Detection and quantification of the toxic marine microalgae *Karlodinium veneficum* and *Karlodinium armiger* using recombinase polymerase amplification and enzyme-linked oligonucleotide assay

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Abstract

Karlodinium is a dinoflagellate responsible for fish-killing events worldwide. In Alfacs Bay (NW Mediterranean Sea), the presence of two *Karlodinium* species (*K. veneficum* and *K. armiger*) with different toxicities has been reported. This work presents a method that combines recombinase polymerase amplification (RPA) with an enzyme-linked oligonucleotide assay (ELONA) to identify, discriminate and quantify these two species. The system was characterised using synthetic DNA and genomic DNA, and the specificity was confirmed by cross-reactivity experiments. Calibration curves were constructed using 10-fold dilutions of cultured cells, attaining a limit of detection of around 50,000 cells/L, far below the *Karlodinium* spp. alert threshold (200,000 cells/L). Finally, the assay was applied to spiked seawater samples, showing an excellent correlation with the spiking levels and light microscopy counts. This approach is more rapid, specific and user-friendly than traditional microscopy techniques, and shows great promise for the surveillance and management of harmful algal blooms.

Keywords: *Karlodinium veneficum*, *Karlodinium armiger*, harmful algal bloom (HAB), recombinase polymerase amplification (RPA), enzyme-linked oligonucleotide assay (ELONA), seawater.

1. Introduction

Harmful algal blooms (HABs) are natural phenomena whose frequency, intensity and geographical extent have increased during recent years. Detection of HABs has become a challenging concern due to the direct impact on marine life, human health and the economy (Anderson *et al.* 2012). The genus *Karlodinium* (initially classified as *Gymnodinium* or *Gyrodinium*) is a widespread ichthyotoxic dinoflagellate implicated in numerous fish mortality events around the world, negatively affecting coastal ecosystems and marine fisheries (Place *et al.* 2012). In Alfacs Bay (NW Mediterranean Sea), *Karlodinium* spp. blooms have been periodically reported since the 1990s and two *Karlodinium* species, characterised as *K. veneficum* and *K. armiger* by morphological and genetic analysis, have been described and have settled in this region (Garces *et al.* 2006). These species produce different haemolytic toxins (Rasmussen *et al.* 2017; Van Wagoner *et al.* 2008) and present different levels of ichthyotoxicity (Berge *et al.* 2012) resulting in different risks to marine organisms, with consequences on the marine-based economy. *Karlodinium* spp. blooms can reach high densities (above 4,000,000 cells/L) and, based on toxicological studies, a level of 200,000 cells/L for *Karlodinium* spp. has been established as a warning level for the fauna in this important fish and shellfish aquaculture area (Fernandez-Tejedor *et al.* 2004).

There are many well stablished monitoring programs which periodically sample for the presence of HAB species in fish and shellfish aquaculture areas. Although not specified in the legislation, current toxic microalgae monitoring is regularly performed via light microscopy using the Utermöhl cell-counting method. However, this technique is time consuming, requires a high level of taxonomic expertise and is based on morphological characteristics, which in some cases are insufficient to discriminate among HAB species. This is the case for *Karlodinium*, since the high degree of morphological similarity between *K. veneficum* and *K. armiger* makes light microscopy inappropriate for discriminating between these species (Bergholtz *et al.* 2006; Garces *et al.* 2006).

Due to the difficulties and limitations of techniques based on morphological identification, there is a demand for new tools to provide a more reliable early warning of HAB events in order to facilitate and implement appropriate preventive measures. In this regard, the use of molecular methods for microalgae identification are being increasingly explored because they are faster and more accurate than microscopic observations (Medlin and Orozco 2017; Penna and Galluzzi 2013). Most molecular techniques have their origin in medical diagnostics and, during the last three decades, these techniques have been tested, modified, and refined for their application in microalgae identification, detection and quantification (Karlson *et al.* 2010). Among the different molecular methods, quantitative PCR (qPCR) has been widely applied to a variety of toxic microalgae, to detect one (Yuan *et al.* 2012), two (Eckford-Soper and Daugbjerg 2016) or more than two (Nishimura *et al.* 2016; Andree *et al.* 2011) species, mainly targeting ribosomal DNA (rDNA). Specifically for *Karlodinium* species, qPCR assays have been developed for *K. veneficum* (Eckford-Soper and Daugbjerg 2015; Park *et al.* 2009; Zhang *et al.* 2008) and more recently to discriminate between *K. veneficum* and *K. armiger* (Toldrà *et al.* 2018). PCR amplification has also been combined with biosensors for the electrochemical detection of *Karenia mikimotoi* (LaGier *et al.* 2007). However, although PCR is the gold standard amplification method, it has limitations, such as the requirement for thermal cycling equipment, which hampers the development of miniaturised and portable analysis systems for in-field application. A possible solution is the use of ribosomal RNA (rRNA) as a target, which may avoid DNA amplification (Orozco and Medlin 2013). This approach has been exploited in microarrays with fluorescence detection (Ahn *et al.* 2006;

Taylor *et al.* 2013) and in sandwich hybridisation systems followed by colorimetric (Cai *et al.* 2006; Diercks *et al.* 2008a) or electrochemical (Diercks *et al.* 2008b; Diercks-Horn *et al.* 2011; Metfies *et al.* 2005) detection. However, the inherent rRNA instability and the high amount of rRNA required could compromise the reliability and sensitivity, respectively, of these RNA-based assays (Bruce *et al.* 2015; Metfies *et al.* 2005). Another possibility to avoid the need for cycling control and power sources is the use of isothermal nucleic acid amplification methods.

