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

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10   **Abstract**

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

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

## 23 1. Introduction

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

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

43 Due to the difficulties and limitations of techniques based on morphological identification, there is a demand for new  
44 tools to provide a more reliable early warning of HAB events in order to facilitate and implement appropriate preventive  
45 measures. In this regard, the use of molecular methods for microalgae identification are being increasingly explored  
46 because they are faster and more accurate than microscopic observations (Medlin and Orozco 2017; Penna and Galluzzi  
47 2013). Most molecular techniques have their origin in medical diagnostics and, during the last three decades, these  
48 techniques have been tested, modified, and refined for their application in microalgae identification, detection and  
49 quantification (Karlson *et al.* 2010). Among the different molecular methods, quantitative PCR (qPCR) has been widely  
50 applied to a variety of toxic microalgae, to detect one (Yuan *et al.* 2012), two (Eckford-Soper and Daugbjerg 2016) or  
51 more than two (Nishimura *et al.* 2016; Andree *et al.* 2011) species, mainly targeting ribosomal DNA (rDNA). Specifically  
52 for *Karlodinium* species, qPCR assays have been developed for *K. veneficum* (Eckford-Soper and Daugbjerg 2015; Park *et al.*  
53 *et al.* 2009; Zhang *et al.* 2008) and more recently to discriminate between *K. veneficum* and *K. armiger* (Toldrà *et al.* 2018).  
54 PCR amplification has also been combined with biosensors for the electrochemical detection of *Karenia mikimotoi* (LaGier  
55 *et al.* 2007). However, although PCR is the gold standard amplification method, it has limitations, such as the requirement  
56 for thermal cycling equipment, which hampers the development of miniaturised and portable analysis systems for in-  
57 field application. A possible solution is the use of ribosomal RNA (rRNA) as a target, which may avoid DNA amplification  
58 (Orozco and Medlin 2013). This approach has been exploited in microarrays with fluorescence detection (Ahn *et al.* 2006;

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

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

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

## 94 **2. Materials and Methods**

### 95 **2.1. Reagents and materials**

96 Potassium phosphate monobasic and dibasic, Trizma<sup>®</sup> base, sodium chloride, sodium acetate, skimmed milk, 6-mercapto-  
97 1-hexanol, tween<sup>®</sup> 20, 3,3',5,5'-tetramethylbenzidine (TMB) liquid substrate, phenol:chloroform:isoamylalcohol  
98 (25:24:1, v:v:v), chloroform, ethanol, dodecyltrimethylammonium bromide (DTAB), ethylenediaminetetraacetic acid  
99 (EDTA), ethidium bromide solution and agarose were all supplied by Sigma-Aldrich (Madrid, Spain). Biomeme Sample  
100 Prep Kit for DNA was obtained from Biomeme Inc. (Philadelphia, USA). Custom oligonucleotides primers and probes were  
101 synthesized by Biomers (Ulm, Germany). TwistAmp Basic kit was purchased from TwistDx (Cambridge, UK). Pierce  
102 maleimide-activated plates, GeneJET PCR purification kit and ultrapure DNase/RNase-free distilled water were supplied  
103 by Thermo Fisher Scientific (Madrid, Spain).

### 104 **2.2. Microalgal cultures**

105 Clonal cultures of *K. veneficum* (strain IRTA-SMM-00-01; GenBank accession number MG642757) and *K. armiger* (strain  
106 K-0668; GenBank accession number MG642758), isolated from Alfacs Bay, were acquired from IRTA Culture Collection of  
107 Algae (Sant Carles de la Ràpita, Spain) and the Scandinavian Culture Collection of Algae and Protozoa (Copenhagen,  
108 Denmark), respectively. Both cultures were maintained at a temperature of  $18 \pm 2$  °C on a 12:12 h light:dark cycle under  
109 a light intensity of  $110 \mu\text{mol photons m}^{-2} \text{ s}^{-1}$ . *K. veneficum* and *K. armiger* cultures were grown in f/2 medium (Guillard  
110 1973; Guillard and Ryther 1962) and L1 + Urea medium (Guillard and Hargraves 1993), respectively, at a practical salinity  
111 of 36. Culture aliquots were fixed with Lugol's iodine (Thronsdén 1978) and counted under an inverted light microscope  
112 (Leica DMIL) following the Utermöhl method (Utermöhl 1958). Cultures were collected at the exponential phase ( $4 \times 10^7$   
113 cells/L and  $3 \times 10^7$  cells/L for *K. veneficum* and *K. armiger*, respectively) and harvested by centrifugation (3,700 g, 25 min).  
114 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  
115 extraction.

