Colorimetric DNA-based assay for the specific detection and quantification of *Ostreopsis* cf. *ovata* and *Ostreopsis* cf. *siamensis* in the marine environment

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Abstract

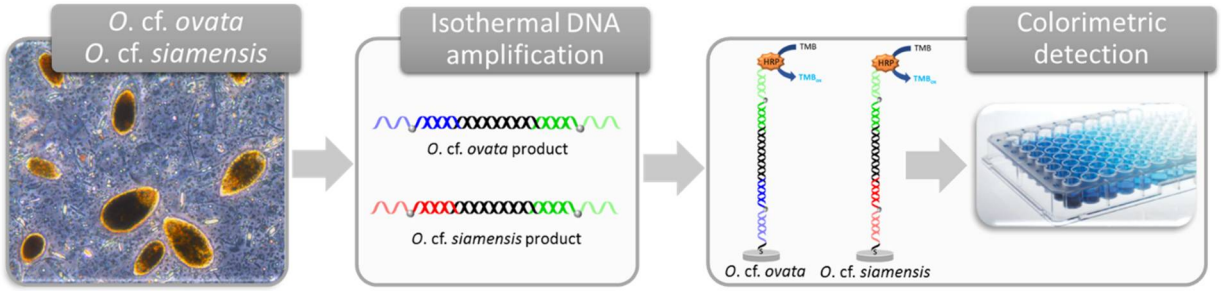
Ostreopsis is a toxic benthic dinoflagellate largely distributed worldwide in tropical and temperate areas. In the Mediterranean Sea, periodic summer blooms have been reported and have become a serious concern due to their direct impact on human health and the environment. Current microalgae identification is performed via light microscopy, which is time-consuming and is not able to differentiate among *Ostreopsis* species. Therefore, there is mature need for rapid, specific and easy-to-use detection tools. In this work, a colorimetric assay exploiting a combination of recombinase polymerase amplification (RPA) and a sandwich hybridisation assay was developed for *O.* cf. *ovata* and *O.* cf. *siamensis* detection and quantification. The specificity of the system was demonstrated by cross-reactivity experiments and calibration curves were successfully constructed using genomic DNA, achieving limits of detection of 10 and 14 pg/µL for *O.* cf. *ovata* and *O.* cf. *siamensis*, respectively. The assay was applied to the analysis of planktonic and benthic environmental samples from different sites of the Catalan coast. Species-specific DNA quantifications were in agreement with qPCR analysis, demonstrating the reliability of the colorimetric approach. Significant correlations were also obtained between DNA quantifications and light microscopy counts. The approach may be a valuable tool to provide timely warnings, facilitate monitoring activities or study population dynamics, and paves the way towards the development of *in situ* tools for the monitoring of harmful algal blooms.

Keywords (6 max): *Ostreopsis* spp.; *O.* cf. *ovata*; *O.* cf. *siamensis*; monitoring; recombinase polymerase amplification (RPA); colorimetric DNA-based assay.

Highlights:

- A colorimetric DNA-based assay to detect two *Ostreopsis* species is developed.
- The assay exploits the isothermal recombinase polymerase amplification technique.
- The assay is specific and enables the determination of 10-14 pg/µL of target DNA.
- The assay was tested in planktonic and benthic samples from the Catalan coast.
- Results of the assay were in agreement with qPCR analysis and light microscopy counts.

Graphical abstract:



1. Introduction

Ostreopsis (Schmidt, 1901) is a genus of toxic epi-benthic marine dinoflagellates generally recorded in tropical and subtropical seas, but its occurrence in temperate areas has increased markedly in the last years. In specific areas of the Mediterranean Sea, periodic *Ostreopsis* blooms have been reported during the summer-autumn season since 2000, especially in shallow waters characterised by rocky substrates where macroalgae attach (Accoroni and Totti, 2016; Berdalet et al., 2017). *Ostreopsis* blooms near certain beaches are associated to respiratory and cutaneous irritations in humans through direct contact with marine aerosols and/or seawater (Gallitelli et al., 2005; Vila et al., 2016). Additionally, some *Ostreopsis* species produce palytoxin (PLTX) and/or PLTX-like compounds, which are related to mass death of benthic marine organisms (e.g. sea urchin) and can bioaccumulate in shellfish (Aligizaki et al., 2008; Amzil et al., 2012; Mangialajo et al., 2011). In this sense, *Ostreopsis*-related seafood poisoning has been reported in tropical regions, but not yet in the Mediterranean (Aligizaki et al., 2011; Berdalet et al., 2017; Vilarino et al., 2018). *O.* cf. *ovata* and *O.* cf. *siamensis* have been recurrently identified in blooms along the Mediterranean coast (Aligizaki and Nikolaidis, 2006; Battocchi et al., 2010; Penna et al., 2005; Vila et al., 2001), where they are typically found together. Whilst *O.* cf. *ovata* is more widely distributed and produces high amounts of PLTX-like compounds (i.e. ovatoxins) (Ciminiello et al., 2012; García-Altares et al., 2015), *O.* cf. *siamensis* strains have been reported as non-toxic (Ciminiello et al., 2013). Recently, *O. fattorussoi* has been described in the eastern Mediterranean coast, and shown to produce low toxin amounts (Accoroni et al., 2016; Tartaglione et al., 2016).

Interest in monitoring *Ostreopsis* spp. abundances has increased recently due to the biogeographical expansion of this genus. Monitoring activities are regularly performed in regions affected by these blooms in order to satisfy the sanitary regulatory requirements for bathing waters. Although no European or international official thresholds have been proposed, some countries such as Italy and Spain, where respiratory and skin symptoms were first described, have defined alarm thresholds for *Ostreopsis* spp. of ~10000-30000 cells/L of seawater and 100000 cells/g fresh weight of macroalgae (fwm). Similarly, a warning threshold of 30000 cells/L and an alarm threshold of 100000 cells/L have been proposed in France (Giussani et al., 2017; Vassalli et al., 2018). The most commonly applied strategy for benthic microalgae monitoring is based on seawater and/or macroalgae sampling, with subsequent microalgae identification and enumeration by light microscopy following the Utermöhl cell-counting method. However, light microscopy requires a high level of taxonomic expertise, in addition to being time intensive and impractical for processing a large number of samples (Vassalli et al., 2018). Furthermore, correct identification of *Ostreopsis* species is extremely difficult due to the wide variability in morphological and morphometric features within each species (Penna et al., 2005).