There are only a few reports detailing isothermal amplification for the detection of toxic microalgae, such as nucleic acid sequence-based amplification (NASBA) (Casper *et al.* 2004; Loukas *et al.* 2017) and loop-mediated isothermal amplification (LAMP). The detection of DNA isothermally amplified using LAMP has mainly been achieved by fluorescence or turbidity measurements (Chen *et al.* 2013; Zhang *et al.* 2009; Zhang *et al.* 2014). A lateral flow (LF) strip exploiting LAMP has also developed for the detection of *K. veneficum* (Huang *et al.* 2017). However, LAMP is highly dependent on extremely careful primer design and NASBA requires an initial DNA melting step (Mayboroda *et al.* 2018). Recombinase polymerase amplification (RPA) is a very attractive alternative that overcomes these drawbacks and it has been chosen in the present study for the detection of two *Karlodinium* species (*K. veneficum* and *K. armiger*). RPA is based on the use of a mixture of polymerases, recombinases and DNA binding proteins that are capable of pairing oligonucleotide primers with homologous sequences in single (ssDNA) and double stranded DNA (dsDNA), typically within 25 min and at a low and constant temperature (37-42 °C) (Piepenburg *et al.* 2006). Recent publications demonstrate that RPA technology has been successfully applied to the detection of viruses (Euler *et al.* 2013), protozoa (Crannell *et al.* 2016) and bacteria (Santiago-Felipe *et al.* 2014), but its application to microalgae has not yet been described. Our RPA strategy exploits the use of tailed primers that result in amplicons of dsDNA flanked by ssDNA tails (Fig. 1a). This is accomplished by a C3 stopper located between the primer and the tail that prevents the polymerase from further elongation (Jauset-Rubio *et al.* 2016; Joda *et al.* 2015). Amplicons obtained after RPA are detected in a colorimetric sandwich enzyme-linked oligonucleotide assay (ELONA) using complementary oligonucleotides: a capture probe (specific for each *Karlodinium* species) immobilised through a thiol group on maleimide-coated microtiter plates and a reporter probe (common for the two species) conjugated to horseradish peroxidase (HRP) (Fig. 1b). Compared to other detection strategies, sandwich formats enhance the specificity of the assays because of the use of two hybridization events (capture and reporter). Additionally, the use of tailed primers avoids the need for primer labelling and/or any post-amplification processing to generate ssDNA thus reducing complexity, time and cost of the assay.

In this work, an RPA-ELONA method has been developed and applied to the detection and quantification of *K. veneficum* and *K. armiger*. The RPA-ELONA method was combined with a rapid and easy DNA extraction commercial kit that meets the criteria to perform analysis in the field due to its ease of use, short time requirement, and no need for specialised equipment. Since two *Karlodinium* species are targeted for detection and discrimination, distinct probes and primers were designed and used. Characterisation and specificity of the method was assessed by cross-reactivity experiments using synthetic ssDNA and genomic DNA, while sensitivity was assessed by constructing calibration curves using serial dilutions of culture cells. Finally, spiked seawater samples were analysed by RPA-ELONA and the results compared with light microscopy counts.

2. Materials and Methods

2.1. Reagents and materials

Potassium phosphate monobasic and dibasic, Trizma® base, sodium chloride, sodium acetate, skimmed milk, 6-mercapto-1-hexanol, tween® 20, 3,3',5,5'-tetramethylbenzidine (TMB) liquid substrate, phenol:chloroform:isoamylalcohol (25:24:1, v:v:v), chloroform, ethanol, dodecyltrimethylammonium bromide (DTAB), ethylenediaminetetraacetic acid (EDTA), ethidium bromide solution and agarose were all supplied by Sigma-Aldrich (Madrid, Spain). Biomeme Sample Prep Kit for DNA was obtained from Biomeme Inc. (Philadelphia, USA). Custom oligonucleotides primers and probes were synthetized by Biomers (Ulm, Germany). TwistAmp Basic kit was purchased from TwistDx (Cambridge, UK). Pierce maleimide-activated plates, GeneJET PCR purification kit and ultrapure DNase/RNase-free distilled water were supplied by Thermo Fisher Scientific (Madrid, Spain).

2.2. Microalgal cultures

Clonal cultures of *K. veneficum* (strain IRTA-SMM-00-01; GenBank accession number MG642757) and *K. armiger* (strain K-0668; GenBank accession number MG642758), isolated from Alfacs Bay, were acquired from IRTA Culture Collection of Algae (Sant Carles de la Ràpita, Spain) and the Scandinavian Culture Collection of Algae and Protozoa (Copenhagen, Denmark), respectively. Both cultures were maintained at a temperature of 18 ± 2 °C on a 12:12 h light:dark cycle under a light intensity of 110 µmol photons m^{-2} s^{-1}. *K. veneficum* and *K. armiger* cultures were grown in f/2 medium (Guillard 1973; Guillard and Ryther 1962) and L1 + Urea medium (Guillard and Hargraves 1993), respectively, at a practical salinity of 36. Culture aliquots were fixed with Lugol's iodine (Throndsen 1978) and counted under an inverted light microscope (Leica DMIL) following the Utermöhl method (Utermöhl 1958). Cultures were collected at the exponential phase ($4x10^7$ cells/L and $3x10^7$ cells/L for *K. veneficum* and *K. armiger*, respectively) and harvested by centrifugation (3,700 g, 25 min). Pellets containing 10^6 cells and 10-fold serial dilutions from 10^6 to 10^2 cells were prepared and stored at -20 °C until DNA extraction.

