD-based reader combined with CdS quantum dot-labeled lateral flow strips for ultrasensitive quantitative detection of CagA. Nanoscale Res. Lett. 9, 57.

Hamilton, B., Hurbungs, M., Vernoux, J.P., Jones, A., Lewis, R.J., 2002. Isolation and characterisation of Indian Ocean ciguatoxin. Toxicon 40, 685–693.

He, Y., Zhang, S., Zhang, X., Baloda, M., Gurung, A.S., Xu, H., Zhang, X., Liu, G., 2011. Ultrasensitive nucleic acid biosensor based on enzyme-gold nanoparticle dual label and lateral flow strip biosensor. Biosens. Bioelectron. 26, 2018–2024.

Jauset-Rubio, M., Svobodová, M., Mairal, T., McNeil, C., Keegan, N., Saeed, A., Abbas, M. N., El-Shahawi, M.S., Bashammakh, A.S., Alyoubi, A.O., O'Sullivan, C.K., 2016. Ultrasensitive, rapid and inexpensive detection of DNA using paper based lateral flow assay. Sci. Rep. 6, 37732.

Jauset-Rubio, M., Tomaso, H., El-Shahawi, M.S., Bashammakh, A.S., Al-Youbi, A.O., O'Sullivan, C.K., 2018. Duplex lateral flow assay for the simultaneous detection of Yersinia pestis and Francisella tularensis. Anal. Chem. 90, 12745–12751.

Kaufmann, M., Böhm-Beck, M., 2013. *Gambierdiscus* and related benthic dinoflagellates from Madeira archipelago (NE Atlantic). Harmful Algae News 47, 18–19.

Lazcka, O., Del Campo, F.J., Muñoz, F.X., 2007. Pathogen detection: a perspective of traditional methods and biosensors. Biosens. Bioelectron. 22, 1205–1217.

Leonardo, S., Gaiani, G., Tsumuraya, T., Hirama, M., Turquet, J., Sagristà, N., Rambla-Alegre, M., Caixach, J., Diogéne, J., O'Sullivan, C.K., Alcaraz, C., Campàs, M., 2020. Addressing the analytical challenges for the detection of ciguatoxins using an electrochemical biosensor. Anal. Chem. 92, 4858–4865.

Lewis, R.J., 2001. The changing face of ciguatera. Toxicon 39, 97–106.

Linares, E.M., Kubota, L.T., Michaelis, J., Thalhammer, S., 2012. Enhancement of the detection limit for lateral flow immunoassays: evaluation and comparison of bioconjugates. J. Immunol. Methods 375, 264–270.

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, e0185776.

Liu, X., Wu, W., Cui, D., Chen, X., Li, W., 2021. Functional micro-/nanomaterials for multiplexed biodetection. Adv. Mater. 33, 2004734.

Lozano-Duque, Y., Richlen, M.L., Smith, T.B., Anderson, D.M., Erdner, D.L., 2018. Development and validation of PCR-RFLP assay for identification of *Gambierdiscus* species in the Greater Caribbean region. J. Appl. Phycol. 30, 3529–3540.

Lyu, Y., Richlen, M.L., Sehein, T.R., Chinain, M., Adachi, M., Nishimura, T., Xu, Y., Parsons, M.L., Smith, T.B., Zheng, T., Anderson, D.M., 2017. LSU rDNA based RFLP assays for the routine identification of *Gambierdiscus* species. Harmful Algae 66, 20–28.

Mao, X., Wang, W., Du, T.E., 2013. Dry-reagent nucleic acid biosensor based on blue dye doped latex beads and lateral flow strip. Talanta 114, 248–253.

McPartlin, D.A., Loftus, J.H., Crawley, A.S., Silke, J., Murphy, C.S., O'Kennedy, R.J., 2017. Biosensors for the monitoring of harmful algal blooms. Curr. Opin. Biotechnol. 45, 164–169.

Medlin, L.K., Orozco, J., 2017. Molecular techniques for the detection of organisms in aquatic environments, with emphasis on harmful algal bloom species. Sensors 17, 1184.

Nagai, S., Miyamoto, S., Ino, K., Tajimi, S., Nishi, H., Tomono, J., 2016. Easy detection of multiple Alexandrium species using DNA chromatography chip. Harmful Algae 51, 97–106.

Nishimura, T., Hariganeya, N., Tawong, W., Sakanari, H., Yamaguchi, H., Adachi, M., 2016. Quantitative PCR assay for detection and enumeration of ciguatera-causing dinoflagellate *Gambierdiscus* spp. (Gonyaulacales) in coastal areasa of Japan. Harmful Algae 52, 11–22.

Noguera, P., Posthuma-Trumpie, G.A., van Tuil, M., van der Wal, F.J., de Boer, A., Moers, A.P.H.A., van Amerongen, A., 2011. Carbon nanoparticles in lateral flow methods to detect genes encoding virulence factors of Shiga toxin-producing Escherichia coli. Anal. Bioanal. Chem. 399, 831–838.

Pasinszki, T., Lako, J., Dennis, T., 2020. Advances in detecting ciguatoxins in fish. Toxins (Basel) 12, 494.

Pitz, K.J., Richlen, M.L., Fachon, E., Smith, T.B., Parsons, M.L., Anderson, D.M., 2021. Development of fluorescence in situ hybridization (FISH) probes to detect and enumerate *Gambierdiscus* species. Harmful Algae 101, 101914.