Progress in molecular taxonomy has favored the development of molecular techniques for microalgae detection. These techniques offer significant advantages compared to conventional optical techniques since they are rapid and species-specific, offering the possibility to provide timely monitoring and to correctly identify *Ostreopsis* species (Penna et al., 2007). Species-specific identification is a critical issue for coastal management given that *Ostreopsis* species present different toxicities and can produce different PLTX-like compounds. In this sense, PCR and quantitative PCR (qPCR) have been used to detect and quantify *Ostreopsis* spp. in different environmental samples including seawater, marine aerosols, macroalgae and mussels. So far, PCR/qPCR assays exist for O. cf. *ovata* (Battocchi et al., 2010; Casabianca et al., 2014;

Perini et al., 2011), *O.* cf. *siamensis* (Battocchi et al., 2010; Casabianca et al., 2013) and *O. fattorussoi* (Vassalli et al., 2018). Despite being increasingly explored for microalgae detection, PCR-based methods inherently require a power supply and precise temperature control, thus hindering its use for *in situ* testing as well as its incorporation in easy-to-use miniaturised devices. Therefore, innovative molecular approaches overcoming such problems are required.

Isothermal DNA amplification methods can address these requirements since they are performed at a constant temperature. In recent years, several isothermal techniques have been described, including nucleic acid sequence-based amplification (NASBA), strand displacement amplification (SDA), rolling circle amplification (RCA), loop-mediated isothermal amplification (LAMP), helicase-dependent amplification (HDA) and recombinase polymerase amplification (RPA) (Deng and Gao, 2015). The latter is particularly attractive due to its rapidity, simplicity, high sensitivity and selectivity (Lobato and O'Sullivan, 2018). It only requires two primers and operates at a low and constant temperature of about 37-42 °C, without the need for an initial thermal denaturation step to generate single stranded DNA (ssDNA) from the double stranded DNA (dsDNA) target. In contrast to PCR, RPA does not employ thermal cycling but a mixture of three core proteins (a recombinase, a single-stranded DNA-binding protein and strand-displacing DNA polymerase) to achieve amplification. The RPA process starts when the recombinase protein binds to primers, forming complexes with homologous DNA in a duplex target, forcing displacement of the non-complementary strand. The displaced DNA strand is stabilised by single-stranded DNA-binding proteins, thus preventing the dissociation of the primer and facilitating hybridisation of the duplex target. Finally, the strand-displacing DNA polymerase binds to the 3' end of the primer and copies the DNA, achieving exponential amplification (Piepenburg et al., 2006). RPA has been used to amplify diverse targets, including RNA, ssDNA and dsDNA, of a wide variety of organisms such as bacteria, virus, protozoa, animals and plants, from diverse sample types. However, reports detailing the use of isothermal amplification methods to detect toxic microalgae are scarce (Toldrà et al., 2018b).

Within this context, we propose a colorimetric assay for the detection of *O.* cf. *ovata* and *O.* cf. *siamensis* that exploits RPA, using species-specific primers designed to bind within the ribosomal DNA (rDNA). These primers are designed to render a dsDNA amplicon with one ssDNA tail at each end (Fig. 1a), which is subsequently detected via a colorimetric sandwich hybridisation assay (i.e. enzyme-linked oligonucleotide assay, ELONA) (Fig. 1b). Detection is achieved using complementary oligonucleotide probes: a thiolated capture probe (specific for each *Ostreopsis* species) immobilised on maleimide-coated microtitre plates and a horseradish peroxidase (HRP)-labelled reporter probe (common for both *Ostreopsis* species), which is used to produce a change in colour following substrate addition. Whilst most sandwich hybridisation assays involve a melting step of the amplified DNA prior to the detection, the use of tailed primers bypasses this step, thus reducing complexity and assay time. The specificity of the RPA-ELONA was assessed by cross-reactivity experiments. Subsequently, limits of detection (LODs) were determined by constructing calibration curves using genomic DNA. Finally, environmental samples collected along the Catalan coast were analysed using our approach and the results compared with qPCR and light microscopy analysis.

2. Materials and Methods

2.1. Reagents and materials

Non-treated polystyrene Nunc flasks, 24-well Nunc microplates, Pierce maleimide-activated plates, GeneJET PCR purification kit and SYBR® Green dye were obtained from Thermo Fisher Scientific (Spain). TwistAmp Basic kit was purchased from TwistDx (UK). Custom DNA oligonucleotides were synthetised by Biomers (Germany). Proteinase K, 6-mercapto-1-hexanol, TWEEN® 20, 3,3',5,5'-tetramethylbenzidine (TMB) liquid substrate, chloroform, phenol:chloroform:isoamylalcohol (25:24:1, v:v:v), ethidium bromide and all other reagents were acquired from Sigma-Aldrich (Spain).

2.2. *Ostreopsis* cultures

Strains IRTA-SMM-16-133 of *O.* cf. *ovata* and IRTA-SMM-16-84 of *O.* cf. *siamensis* were isolated from macroalgae samples, mostly *Jania rubens* and *Corallina elongata*, collected in La Fosca (northern coast of Catalonia, Spain) in August 2016. Strain IRTA-SMM-16-135 of *O. fattorussoi* was isolated from seawater samples collected in Rhodes (Greece) in August 2016. Cells were isolated with a glass pipette by the capillary method and cultivated, first in 24-well microplates and then in polystyrene flasks. Clonal cultures were grown in 5-fold (Guillard, 1973; Guillard and Ryther, 1962) diluted f/2 medium at a practical salinity of 36. Cultures were maintained at a temperature of 24 ± 2 °C with a photon irradiance of 110 µmol photons m^{-2} s^{-1} under a 12:12 h light:dark photoperiod. Culture aliquots were fixed with 3% lugol's iodine and counted under an inverted light microscope (Leica DMIL, Spain) following the Utermöhl method (Utermöhl, 1958). All cultures were collected at the exponential phase (7 days). Pellets containing 10^5 cells were prepared by centrifugation (4500 rpm, 25 min) and stored at -20 °C until DNA extraction.