2.3. Spiked environmental samples

Natural seawater (10 L) was collected in June 2017 from L'Ametlla de Mar (40°49'51.42"N; 0°45'6.90"E; Catalonia, Spain) and subsequently analysed using light microscopy to confirm the presence and absence of *Karlodinium* spp. as well as other phytoplankton species. For this purpose, a volume of 50 mL was settled in sedimentation chambers for 24 h and counted using the Utermöhl method after fixation in Lugol's iodine. Spiked samples of seawater (1 L) containing *Karlodinium* species (previously counted as stated in section 2.2) were prepared at the warning level (200,000 cells/L) and at the fish mortality level (1,000,000 cells/L). Spiked concentrations were as follows: *K. veneficum* (200,000 cells/L and 1,000,000 cells/L), *K. armiger* (200,000 cells/L and 1,000,000 cells/L) and equal mixture of *K. veneficum* and *K. armiger* (400,000 cells/L and 2,000,000 cells/L). The spiked samples were fixed with Lugol's iodine solution. For light microscopy counts, a volume of 50 mL was settled in sedimentation chambers for 24 h and counted following the Utermöhl method. For RPA-ELONA analysis, 50-mL aliquots were centrifuged at 3,700 g for 25 min and maintained at -20 °C until DNA extraction.

128 **2.4. DNA extraction**

129 Two different extraction methods were used in this study. The phenol/chloroform/isoamylalcohol (PCI) method was used
130 to extract genomic DNA from pellets containing 10^6 cells for the subsequent construction of calibration curves, whereas
131 the Biomeme kit was used to extract the DNA from "10-fold serial dilutions of cells" and from "spiked samples".
132 Extractions were performed following the protocol described by Toldrà *et al.* (2018). Briefly, for the PCI method, cell
133 pellets were resuspended in lysis buffer (1 M NaCl, 70 mM Tris, 30 mM EDTA, pH 8.6), 10% w/v DTAB and chloroform,
134 and then disrupted using a BeadBeater-8 (Biospec, USA). After centrifugation, the DNA from the resulting aqueous phase
135 was extracted by the standard phenol/chloroform/isoamylalcohol procedure (Sambrook, 1989), followed by sodium
136 acetate/ethanol DNA precipitation. The DNA was rinsed with 70% v/v ethanol and dissolved in 50 µL of molecular biology-
137 grade water. For the Biomeme method, DNA was extracted according to the manufacturer's guidelines, but with some
138 adjustments. Cell pellets were resuspended in lysis buffer and disrupted using a bead beater. The homogenised samples
139 were mixed with Biomeme Lysis Buffer (500 µL), which was pumped through a syringe with an ion-exchange membrane
140 attached. The membrane was washed firstly with Biomeme Protein Wash (500 µL) and then with Biomeme Wash Buffer
141 (750 µL), and then air-dried. Purified DNA was eluted in 500 µL of Biomeme Elution Buffer. Finally, genomic DNA extracted
142 by both methods was quantified using a NanoDrop 2000 spectrophotometer (Thermo Fisher Scientific, Madrid, Spain)
143 and stored at -20 ˚C until analysis.

144 **2.5. Primers and probes design and specificity**

145 Primers used in this study were based on the two species-specific primers for *K. veneficum* and *K. armiger* previously
146 designed within the ITS1 rDNA region for qPCR assay (Toldrà *et al.* 2018). The design aimed at minimising the number of
147 required primers: one genus-specific (for *Karlodinium*) and two species-specific (for *K. veneficum* and *K. armiger*) primers.
148 The genus-specific primer described by Toldrà *et al.* (2018) was slightly modified 4 bp downstream in order to avoid
149 primer-dimer formation. Primers for *K. veneficum* amplified a product of 139 bp, whereas primers for *K. armiger* amplified
150 a product of 153 bp. Primers were modified by adding oligonucleotide tails, resulting in amplicons with one ssDNA tail at
151 each end, which enable subsequent detection via sandwich ELONA through complementary capture and reporter probes
152 (Fig. 1). All primers and probe sequences are shown in Table 1. Primer specificity was tested by electrophoresis of the
153 RPA products in 2% w/v agarose gel.

154 **2.7. Recombinase polymerase amplification (RPA) reaction**

155 RPA was performed following the indications provided in the TwistAmp Basic kit with some minor modifications. The RPA
156 conditions including reagent concentration (primers, rehydration buffer and enzyme pellet), reaction time and the
157 requirement for a final purification step were systematically optimised. Following optimisation, each RPA reaction (50
158 µL) contained: 480 nM of each primer, 14 mM magnesium acetate, 0.5x rehydration buffer, 0.5x enzyme pellet and 5 µL
159 of DNA, this DNA being: a) synthetic ssDNA and genomic DNA to check the specificity of the subsequent ELONA, b)
160 synthetic ssDNA, genomic DNA and genomic DNA extracted from cell dilutions for the calibration curves, and c) genomic
161 DNA extracted from spiked samples. All reagents except the DNA and magnesium were prepared in a master mix, which
162 was distributed into 0.2-mL reaction tubes. The DNA was then added into the tubes, and magnesium added to initiate

the RPA reaction. The tubes were immediately placed into a Nexus Gradient Thermal Cycler (Eppendorf Ibérica, Madrid, Spain) at 37 ˚C for 30 min. Subsequently, RPA products were purified using GeneJET PCR purification kit following manufacturer instructions, with a final elution with 50 µL of TE buffer. RPA reactions were performed in triplicate and blanks (no DNA) were included in all cases.