### 116 **2.3. Spiked environmental samples**

117 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)  
118 and subsequently analysed using light microscopy to confirm the presence and absence of *Karlodinium* spp. as well as  
119 other phytoplankton species. For this purpose, a volume of 50 mL was settled in sedimentation chambers for 24 h and  
120 counted using the Utermöhl method after fixation in Lugol's iodine. Spiked samples of seawater (1 L) containing  
121 *Karlodinium* species (previously counted as stated in section 2.2) were prepared at the warning level (200,000 cells/L)  
122 and at the fish mortality level (1,000,000 cells/L). Spiked concentrations were as follows: *K. veneficum* (200,000 cells/L  
123 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*  
124 (400,000 cells/L and 2,000,000 cells/L). The spiked samples were fixed with Lugol's iodine solution. For light microscopy  
125 counts, a volume of 50 mL was settled in sedimentation chambers for 24 h and counted following the Utermöhl method.  
126 For RPA-ELONA analysis, 50-mL aliquots were centrifuged at 3,700 g for 25 min and maintained at -20 °C until DNA  
127 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  $\mu$ L 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  $\mu$ L), which was pumped through a syringe with an ion-exchange membrane  
140 attached. The membrane was washed firstly with Biomeme Protein Wash (500  $\mu$ L) and then with Biomeme Wash Buffer  
141 (750  $\mu$ L), and then air-dried. Purified DNA was eluted in 500  $\mu$ L 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 *Karlotidium*) 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  $\mu$ L) contained: 480 nM of each primer, 14 mM magnesium acetate, 0.5x rehydration buffer, 0.5x enzyme pellet and 5  $\mu$ L  
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

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

## 167 **2.8. Enzyme Linked Oligonucleotide Assay (ELONA) detection**

168 Maleimide-activated plates were rinsed three times with 200 µL of washing buffer (100 mM potassium phosphate, 150  
169 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  
170 phosphate, 150 mM NaCl, pH 7.4) were then added and left to incubate overnight at 4 °C. Any remaining maleimide  
171 groups were subsequently blocked with 100 µM 6-mercapto-1-hexanol in MiliQ water adding 200 µL per well, and an  
172 additional blocking step was carried out via addition of 200 µL of 5% w/v skimmed milk in binding buffer. RPA product  
173 was added to the functionalised maleimide plates (45 µL per well) and 50 µL of 10 nM reporter probe labeled with HRP  
174 in washing buffer were added to the wells. Finally, after incubation with 50 µL of TMB liquid substrate, the absorbance  
175 was read at 620 nm with a Microplate Reader KC4 (BIO-TEK Instruments Inc., Vermont, USA). After each step, wells were  
176 rinsed three times with 200 µL of washing buffer and during incubations microtitre plates were placed on a plate shaker  
177 for mixing. With the exception of the capture probe immobilisation step, which was performed at 4 °C overnight, all steps  
178 were conducted at room temperature for 30 min.

## 179 **2.9 Data analysis and statistics**

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

## 188 **3. Results and discussion**

### 189 **3.1. Primer specificity**

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

### 197 **3.2. Optimisation of RPA conditions**

198 RPA conditions were optimised using synthetic ssDNA and subsequent ELONA detection. RPA was performed maintaining  
199 some conditions (at 37 °C for 30 min with purification step) and concentrations (14 mM magnesium acetate and 5 µL of  
200 DNA), whilst varying primer (240-480 nM), rehydration buffer (0.5-1x) and enzyme pellet (0.5-1x) concentrations. Results  
201 demonstrated that the LOD was remarkably decreased 110-fold using optimised RPA component concentrations.  
202 Furthermore, in an effort to shorten assay time, different RPA reaction times (5, 10, 20, 30 and 40 min) were evaluated.  
203 As expected, higher absorbance values were obtained at longer reaction times. Nevertheless, since the LODs obtained at  
204 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  
205 as the optimum amplification time. Finally, the need for an RPA product purification step prior to detection was  
206 evaluated. Results showed that without a purification step the LOD increased significantly (52-fold higher). This lower  
207 performance might be explained by the presence of proteins and primers in the RPA reaction that could hinder  
208 hybridisation events and/or increase the nonspecific adsorption. Optimised RPA conditions (see section 2.7) were used  
209 in subsequent experiments.