The ITS and 5.8 regions of *Ostreopsis* species rDNA genes was PCR-amplified using ITSA/ITSB primers (Sato et al., 2011), bi-directionally sequenced (Sistemas Genómicos, LLC, Spain), edited using BioEdit v7.0.5.2 and phylogenetically analysed using MEGA 5.1. *O.* cf. *ovata* and *O.* cf. *siamensis* were grouped within the Atlantic/Mediterranean/Pacific and the Atlantic/Mediterranean clade, respectively. Sequences were deposited in GenBank (IRTA-SMM-16-133: MH790463, IRTA-SMM-16-84: MH790464, IRTA-SMM-16-135: MH790465).

2.3. Environmental samples

Sampling was performed in August 2017 at 9 sampling stations, distributed in 4 locations of the Catalan coast where *Ostreopsis* spp. blooms commonly occur (Fig. 2 and Table 1 SI). In each station, seawater (planktonic) samples and macroalgae (benthic) samples were taken, except for stations 4 and 5 where only seawater samples were collected. First, seawater samples (2 L) were collected at approximately 50 cm above the macroalgae substrates (< 1.5 m depth). Macroalgae substrates (100-200 g fwm) were collected by hand and placed in a polystyrene bottle containing 2 L of seawater. Bottles were vigorously shaken for 1 min to release the epiphytic cells. Samples were then filtered through a 200-µm mesh to remove larger particles. Planktonic and benthic samples were fixed in 3% lugol's iodine solution. For each sample, 50 mL were centrifuged (4500 rpm, 25 min) and pellets stored at -20 °C until DNA extraction and subsequent molecular analysis by RPA-ELONA and qPCR, and 50 mL were stored at 4 °C until microscopy analysis.

2.4. DNA extraction

Extraction of genomic DNA from cultures and environmental samples was carried out using the phenol/chloroform/isoamylalcohol method as described in (Toldrà et al., 2018a). In short, cell pellets were re-suspended in 200 µL of lysis buffer (1 M NaCl, 70 mM Tris, 30 mM EDTA, pH 8.6), 25 µL of 10% w/v DTAB and 200 µL of chloroform, and then disrupted using a BeadBeater-8 (BioSpec, USA) pulsed for 45 s at full speed. After centrifugation, the aqueous phase was transferred to a fresh tube and DNA was extracted using standard phenol/chloroform procedures (Sambrook et al., 1989). Precipitation of the DNA from the final aqueous solution was obtained by the addition of 2 volumes of absolute ethanol and 0.1 volume of 3 M sodium acetate (pH 8). The DNA was rinsed with 70% v/v ethanol and dissolved in 50 µL of molecular biology-grade water. Genomic DNA was quantified and checked for its purity using a NanoDrop 2000 spectrophotometer (Thermo Fisher Scientific, Spain), and stored at -20 °C until analysis.

2.5. Primer design

Primers used in this study were based on PCR species-specific primers for *O.* cf. *ovata* and *O.* cf. *siamensis* designed within the ITS1-5.8S rDNA region reported by Battocchi et al. (2010), which include: one genus-specific (for *Ostreopsis*) and two species-specific (for *O.* cf. *ovata* and *O.* cf. *siamensis*) primers. Primers were elongated to have a length of 26 bp and a GC content of about 45% and were modified with oligonucleotide tails, resulting in amplicons of dsDNA flanked by ssDNA tails, which allow detection of the RPA product through complementary capture and reporter probes (Fig. 1). Both primer sets amplified a product of 148 bp. Primer and probe sequences are detailed in Table 1. Primers, tails and probes were examined *in silico* using BLAST analysis. The specificity of the primers was tested by electrophoresis of the RPA products in 2% w/v agarose gel using purified genomic DNA.

2.6. Colorimetric DNA-based assay

RPA reaction was performed with the TwistAmp Basic kit. Briefly, each 50-µL RPA reaction contained: 2.4 µL of each forward and reverse primer (10 µM for *O.* cf. *ovata* and 5 µM for *O.* cf. *siamensis*), 2.5 µL of magnesium acetate (480 mM), 14.75 µL of rehydration buffer, 22.95 µL of molecular biology-grade water, 1/2 enzyme pellet and 5 µL of genomic DNA extracted from: a) cultures, for the specificity tests (1 ng/µL) and for the calibration curves (4-fold serial dilutions: from 10 to 0.002 ng/µL); and b) environmental samples. All reagents were prepared in a master mix with the exception of the DNA and magnesium, which was added to initiate the reaction. The reaction took place in a Nexus Gradient Thermal Cycler (Eppendorf Ibérica, Spain) with a fixed temperature of 37 °C for 30 min. RPA reactions were performed in triplicate and positive controls and blanks (NTC = no template control) were included. To evaluate the need to clean-up the RPA product before ELONA detection, two treatments were tested. In the first treatment, RPA products were purified using GeneJET PCR purification kit following the manufacturer instructions, with a final elution step with 50 µL of TE buffer. In the second treatment, proteins were digested by adding 5 µL of proteinase K (2 mg/mL) to the 50-µL RPA product following incubation at 37 °C for 10 min further 80 °C for 10 min.

For the ELONA, maleimide-coated microtitre plates were rinsed three times with 200 µL of PBS-Tween (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 PBS

(100 mM phosphate, 150 mM NaCl, pH 7.4) were added. Blocking of any non-functionalised maleimide groups was achieved via incubation with 200 µL of 100 µM 6-mercapto-1-hexanol in Milli-Q water. A subsequent blocking step was performed by the addition of 200 µL of 5% w/v skimmed milk in PBS. Subsequently, 45 µL of RPA product (50 µL when proteinase K was added) were dispensed into each well and, in the following step, 50 µL of 10 nM HRP-conjugated reporter probe in PBS-Tween were added. Finally, 100 µL of TMB liquid substrate were added and, after 10 min, the absorbance was read at 620 nm using a Microplate Reader KC4 (BIO-TEK Instruments Inc., USA). All steps were performed with agitation at room temperature for 30 min, except for the thiolated capture probe immobilisation step, which was incubated at 4 °C overnight. After each step, microtitre plates were washed three times with 200 µL of washing buffer. Quantifications of 50-mL environmental samples are expressed as ng/µL of genomic DNA of specific *Ostreopsis* species in 50 µL of extracted DNA.