2.8. Enzyme Linked Oligonucleotide Assay (ELONA) detection

Maleimide-activated plates were rinsed three times with 200 µL of washing buffer (100 mM potassium phosphate, 150 mM NaCl, 0.05 % v/v Tween-20, pH 7.4) and 50 µL of 500 nM thiolated capture probe in binding buffer (100 mM phosphate, 150 mM NaCl, pH 7.4) were then added and left to incubate overnight at 4 ˚C. Any remaining maleimide groups were subsequently blocked with 100 µM 6-mercapto-1-hexanol in MiliQ water adding 200 µL per well, and an additional blocking step was carried out via addition of 200 µL of 5% w/v skimmed milk in binding buffer. RPA product was added to the functionalised maleimide plates (45 µL per well) and 50 µL of 10 nM reporter probe labeled with HRP in washing buffer were added to the wells. Finally, after incubation with 50 µL of TMB liquid substrate, the absorbance was read at 620 nm with a Microplate Reader KC4 (BIO-TEK Instruments Inc., Vermont, USA). After each step, wells were rinsed three times with 200 µL of washing buffer and during incubations microtitre plates were placed on a plate shaker for mixing. With the exception of the capture probe immobilisation step, which was performed at 4 ˚C overnight, all steps were conducted at room temperature for 30 min.

2.9 Data analysis and statistics

Calibration curves using dilutions of synthetic ssDNA, genomic DNA and cultured cells were adjusted to a sigmoidal logistic four-parameter equation using SigmaPlot software 12.0 (Systat Software Inc., California, USA). The limit of detection (LOD) was defined as the blank (no DNA) value plus three times the standard deviation (SD) of the blank. Spiked samples were quantified from the equation obtained using the standard curves from cell dilutions. To evaluate differences in genus-level cell quantifications provided by RPA-ELONA and light microscopy and also differences in species-level cell quantifications between RPA-ELONA and spiking levels, a paired t test was conducted using SigmaStat software 3.1 (Systat Software Inc., California, USA). Prior to analysis, a normality and equal variance test was performed. Differences in the results were considered statistically significant at the 0.05 level.

3. Results and discussion

3.1. Primer specificity

Primer specificity is critical when detecting harmful algae because seawater samples commonly contain a wide range of microorganisms. Specific primer sets for *K. veneficum* and *K. armiger* were previously developed by Toldrà *et al.* (2018), and were demonstrated to be highly specific for the qPCR assay, and also deemed suitable for RPA according to the RPA primer design manual (TwistDx). However, electrophoresis of the RPA products revealed the presence of primer-dimers for *K. armiger*, and consequently the primers were slightly modified. Following primer optimisation, the estimated molecular weight of the products as visualised using gel electrophoresis was as expected, and no other bands were observed (Fig. 1 Supplementary data).

3.2. Optimisation of RPA conditions

RPA conditions were optimised using synthetic ssDNA and subsequent ELONA detection. RPA was performed maintaining some conditions (at 37 ˚C for 30 min with purification step) and concentrations (14 mM magnesium acetate and 5 µL of DNA), whilst varying primer (240-480 nM), rehydration buffer (0.5-1x) and enzyme pellet (0.5-1x) concentrations. Results demonstrated that the LOD was remarkably decreased 110-fold using optimised RPA component concentrations. Furthermore, in an effort to shorten assay time, different RPA reaction times (5, 10, 20, 30 and 40 min) were evaluated. As expected, higher absorbance values were obtained at longer reaction times. Nevertheless, since the LODs obtained at 30 and 40 min were very similar (the LOD at 40 min was only ~2-fold lower than the LOD at 30 min), 30 min was chosen as the optimum amplification time. Finally, the need for an RPA product purification step prior to detection was evaluated. Results showed that without a purification step the LOD increased significantly (52-fold higher). This lower performance might be explained by the presence of proteins and primers in the RPA reaction that could hinder hybridisation events and/or increase the nonspecific adsorption. Optimised RPA conditions (see section 2.7) were used in subsequent experiments.

3.3. Specificity of RPA-ELONA

To assess the specificity of the RPA-ELONA for the detection of *K. veneficum* and *K. armiger*, cross-reactivity experiments using synthetic ssDNA and genomic DNA at high concentrations (1 nM and 2.3 ng/µL, respectively), with both single and mixed DNA samples, were performed. A combination of different capture probes, primers and DNA were tested. Absorbance values showed the same trend for *K. veneficum* and *K. armiger*, using both synthetic ssDNA (Fig. 2a) and genomic DNA (Fig. 2b).

Specific detection was obtained for both *K. veneficum* and *K. armiger* when using their corresponding capture probe, primers and target DNA (in single or mixed DNA samples), using both synthetic ssDNA and genomic DNA (Fig. 2, bars with arrows). When species-specific primers and capture probes were used with non-target DNA (i.e. *K. armiger* primers and capture probes with *K. veneficum* DNA, and vice versa), non-specific detection was obtained using synthetic ssDNA (Fig. 2a, bars with asterisks), which did not appear using genomic DNA. This finding could be explained by the presence of common bases between the two species-specific primers because of the high similarity between *K. veneficum* and *K.*

armiger ITS1-5.8S-ITS2 rDNA sequences. When using short (150bp) synthetic oligonucleotides, the upstream species-specific primers may bind to the non-target DNA and, together with the downstream genus-specific primer binding, non-specific dsDNA amplicons may be generated and subsequently detected. For example, when *K. armiger* primers are combined with *K. veneficum* synthetic ssDNA, non-specific amplification occurs, generating a product flanked by *K. armiger* tails, which are complementary to the *K. armiger* capture probes, thus resulting in non-specific detection. This effect was not observed when using mixed DNA samples, probably because species-specific primers have a preference for their target DNA and non-specific amplification is hindered.