Posthuma-Trumpie, G.A., Wichers, J.H., Koets, M., Berendsen, L.B.J.M., van Amerongen, A., 2012. Amorphous carbon nanoparticles: a versatile label for rapid diagnostic (immuno)assays. Anal. Bioanal. Chem. 402, 593–600.

Reverté, L., Soliño, L., Carnicer, O., Diogéne, J., Campàs, M., 2014. Alternative methods for the detection of emerging marine toxins: biosensors, biochemical assays and cell-based assays. Mar. Drugs 12, 5719–5763.

Reverté, L., Toldrà, A., Andree, K.B., Fraga, S., de Falco, G., Campàs, M., Diogéne, J., 2018. Assessment of cytotoxicity in ten strains of *Gambierdiscus australes* from Macaronesian Islands by neuro-2a cell-based assays. J. Appl. Phycol. 30, 2447–2461.

Rodríguez, F., Fraga, S., Ramilo, I., Rial, P., Figueroa, R.I., Riobó, P., Bravo, I., 2017. Canary Islands (NE Atlantic) as a biodiversity 'hotspot' of *Gambierdiscus*: implications for future trends of ciguatera in the area. Harmful Algae 67, 131–143.

Rossignoli, A.E., Tudó, A., Bravo, I., Díaz, P.A., Diogène, J., Riobó, P., 2020. Toxicity characterisation of *Gambierdiscus* species from the Canary Islands. Toxins (Basel) 12, 134.

Takalkar, S., Baryeh, K., Liu, G., 2017. Fluorescent carbon nanoparticle-based lateral flow biosensor for ultrasensitive detection of DNA. Biosens. Bioelectron. 98, 147–154.

Global distribution of the genera Gambierdiscus and Fukuyoa Tester, P., Wickliffe, L., Jossart, J., Rhodes, L., Enevoldsen, H., Adachi, M., Nishimura, T., Rodriguez, F., Chinain, M., Litaker, W., 2020. Global distribution of the genera Gambierdiscus and Fukuyoa. In: Hess, P. (Ed.), Harmful Algae 2018 – from ecosystems to socioecosystems. Proceedings of the 18th Intl. Conf. on Harmful Algae. International Society for the Study of Harmful Algae, Nantes, pp. 138–143.

Toldrà, A., Jauset-Rubio, M., Andree, K.B., Fernández-Tejedor, M., Diogéne, J., Katakis, I., O'Sullivan, C.K., Campàs, M., 2018. Detection and quantification of the toxic marine microalgae *Karlodinium veneficum* and *Karlodinium armiger* using recombinase polymerase amplification and enzyme-linked oligonucleotide assay. Anal. Chim. Acta 1039, 140–148.

Toldrà, A., Alcaraz, C., Andree, K.B., Fernández-Tejedor, M., Diogéne, J., Katakis, I., O'Sullivan, C.K., Campàs, M., 2019. Colorimetric DNA-based assay for the specific detection and quantification of *Ostreopsis* cf. *ovata* and *Ostreopsis* cf. *siamensis* in the marine environment. Harmful Algae 84, 27–35.

Toldrà, A., O'Sullivan, C.K., Diogène, J., Campàs, M., 2020. Detecting harmful algal blooms with nucleic acid amplification-based biotechnological tools. Sci. Total Environ. 749, 141605.

Tsumuraya, T., Fujii, I., Hirama, M., 2014. Preparation of anti-ciguatoxin monoclonal antibodies using synthetic haptens: sandwich ELISA detection of ciguatoxins. J. AOAC Intl. 97, 373.

Tudó, A., Toldrà, A., Andree, K.B., Rey, M., Fernández-Tejedor, M., Campàs, M., Diogène, J., 2018. First report of Gambierdiscus in the Western Mediterranean Sea (Balearic Islands). Harmful Algae News 59, 22–23.

Tudó, A., Gaiani, G., Ray, Varela, M, Tsumuraya, T, Andree, K.B, Fernández-Tejedor, M, Campàs M., Diogéne, J., 2020. Further advances of *Gambierdiscus* species in the Canary Islands, with the first report of *Gambierdiscus belizeanus*. Toxins (Basel) 12, 692.

Vandersea, M.W., Kibler, S.R., Holland, W.C., Tester, P.A., Schultz, T.F., Faust, M.A., Holmes, M.J., Chinain, M., Litaker, R.W., 2012. Development of semi-quantitative PCR assays for the detection and enumeration of *Gambierdiscus* species (Gonyaulacales, Dinophyceae). J. Phycol. 48, 902–915.

Velusamy, V., Arshak, K., Korostynska, O., Oliwa, K., Adley, C., 2010. An overview of foodborne pathogen detection: in the perspective of biosensors. Biotechnol. Adv. 28, 232–254.
Yan, W., Wang, K., Xu, H., Huo, X., Jin, Q., Cui, D., 2019. Machine learning approach to enhance the performance of MNP-labeled lateral flow immunoassay. Nano-Micro Lett 11, 7.
Yasumoto, T., 2005. Chemistry, etiology, and food chain dynamics of marine toxins. Proc. Jpn. Acad. 81, 43–51.
Zheng, C., Wang, K., Zheng, W., Cheng, Y., Li, T., Cao, B., Jin, Q., Cui, D., 2021. Rapid development in lateral flow immunoassay for nucleic acid detection. Analyst 146, 1514–1528.