2.7. qPCR and light microscopy analysis

For the qPCR assay, species-specific primers for *O.* cf. *ovata* and *O.* cf. *siamensis* described in Battocchi et al. (2010) were used. The qPCR conditions included 45 cycles of amplification following a three-step protocol (94 °C for 20 s, 54 °C for 30 s and 72 °C for 30 s) and a final step for melting temperature curve analysis at 60 °C for 1 min with a gradual increase of temperature (1 °C/15 s) (Carnicer et al., 2015). Reactions were performed using an ABI 7300 thermocycler (Thermo Fisher Scientific) in a final volume of 20 µL that contained: 10 µL of 2X SYBR Green dye, primers (final concentration 0.5 µM) and 2 µL of extracted genomic DNA. Each qPCR reaction was performed in triplicate and blanks (NTC) were included. Quantifications of 50-mL environmental samples are expressed as ng/µL of genomic DNA of specific *Ostreopsis* species in 50 µL of extracted DNA.

For light microscopy counts, fixed environmental samples were counted following the Utermöhl method (Utermöhl, 1958) under an inverted microscope, as implemented in the monitoring program. Planktonic samples were settled in 50-mL chambers for 24 h and benthic samples in 3-mL or 10-mL chambers for 4 or 8h, respectively. Counting was performed across transects or in the whole chamber to count a minimum number of 100 *Ostreopsis* spp. cells per sample (when possible). *Ostreopsis* spp. (at genus level) and other planktonic and benthic species were considered. Cell abundance is reported as cells/L for planktonic samples and cells/g fwm for benthic samples. LODs were 20 cells/L (50-mL chambers), 100 cells/L (10-mL chambers) and 336 cells/L (3-mL chambers).

2.8. Data analysis and statistics

RPA-ELONA calibration curves using dilutions of genomic DNA were fitted to a sigmoidal logistic four-parameter equation using SigmaPlot 12.0 (Systat Software Inc., California, USA). The LOD was the concentration of DNA that increased absorbance above the blank (NTC) value plus three times its standard deviation (SD). Environmental samples were quantified using the obtained equations. For the qPCR assay, calibration curves using dilutions of genomic DNA were constructed and accepted when the slope was between 3.2 and 3.4 (95-105% efficiency). LODs for the qPCR assay were 1 pg/µL of genomic DNA for *O.* cf. *ovata* and *O.* cf. *siamensis*.

Correlation between RPA-ELONA and qPCR measurements was assessed using Pearson's correlation coefficient (*r*). Quadratic polynomial regression was used to determine the relationship between RPA-ELONA and light microscopy

206 counts for both benthic (cells/g fwm) and planktonic (cells/L) samples. Due to the different type of samples and sampling
207 methodology, the regression analysis was performed separately for planktonic and benthic samples. Predicted cell
208 abundances were obtained from the regression model. The correlation between predicted and observed values was then
209 analysed using Pearson's correlation coefficient (*r*). Data analyses were performed with IBM SPSS Statistics 23.0 (IBM
210 Corp., New York, USA).

3. Results and discussion

3.1. Primer design

There are several reports demonstrating that PCR primers can be also used in RPA (Mayboroda et al., 2016; Toldrà et al., 2018b; Yamanaka et al., 2017). However, when species-specific PCR primers for *O*. cf. *ovata* and *O*. cf. *siamensis* (20-22 bp in length) were used in RPA, extremely high LODs (> 1 ng/µL) were obtained in the ELONA. Consequently, primers were re-designed following RPA primer design recommendations (Appendix to the TwistAmp reaction kit manuals): primers of 30-35 bp in length with a GC content between 40-60% that amplify targets between 100 and 200 bp. Following optimisation, primers were 26 bp in length with a GC content of 45%, amplifying targets of 148 bp. Longer primers could not be designed due to the potential presence of primer-dimers as checked using Multiple Primer analyser software (Themo Fisher Scientific). Results demonstrated that the LODs were remarkably improved (10 pg/µL in front of 981 pg/µL; see these LODs in section 3.4) when using the re-designed primers. The need for longer primers in RPA may be explained because of the different mechanisms for amplification: thermal versus isothermal for PCR and RPA, respectively.

3.2. Purification of RPA products

DNA purification of RPA products is generally required before detection, since LODs are usually lower. Nevertheless, to simplify the assay and reduce the use of reagents and equipment, the requirement for a cleaning step after amplification in our assay was evaluated. Results are shown in Fig 3. Although no significant differences were observed in the specific signal, non-specific detection varied depending on the cleaning process, being more evident for *O*. cf. *siamensis*. The highest non-specific values were obtained when no treatment was performed, which may be attributed to the presence of proteins and residual primers. The use of proteinase K decreased this background, although it was higher than when using the commercial kit, suggesting that proteinase K properly digested proteins but had no effect on removing excess primers. Nonetheless, LODs were not significantly different between treatments or even if no treatment was applied, and therefore subsequent experiments were carried out without treatment. In an effort to decrease the non-specific adsorption for *O*. cf. *siamensis*, which was very high in absolute absorbance values, the use of lower primer concentrations in the RPA reaction was tested. As can be seen in Fig. 3b, non-specific adsorption was notably decreased when using a primer concentration reduced by half, and thus these conditions were selected for *O*. cf. *siamensis*.

3.3. Specificity of the RPA-ELONA

To evaluate the specificity of the RPA-ELONA assay, cross-reactivity experiments were performed, where different primers (*O*. cf. *ovata* and *O*. cf. *siamensis* primers) were tested with different capture probes (*O*. cf. *ovata* and *O*. cf. *siamensis* capture probes) in the presence of considerable amounts (1 ng/µL) of genomic DNA from various *Ostreopsis* species (*O*. cf. *ovata*, *O*. cf. *siamensis* and *O. fattorussoi*). For *O*. cf. *ovata* (Fig. 4a), specific detection was achieved when combining *O*. cf. *ovata* primers, *O*. cf. *ovata* capture probe and target *O*. cf. *ovata* DNA, either using single *O*. cf. *ovata* DNA or a mixture of genomic DNA from *O*. cf. *ovata* and *O*. cf. *siamensis*. On the other hand, no significant responses were observed when non-target genomic DNA from other *Ostreopsis* species (i.e. *O*. cf. *siamensis* and *O. fattorussoi*) or NTC were used. Furthermore, all other combinations of primers and capture probes also provided no significant

responses. Similar results were obtained for *O*. cf. *siamensis* (Fig 4b): specific detection was only achieved when the corresponding *O*. cf. *siamensis* primers and capture probes were used with target *O*. cf. *siamensis* DNA, without any other signal observed using non-target DNA (i.e. *O*. cf. *ovata* and *O. fattorussoi*) and NTC. These results demonstrate the high specificity of the system and the ability to discriminate between *O*. cf. *ovata* and *O*. cf. *siamensis* in the presence of background genomic DNA from non-target *Ostreopsis* species present in the Mediterranean.