On the other hand, genomic DNA is a more complex and a larger matrix, in which the target represents a miniscule part of the DNA. Consequently, primers might find more partial-complementary sites and the formation of non-specific dsDNA amplicons is negligible and subsequently not detected. Additionally, no significant differences were observed between single and mixed DNA samples, which indicates that the assay is highly specific for genomic DNA regardless of the simultaneous presence of a non-specific target.

Finally, all other combinations gave negative results, showing no significant differences compared to the blanks (no DNA bars). We clearly demonstrated the strong specificity of the assay for *K. veneficum* and *K. armiger* using genomic DNA, and thus the cross-reactivity observed with short synthetic DNA does not hinder the in-field application of the developed technique. Since in a real world application genomic and not synthetic DNA is targeted, the reason for the anomaly observed with synthetic DNA target is relatively irrelevant and the explanations mentioned in the previous paragraph are subject to further investigations.

3.5. Calibration curves and LOD determination

Synthetic ssDNA and genomic DNA were initially used as targets to demonstrate the feasibility of the approach. Calibration curves using 10-fold serial dilutions of synthetic ssDNA for both *K. veneficum* and *K. armiger* were obtained (Fig. 3a), achieving LODs of 0.043 fM and 0.7 fM, respectively. Calibration curves using dilutions of total genomic DNA extracted from both *Karlodinium* species were then constructed (Fig. 3b), which provided similar LODs: 12 pg/µL for *K. veneficum* and 11 pg/µL for *K. armiger*. Afterwards, standard curves based on cell dilutions were constructed to allow quantification of the number of *Karlodinium* cells in a sample. These calibration curves were prepared using genomic DNA extracted (using the Biomeme method) from 10-fold serial dilutions of cultured cells (Fig. 3c) and the LODs attained were of the same order of magnitude than with genomic DNA: 2,483 cells for *K. veneficum* and 2,417 cells for *K. armiger*. Taking into account that the protocol involves the analysis of 50-mL samples, the LODs can be translated to 49,660 cells/L for *K. veneficum* and 48,340 cells/L for *K. armiger*.

There are only a few reports detailing the use of molecular methods for the detection of *Karlodinium* species. In the qPCR assays described, cell dilutions have been most commonly used to construct calibration curves, achieving LODs of 100 cells for *K. veneficum* and *K. armiger* (Toldrà *et al.* 2018) and 10 cells for *K. veneficum* (Eckford-Soper and Daugbjerg 2015). On the other hand, LAMP-LF used genomic DNA to determine the LOD of the assay, which was 7 pg/µL of *K. veneficum* genomic DNA (Huang *et al.* 2017). However, it was only qualitatively applied to the analysis of field samples, without being correlated with the number of cells. A limited number of molecular methods to detect microalgae without

a prior amplification step have been reported, but they have not been applied to *Karlodinium* and they are limited by poor sensitivity. For example, the electrochemical DNA-biosensor for the detection of *Alexandrium ostenfeldii* achieved an LOD of 16 ng/µL (Metfies *et al.* 2005). Our RPA-ELONA method, although it has a lower sensitivity compared to some of the described molecular methods, facilitates the quantification of *Karlodinium* species below the proposed alert threshold of 200,000 cells/L, which will enable early warnings of *Karlodinium* spp. blooms before they proliferate to critical levels. It is important to mention that this quantification limit could be reduced by centrifuging a larger sample volume or reducing the volume of elution buffer used for the DNA extraction.

3.6. Analysis of environmental spiked samples

To assess the performance of the RPA-ELONA method in a natural sample matrix, cultures of *K. veneficum* and *K. armiger* were used to spike natural seawater at two different levels of environmentally relevant concentrations. A prior study by light microscopy for the presence and abundance of phytoplankton in seawater did not reveal the presence of *Karlodinium* species, although high densities of potentially toxic species (i.e. 40,000 cells/L of *Pseudo-nitzschia* spp.) and negligible densities of other toxic species (i.e. 60 cells/L of *Dinophysis* spp. and *Prorocentrum* spp.) were detected. Natural seawater samples spiked with known abundances of *Karlodinium* cells were analysed using both RPA-ELONA and light microscopy (Table 2). Whereas *Karlodinium* species cannot be discriminated by light microscopy, the RPA-ELONA assay is able to identify, discriminate and quantify these two *Karlodinium* species.

The RPA-ELONA results for each *Karlodinium* species were compared with the spiking level concentrations (prepared by cell counting using light microscopy) and no significant differences ($p > 0.05$) were observed. As mentioned above, total *Karlodinium* species were counted using the standard Utermöhl cell-counting method. Total cell quantifications provided by the two methods (the sum of two RPA-ELONA assays for species-level and light microscopy for genus-level) were the same at the confidence level specified ($p > 0.05$). This agreement between techniques also indicated that the RPA-ELONA method is highly specific in the quantification of *Karlodinium* species, even in the presence of other microalgae species at high densities. The developed RPA-ELONA method showed an excellent agreement with the microscopic method in the analysis of seawater spiked with cultured cells.