### 210 **3.3. Specificity of RPA-ELONA**

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

216 Specific detection was obtained for both *K. veneficum* and *K. armiger* when using their corresponding capture probe,  
217 primers and target DNA (in single or mixed DNA samples), using both synthetic ssDNA and genomic DNA (Fig. 2, bars with  
218 arrows). When species-specific primers and capture probes were used with non-target DNA (i.e. *K. armiger* primers and  
219 capture probes with *K. veneficum* DNA, and vice versa), non-specific detection was obtained using synthetic ssDNA (Fig.  
220 2a, bars with asterisks), which did not appear using genomic DNA. This finding could be explained by the presence of  
221 common bases between the two species-specific primers because of the high similarity between *K. veneficum* and *K.*



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

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

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

### 240 **3.5. Calibration curves and LOD determination**

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

251 There are only a few reports detailing the use of molecular methods for the detection of *Karlodinium* species. In the qPCR  
252 assays described, cell dilutions have been most commonly used to construct calibration curves, achieving LODs of 100  
253 cells for *K. veneficum* and *K. armiger* (Toldrà *et al.* 2018) and 10 cells for *K. veneficum* (Eckford-Soper and Daugbjerg  
254 2015). On the other hand, LAMP-LF used genomic DNA to determine the LOD of the assay, which was 7 pg/μL of *K.*  
255 *veneficum* genomic DNA (Huang *et al.* 2017). However, it was only qualitatively applied to the analysis of field samples,  
256 without being correlated with the number of cells. A limited number of molecular methods to detect microalgae without

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

### 264 **3.6. Analysis of environmental spiked samples**

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

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

#### 281 **4. Conclusions**

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

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

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#### 306 **Statement of contributions**

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

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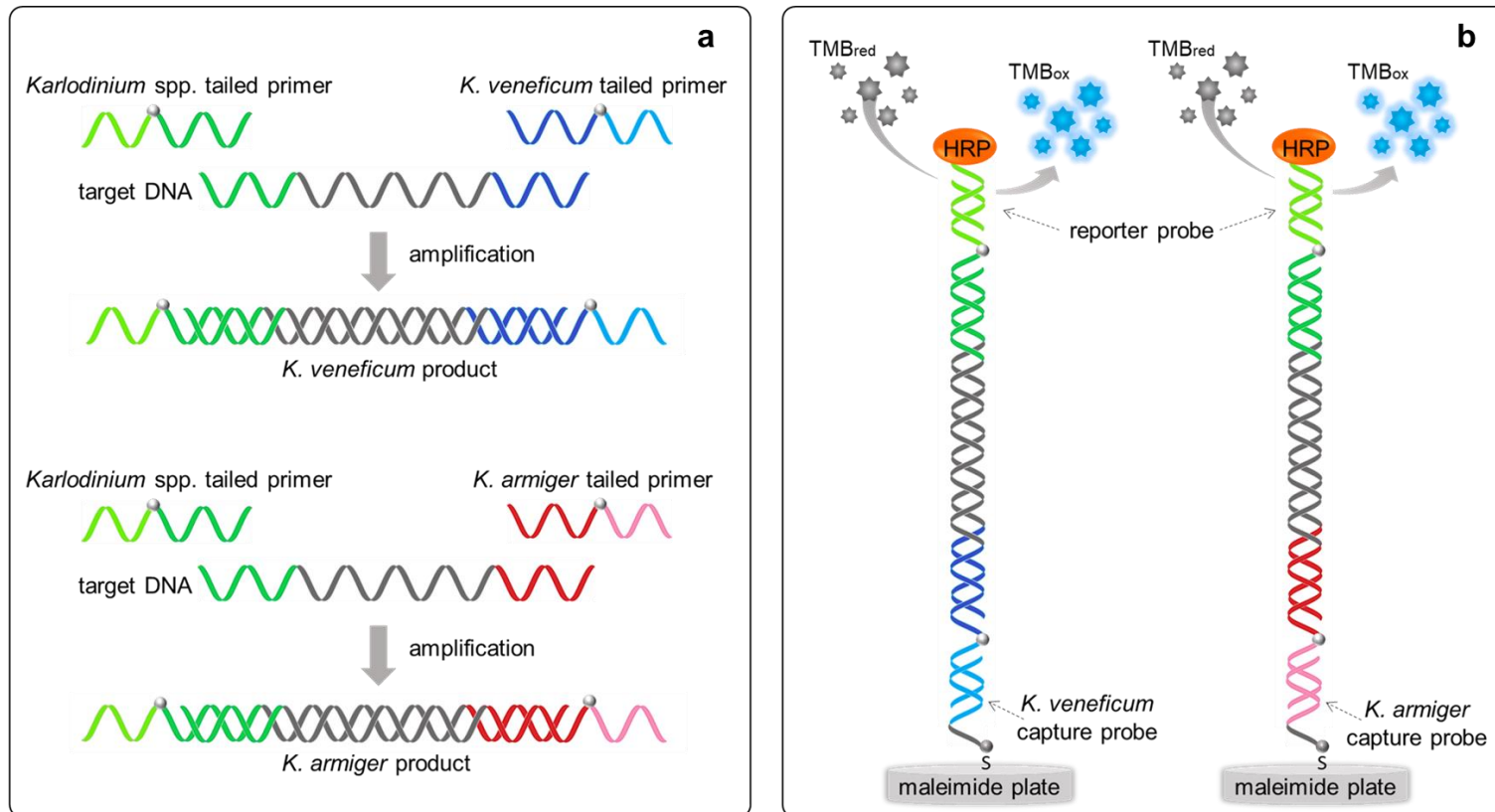
**Table 1** Summary of probes and primers used in this study.