3.4. Calibration curves

The sensitivity of the colorimetric assay was assessed using serial dilutions of genomic DNA extracted from clonal cultures of *O*. cf. *ovata* and *O*. cf. *siamensis*. Based on the calibration curves obtained (Fig. 5), the LODs achieved for *O*. cf. *ovata* and for *O*. cf. *siamensis* were 10 pg/µL (50 pg) and 14 pg/µL (70 pg) per well, respectively. Using *Ostreopsis* cultures, it was experimentally found that the DNA content per cell was 12 pg for *O*. cf. *ovata* and 4 pg for *O*. cf. *siamensis,* thereby LODs for the colorimetric assay could be expressed as 4 cells for *O*. cf. *ovata* and 19 cells for *O*. cf. *siamensis*. Taking into account that these values are obtained from 5 µL of extracted DNA, LODs were 40 cells for *O*. cf. *ovata* and 190 cells for *O*. cf. *siamensis* in the 50-mL samples, which correspond to 800 cells/L and 3800 cells/L, respectively, both far below the Spanish alarm thresholds.

The use of genomic DNA to construct calibration curves and subsequently determine the LOD of the assay has been used in molecular methods for microalgae detection. In this work, LODs for the qPCR assay were 1 pg/µL (2 pg) of genomic DNA for *O*. cf. *ovata* and *O*. cf. *siamensis*. The PCR-based assay described by (Battocchi et al., 2010), showed LODs of 1 pg of genomic DNA for both species. Other approaches involving the use of plasmid DNA to construct calibration curves have also been reported. In this regard, LODs of 10 and 2 rDNA copies were achieved by PCR (Battocchi et al., 2010) and qPCR (Casabianca et al., 2013), respectively, for *O*. cf. *ovata* and *O*. cf. *siamensis.* Although LODs of the colorimetric assay are not as low as those achieved by qPCR and despite the difficulty to compare them with approaches based on DNA copies, the assay can be used as an early warning system able to respond to current thresholds. In addition, the simplicity of the assay would allow eventually its field application, something much more difficult to envisage with qPCR.

3.5. Analysis of environmental samples

The applicability of the DNA-based assay was evaluated using 16 environmental samples (9 planktonic and 7 benthic samples) collected along the Catalan coast (Fig. 2). The samples were analysed using the colorimetric DNA-based assay, qPCR and light microscopy. Light microscopy allowed the identification of the genus *Ostreopsis*, whereas species-specific identification of *O.* cf. *ovata and O.* cf. *siamensis* was achieved using the molecular methods (colorimetric DNA-based assay and qPCR). Data are presented in Table 2.

The colorimetric RPA-ELONA assay revealed the presence of *O.* cf. *ovata* DNA in the majority of the analysed environmental samples, with the exception of planktonic samples from Palamós (stations 1 and 2) and Les Cases d'Alcanar (stations 8 and 9), and benthic samples from stations 2 and 9 (Fig. 2 and Table 2), whilst *O*. cf. *siamensis* DNA was not detected. These results are in agreement with previous studies, which report that the *O*. cf. *ovata* genotype is predominant and is found in greater frequency and abundance than that of *O*. cf. *siamensis* along the Mediterranean coasts (Battocchi et al., 2010).

Regarding DNA quantification by qPCR, as for the colorimetric assay, *O*. cf. *ovata* DNA was not detected in any of the samples from stations 2 and 9. Instead, *O*. cf. *ovata* DNA was detected in planktonic samples from stations 1 and 8. Additionally, *O.* cf. *siamensis* was present in one benthic sample (station 1), in which the two *Ostreopsis* species co-occurred. These two species have also been found together as described in other works (Battocchi et al., 2010; Vila et al., 2001). Such qPCR quantifications were below the LODs of the colorimetric assay, thereby they were not detected using the latter. When comparing both *O*. cf. *ovata* DNA quantifications (Fig. 6), an excellent agreement between molecular techniques was achieved (Pearson's $r = 0.99$; $N = 16$, $P < 0.0001$), highlighting the reliability of the colorimetric approach.

Environmental samples were analysed using light microscopy at the genus level for *Ostreospis* spp. The target *Ostreopsis* spp. was not the main component of the natural planktonic and benthic communities, which were largely dominated by diatoms (Table 2 SI). *Ostreopsis* spp. abundances in planktonic samples were always below the Spanish alarm threshold established for *Ostreopsis* spp. in seawater (10000 - 30000 cells/L) and ranged from 300 to 7600 cells/L, with the highest abundances being detected in Sant Andreu de Llavaneres (stations 3 and 4). Rough sea conditions were observed in this locality during the sampling, suggesting detachment of cells from the substrate to the water column. *Ostreopsis* spp. densities in benthic samples were also lower than the threshold of 100000 cells/g fwm, and ranged from 210 to 60710 cells/g fwm, with the highest density observed in La Fosca (station 2).

Herein, genomic DNA from cultures has been used to construct calibration curves, although the use of plasmids or cells from cultures has also been described (Andree et al., 2011; Nishimura et al., 2016; Zhang et al., 2016). Since the rDNA copy number per microalgae cell may vary depending on the species, strains, growth phase and/or environmental conditions, it is challenging to provide cell quantifications in field samples using molecular methods (Galluzzi et al., 2010; Perini et al., 2011). To increase their reliability, strategies including the use of site-specific environmental calibration curves have been described (Casabianca et al., 2014; Perini et al., 2011). Despite the good agreement achieved, such strategies are time-consuming and not useful when a rapid response is required. In this work, we propose an alternative approach to obtain cell quantifications. A quadratic polynomial regression model was used to analyse the association between *O*. cf. *ovata* DNA colorimetric quantifications and *Ostreospis* spp. light microscopy counts (all cells considered to be *O*. cf. *ovata*). Samples that were negative for both *Ostreopsis* spp. and *O.* cf. *ovata* DNA (station 8) were not included in the analysis, nor the planktonic sample from station 1, which was considered as an outlier although it did not significantly affect the overall model performance (Pearson's $r = 0.81$; $P = 0.015$). The regression model was used to predict cell abundances in the environmental samples. The relationship between the model-predicted and observed cell abundances (Fig. 7) was highly significant for both planktonic (Pearson's $r = 0.96$; $P < 0.001$) and benthic samples (Pearson's $r = 0.97$; $P = 0.001$). These results indicate the capability of the DNA-based assay to properly estimate *Ostreopsis* cell abundances, even below the proposed thresholds and regardless of the presence of other microalgae species at high concentrations, again highlighting the specificity of the method described here (Figure 7).