4. Conclusions

The present work reports the development of an RPA-ELONA method for the detection, discrimination and quantification of two *Karlodinium* species (*K. veneficum* and *K. armiger*). This approach is applied for the first time to the detection of harmful algae. The method showed high specificity and, under the current experimental conditions, attained a sensitivity around 50,000 cells/L for both species, a concentration that is below the proposed alert threshold (200,000 cells/L) in seawater. An excellent degree of correlation between cell concentrations determined by RPA-ELONA with spiking levels and light microscopy counts confirmed the reliability and applicability of the method.

This assay presents multiple benefits. It is species-specific and avoids the need for taxonomic expertise. In particular, RPA-ELONA can discriminate between *K. veneficum* and *K. armiger* while this is not possible using light microscopy. The discrimination between *K. veneficum* and *K. armiger* is crucial because these two species present different levels of ichthyotoxicity, which poses different risks to marine organisms and the marine-based economy. Additionally, it is more rapid than traditional light microscopy techniques that use the Utermöhl method to estimate microalgae species abundances, and it allows high throughput analysis with reduced cost. In addition to these advantages, the RPA-ELONA is a versatile approach that opens up the possibility to be easily adapted to many other microalgae, to be exploited with other detection systems (e.g. electrochemical), to be formatted in a multiplex configuration and to be subsequently integrated into miniaturised and automated devices. Thus, the combination of the RPA-ELONA with the rapidity and ease of the Biomeme DNA extraction kit paves the way towards the deployment of portable platforms for *in situ* detection of microalgae.

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Statement of contributions

Anna Toldrà has developed the RPA-ELONA assay for these microalgae and has performed all the experimental work; she has written the manuscript draft. **Míriam Jauset-Rubio** has contributed to the RPA optimisation. **Karl B. Andree** has contributed to design the primers. **Margarita Fernández-Tejedor** has coordinated the seawater sampling and the phytoplankton work. **Jorge Diogène** has participated in the discussion of the results. **Ioanis Katakis** has supervised the experimental work and has participated in the discussion of the results. **Ciara K. O'Sullivan** has provided expertise on RPA-ELONA for application to microalgae in this work, supervised the experimental work and discussed the obtained results. **Mònica Campàs** has designed the experimental plan, supervised the experimental work and discussed the obtained results. All authors have critically reviewed the manuscript. Ciara K. O'Sullivan and Mònica Campàs have contributed equally to the work as corresponding authors.

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Table 1 Summary of probes and primers used in this study.

name	Sequence (5'-3')
K. veneficum capture probe	gtc gtg act ggg aaa act ttt ttt ttt ttt tt-C6 thiol
K. armiger capture probe	ttc att gag ttc gtc gta att ttt ttt ttt ttt tt-C6 thiol
Reporter probe	HRP-act ggc cgt cgt ttt aca
Forward *Karlodinium* spp. primer	tgt aaa acg acg gcc agt-C3-aca cac atc caa cca tyt cac tg
Reverse *K. veneficum* primer	gtt ttc cca gtc acg ac-C3-ata gct tcg cag aca aag gtg aat c
Reverse *K. armiger* primer	att acg acg aac tca atg aa-C3- ata gct tca cag cag agg tta caa c
K. veneficum ssDNA	ata gct tcg cag aca aag gtg aat ccc aat gct gct cca cta ccc gcg aac tgc taa cgc cag ggt gcg gaa gag aac tac ccc aac ccc cgc gca aga gct cac aaa gaa gtt cac agt gaa atg gtt gga tgt gtg t
K. armiger ssDNA	ata gct tca cag cag agg tta caa cac caa tgc tgc tcc gct acc cgc gat ctc atg cac cag gga gcg gca aga agc cag agc ttc aag aca ccc cta ccc ccg tgc agg agc tca caa aga aag ttc aca gtg aga tgg ttg gat gtg tgt

Table 2 Specific determination of *K. veneficum* and *K. armiger* concentrations in seawater samples by RPA-ELONA (n = 3) and light microscopy (LM).

	***Karlodinium* species**	**Spiking level (cells/L)**	**LM* (cells/L)**	**RPA-ELONA (cells/L)**	**LM/Spiking level (%)**	**RPA-ELONA/Spiking level (%)**	**RPA-ELONA/LM (%)**
Sample 1	*K. veneficum*	1,000,000	1,293,906	855,205 ± 63,224	129	86	66
Sample 2	*K. veneficum*	200,000	152,544	186,071 ± 30,541	76	93	122
Sample 3	*K. armiger*	1,000,000	833,016	959,268 ± 130,310	83	96	115
Sample 4	*K. armiger*	200,000	115,668	157,114 ± 39,207	58	79	136
Sample 5	*K. veneficum* *K. armiger*	1,000,000 1,000,000	1,864,044	936,032 ± 108,723 700,547 ± 48,050	93	94 70	89
Sample 6	*K. veneficum* *K. armiger*	200,000 200,000	447,234	220,842 ± 27,283 214,838 ± 34,065	112	110 107	97

*Spiked samples were analysed singular by LM; during the intra laboratory validation of this method the repeatability error was 41.47%.