name	Sequence (5'-3')
<i>K. veneficum</i> capture probe	gtc gtc act ggg aaa act ttt ttt ttt ttt tt-C6 thiol
<i>K. armiger</i> 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 <i>Karlodinium</i> spp. primer	tgt aaa acg acg gcc agt-C3-aca cac atc caa cca tyt cac tg
Reverse <i>K. veneficum</i> primer	gtt ttc cca gtc acg ac-C3-ata gct tcg cag aca aag gtg aat c
Reverse <i>K. armiger</i> primer	att acg acg aac tca atg aa-C3- ata gct tca cag cag agg tta caa c
<i>K. veneficum</i> 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
<i>K. armiger</i> 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).

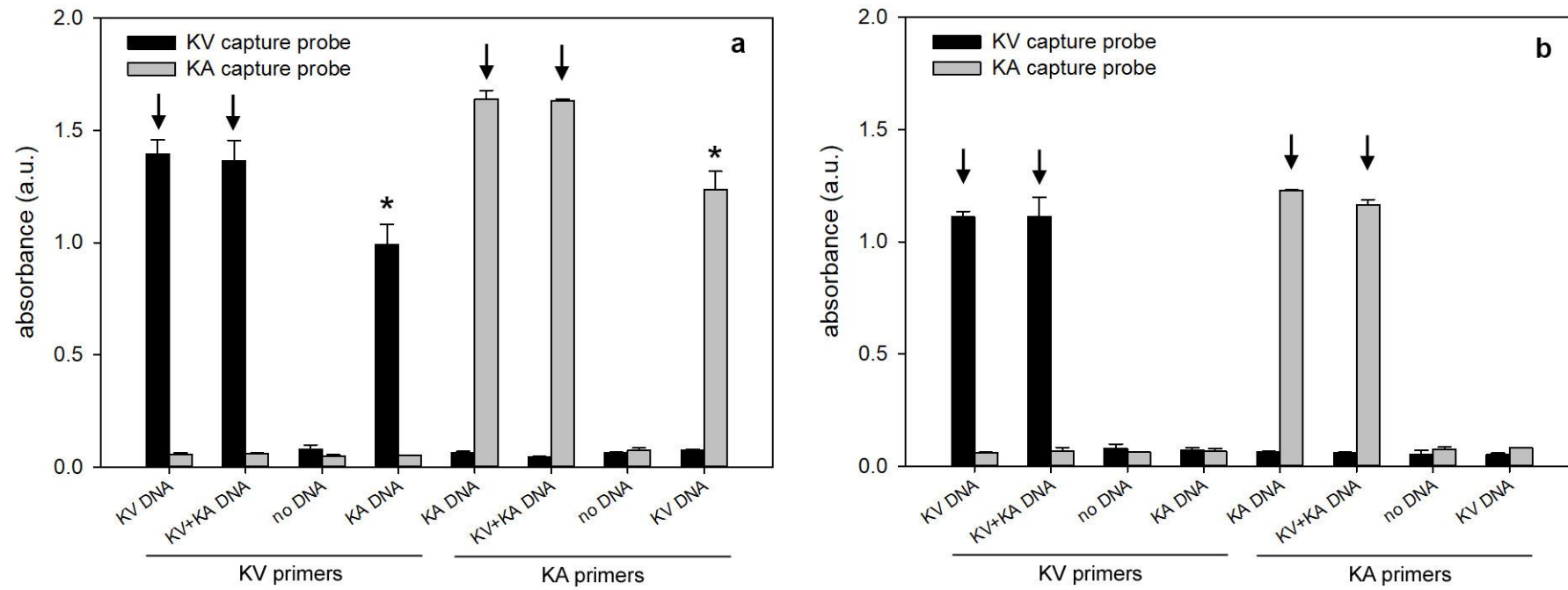
	<i>Karlodinium</i> species	Spiking level (cells/L)	LM* (cells/L)	RPA-ELONA (cells/L)	LM/Spiking level (%)	RPA-ELONA/Spiking level (%)	RPA-ELONA/LM (%)
Sample 1	<i>K. veneficum</i>	1,000,000	1,293,906	855,205 ± 63,224	129	86	66
Sample 2	<i>K. veneficum</i>	200,000	152,544	186,071 ± 30,541	76	93	122
Sample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