4. Conclusions

This study reports the development of a colorimetric approach for the detection of *O*. cf. *ovata* and *O*. cf. *siamensis*, with specificity and limits of detection sufficient to be used as an early warning protocol for toxic algae blooms. The method provided comparable results with qPCR in the quantification of *O*. cf. *ovata* and *O*. cf. *siamensis* DNA. Moreover, the approach was demonstrated to be a useful and reliable tool to estimate cell abundances in environmental planktonic and benthic samples. However, analyses of environmental samples over an extended period of time and including other geographical sites should be carried out to validate the robustness of the assay.

This method offers important advantages over traditional counting techniques: it enables species-specific identification of two significant *Ostreopsis* species, it does not require highly trained personnel, it is rapid and it allows high sample throughput. Additionally, due to the use of isothermal DNA amplifications techniques, it could be easily integrated into portable biosensor systems. The method can help to understand the dynamics of toxic microalgae blooms and improve current monitoring programs (as a tool complementary to light microcopy), which would facilitate management activities and prevent health and economic risks related to *Ostreopsis* blooms in coastal areas. The combination of rapid and specific analytical tools with adequate sampling strategies, particularly for benthic species, has great potential for *in situ* environmental monitoring.

Acknowledgements

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Table 1 Primers (underlined) and probes used in this study.

Name	Sequence (5'-3')
Forward *O.* cf. *ovata* primer with tail	gtt ttc cca gtc acg ac-C3-aca atg ctc atg cca atg atg ctt gg
Forward *O.* cf. *siamensis* primer with tail	att acg acg aac tca atg aa-C3-tga gtt tgt gtg tat ctt gca cat gc
Reverse *Ostreopsis* spp. primer with tail	tgt aaa acg acg gcc agt-C3-gca wtt ggc tgc act ctt cat aty gt
O. cf. *ovata* capture probe	gtc gtg act ggg aaa act ttt ttt ttt ttt tt-C3 thiol
O. cf. *siamensis* capture probe	ttc att gag ttc gtc gta att ttt ttt ttt ttt tt-C3 thiol
Reporter probe	HRP-act ggc cgt cgt ttt aca

Table 2 *O.* cf. *ovata* and *O.* cf. *siamensis* DNA quantifications in planktonic and benthic samples by RPA-ELONA and qPCR assay. Results (mean ± SD, n = 3) are expressed as ng/µL of specific *Ostreopsis* species in 50 µL of extracted DNA (from 50-mL samples). Samples were analysed singular by light microscopy for planktonic (cells/L) and benthic (cells/g fwm) *Ostreopsis* spp. abundances.

Station number and sample type	*O.* cf. *ovata* (ng/µL)		*O.* cf. *siamensis* (ng/µL)		*Ostreopsis* spp. abundances
	RPA-ELONA	qPCR	RPA-ELONA	qPCR	Light microscopy
1, planktonic	n.d.	0.010 ± 0.002	n.d.	n.d.	2840
1, benthic	63.721 ± 11.896	78.781 ± 6.367	n.d.	0.003 ± 2E-04	60710
2, planktonic	n.d.	n.d.	n.d.	n.d.	360
2, benthic	n.d.	n.d.	n.d.	n.d.	210
3, planktonic	0.083 ± 0.033	0.063 ± 0.021	n.d.	n.d.	7600
3, benthic	1.369 ± 0.185	2.748 ± 0.248	n.d.	n.d.	32831
4, planktonic	0.149 ± 0.069	0.098 ± 0.016	n.d.	n.d.	6620
5, planktonic	0.025 ± 0.011	0.019 ± 0.001	n.d.	n.d.	600
6, planktonic	0.064 ± 0.024	0.056 ± 0.022	n.d.	n.d.	1680
6, benthic	0.082 ± 0.016	0.083 ± 0.021	n.d.	n.d.	667
7, planktonic	0.020 ± 0.005	0.022 ± 5E-05	n.d.	n.d.	480
7, benthic	0.139 ± 0.042	0.250 ± 0.009	n.d.	n.d.	2071
8, planktonic	n.d.	0.005 ± 2E-04	n.d.	n.d.	300
8, benthic	4.220 ± 0.855	3.918 ± 0.257	n.d.	n.d.	6015
9, planktonic	n.d.	n.d.	n.d.	n.d.	n.d.
9, benthic	n.d.	n.d.	n.d.	n.d.	n.d.

n.d.: not detected

Figure 1 Principle of the colorimetric DNA-based assay. The assay involves two steps: (**a**) recombinase polymerase amplification and (**b**) colorimetric detection.