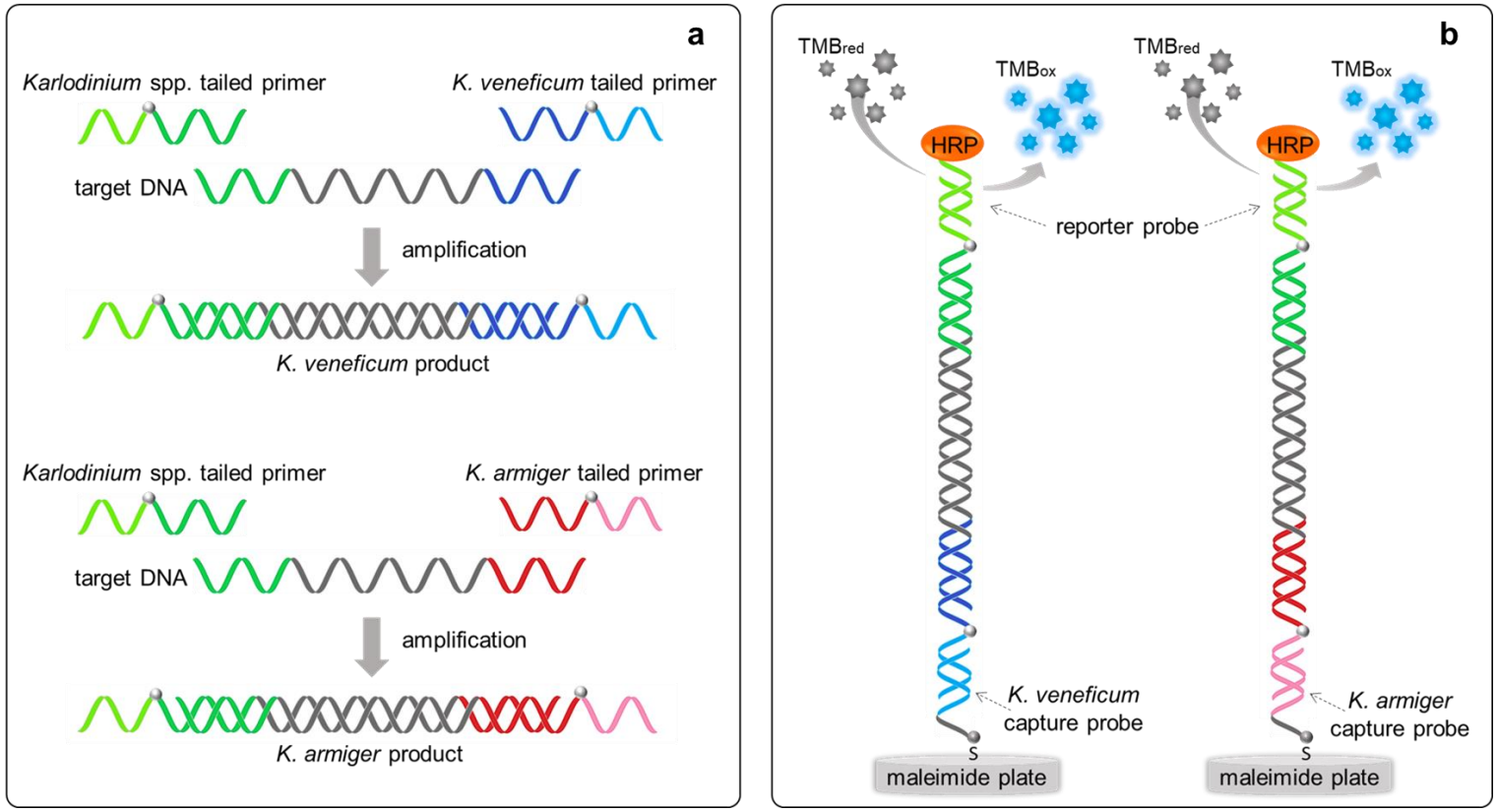

Figure 1. Schematic representation of the RPA-ELONA method for *K. veneficum* and *K. armiger*: (**a**) RPA with tailed primers and (**b**) ELONA detection.