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

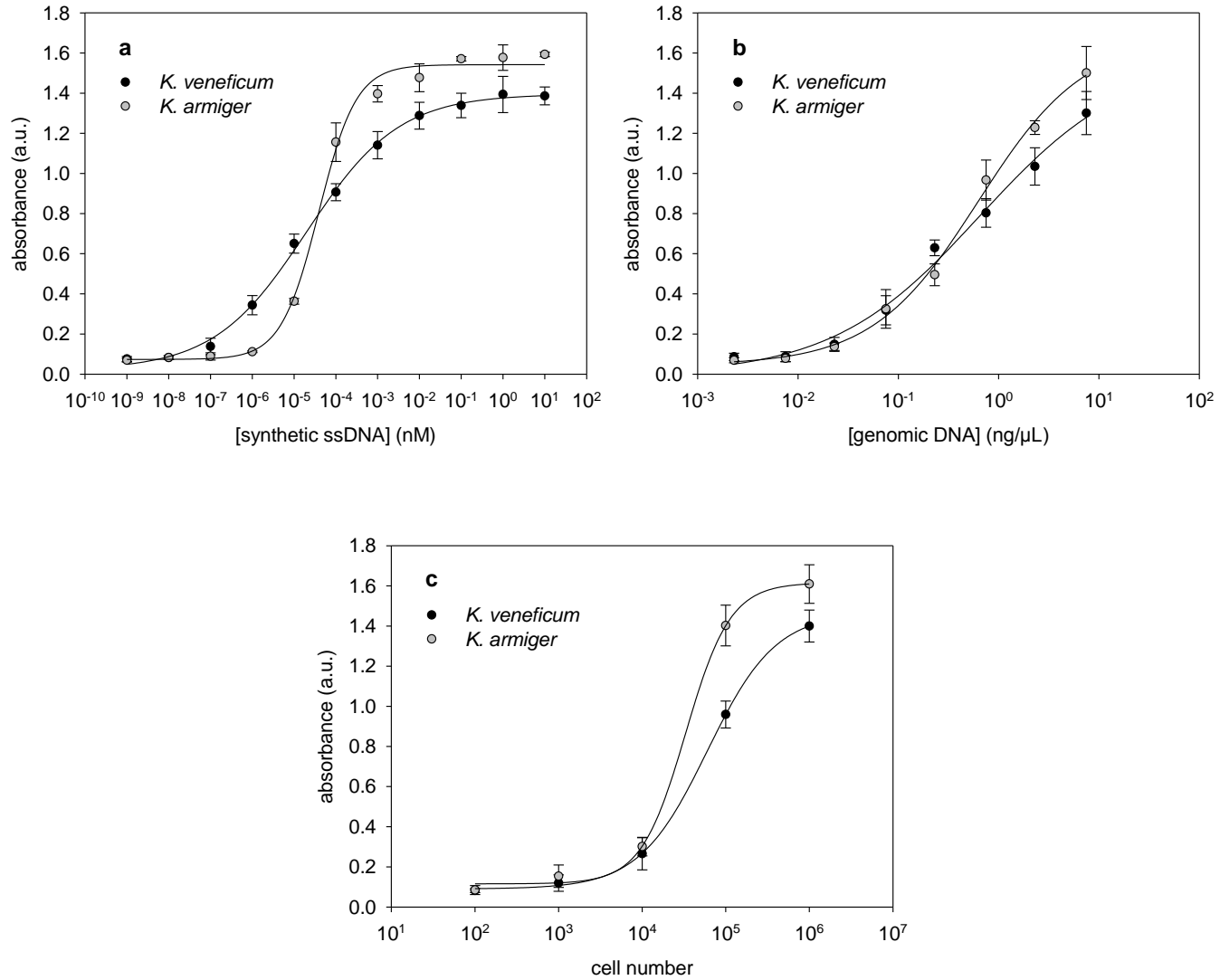


**Figure 1.** Schematic representation of the RPA-ELONA method for *K. veneficum* and *K. armiger*: (a) RPA with tailed primers and (b) ELONA detection.





**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.