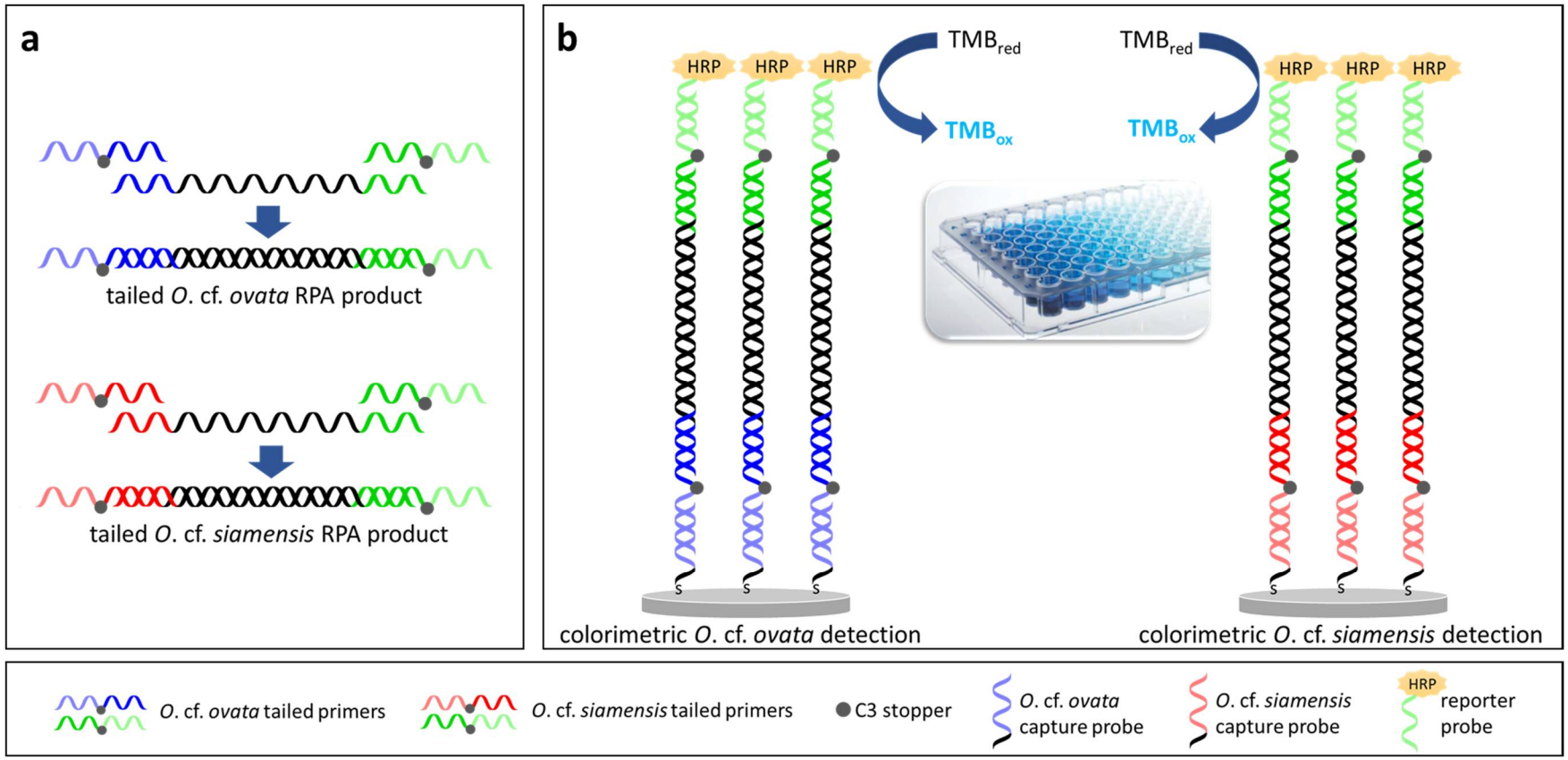


Figure 2 Location of the sampling stations in the Catalan coast. At each station, planktonic and benthic samples were collected (see Table 1. SI for geographic coordinates).

Figure 3 Comparison of different cleaning treatments (commercial kit, CK; proteinase K, PK; without treatment, WT) on RPA-ELONA results: (**a**) *O.* cf. *ovata* and (**b**) *O.* cf. *siamensis*. Target genomic DNA (1 ng/μL) and NTC were tested. *O.* cf. *siamensis* results without treatment and using half primer concentration (WT-1/2) are also shown.

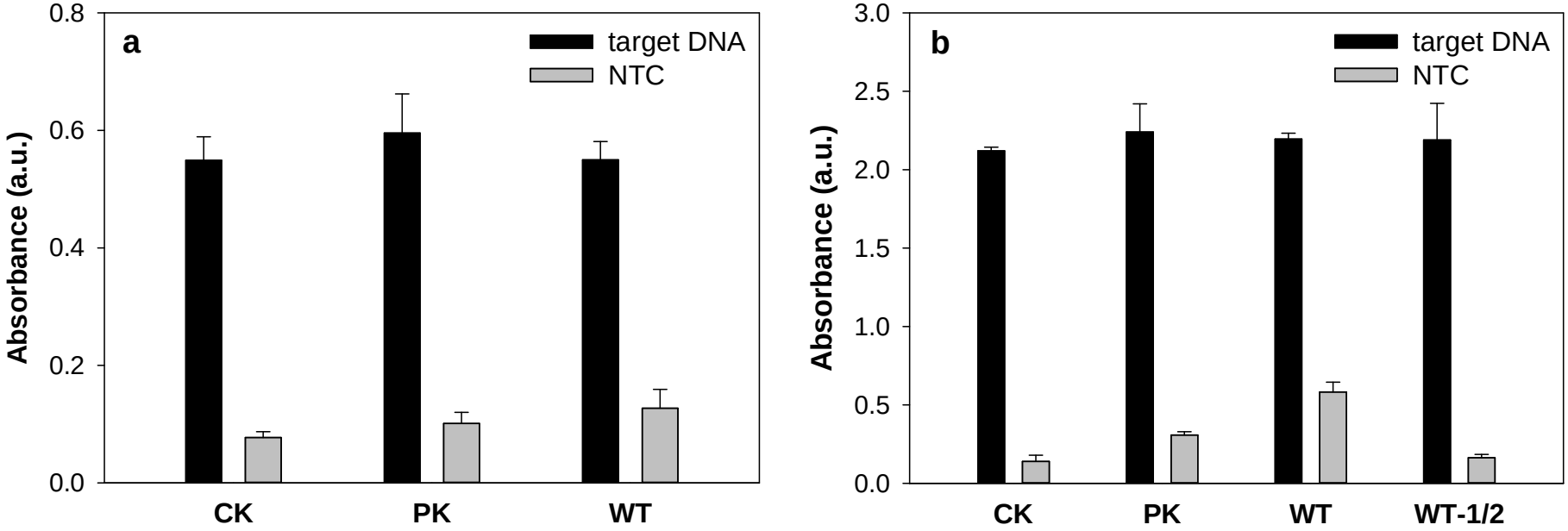


Figure 4 RPA-ELONA cross-reactivity experiments. A combination of different capture probes (*O.* cf. *ovata* in **a** and *O.* cf. *siamensis* in **b**), primers and genomic DNA (1 ng/µL) was tested. Error bars represent the standard deviation for 3 replicates. OO = *O.* cf. *ovata*, OS = *O.* cf. *siamensis*, OF= *O. fattorussoi*, NTC = no template control.