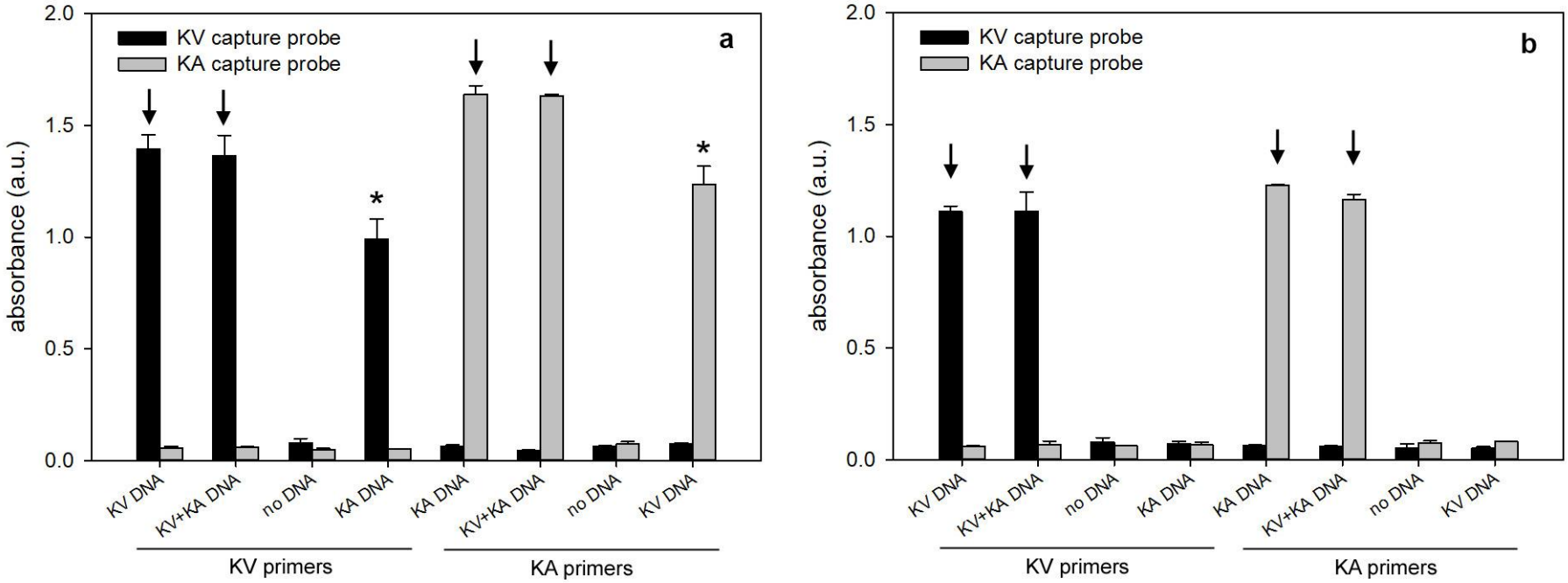

Figure 2. RPA-ELONA results using different capture probes, primers and DNA: (**a**) synthetic ssDNA at 1nM and (**b**) genomic DNA at 2.3 ng/µL. Error bars represent the standard deviation for 3 replicates. Arrows and asterisks represent specific and non-specific detection, respectively. KV = *K. veneficum*, KA = *K. armiger*.

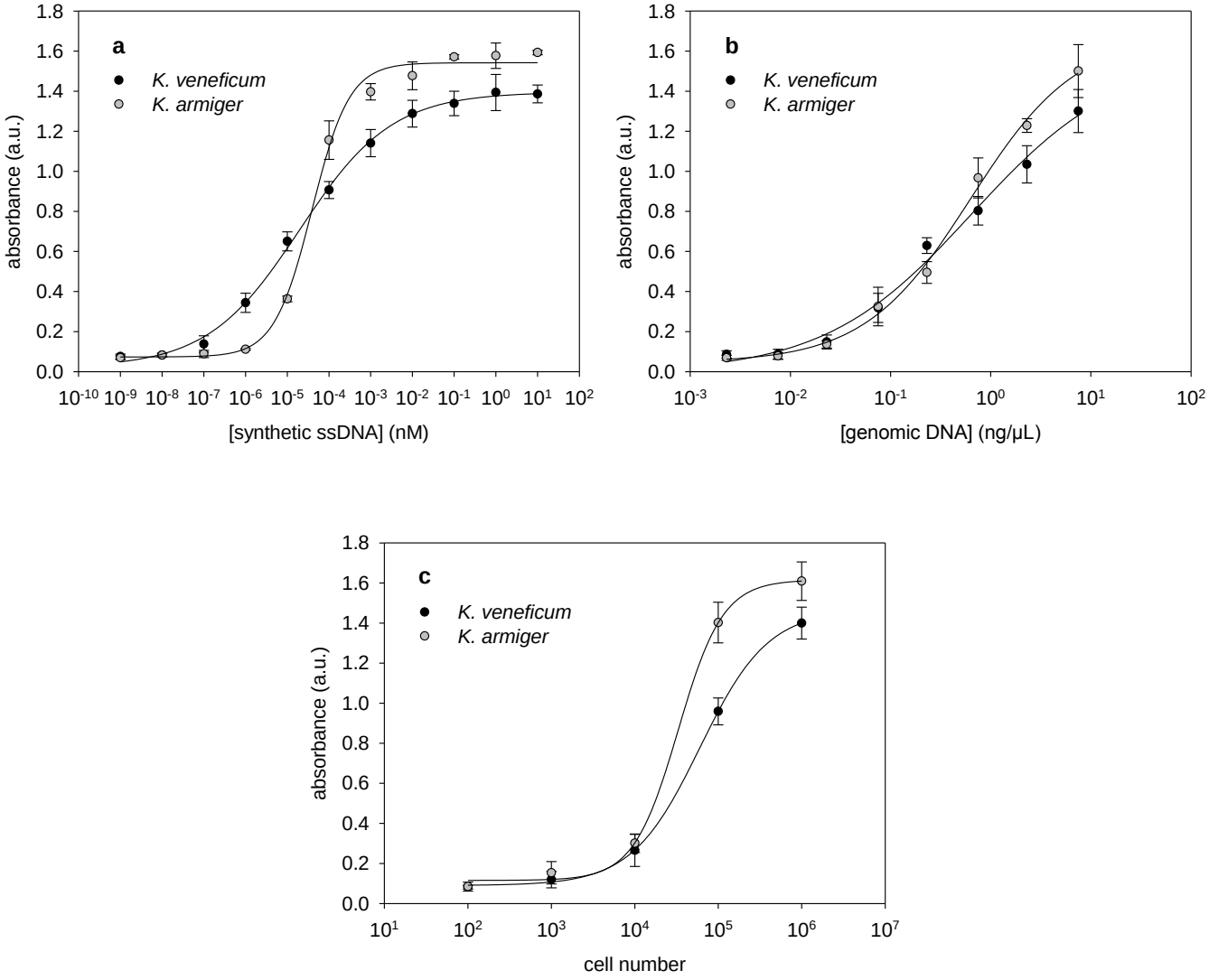

Figure 3. Calibration curves for *K. veneficum* and *K. armiger* obtained with different concentrations of: (**a**) synthetic ssDNA, (**b**) genomic DNA and (**c**) cultured cells. Errors bars represent the standard deviation for 3 replicates.

SUPPLEMENTARY DATA

Figure 1. Gel electrophoresis analysis of target synthetic ssDNA (100nM) amplified by RPA. KV = *K. veneficum*, KA = *K. armiger*, L = 1 Kb Plus DNA Ladder.