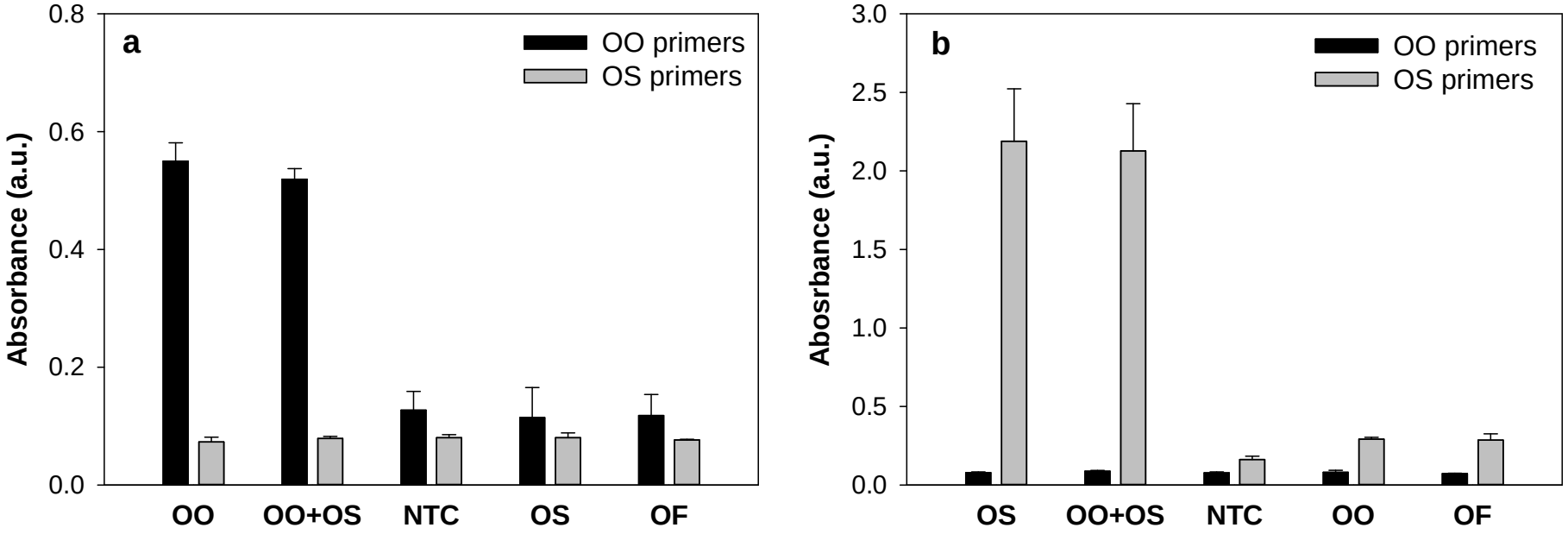


Figure 5 Calibration curves obtained using different concentrations of genomic DNA: (**a**) *O.* cf. *ovata* and (**b**) *O.* cf. *siamensis*. Errors bars are the standard deviation (3 replicates).

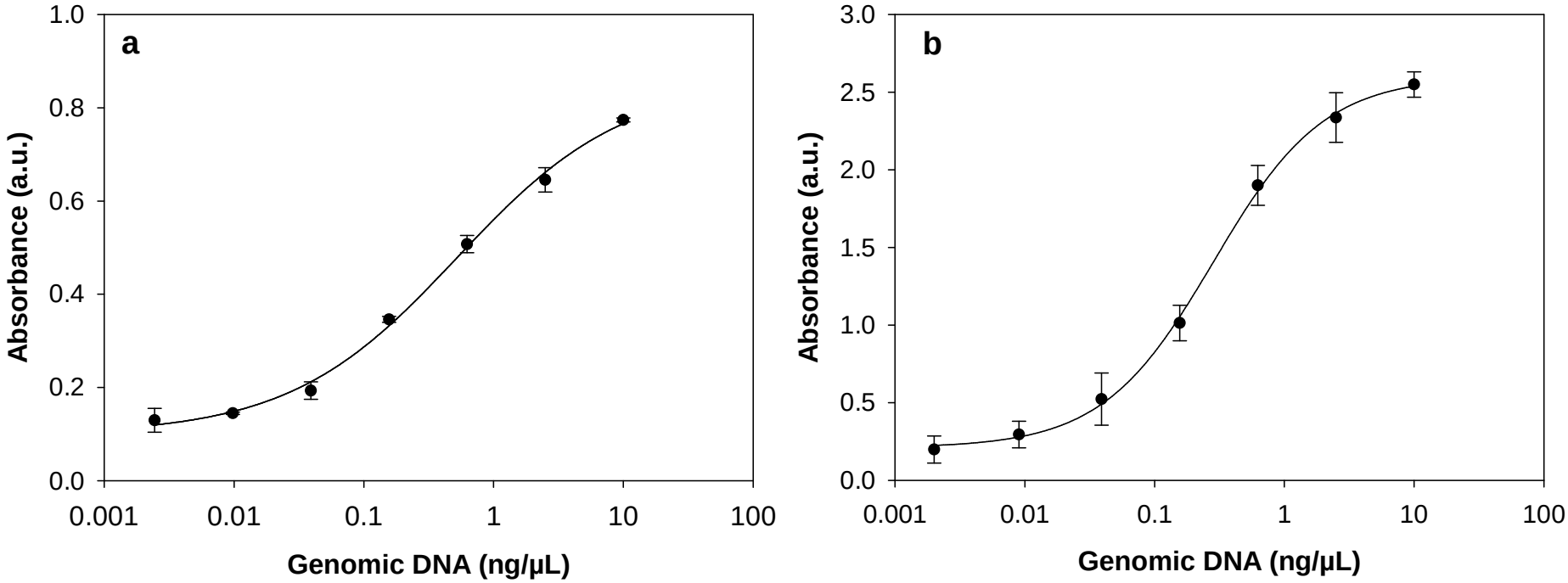


Figure 6 Correlation between *O.* cf. *ovata* DNA quantifications obtained by RPA-ELONA and qPCR in all examined planktonic and benthic samples. Pearson's correlation coefficient (*r*) is shown.

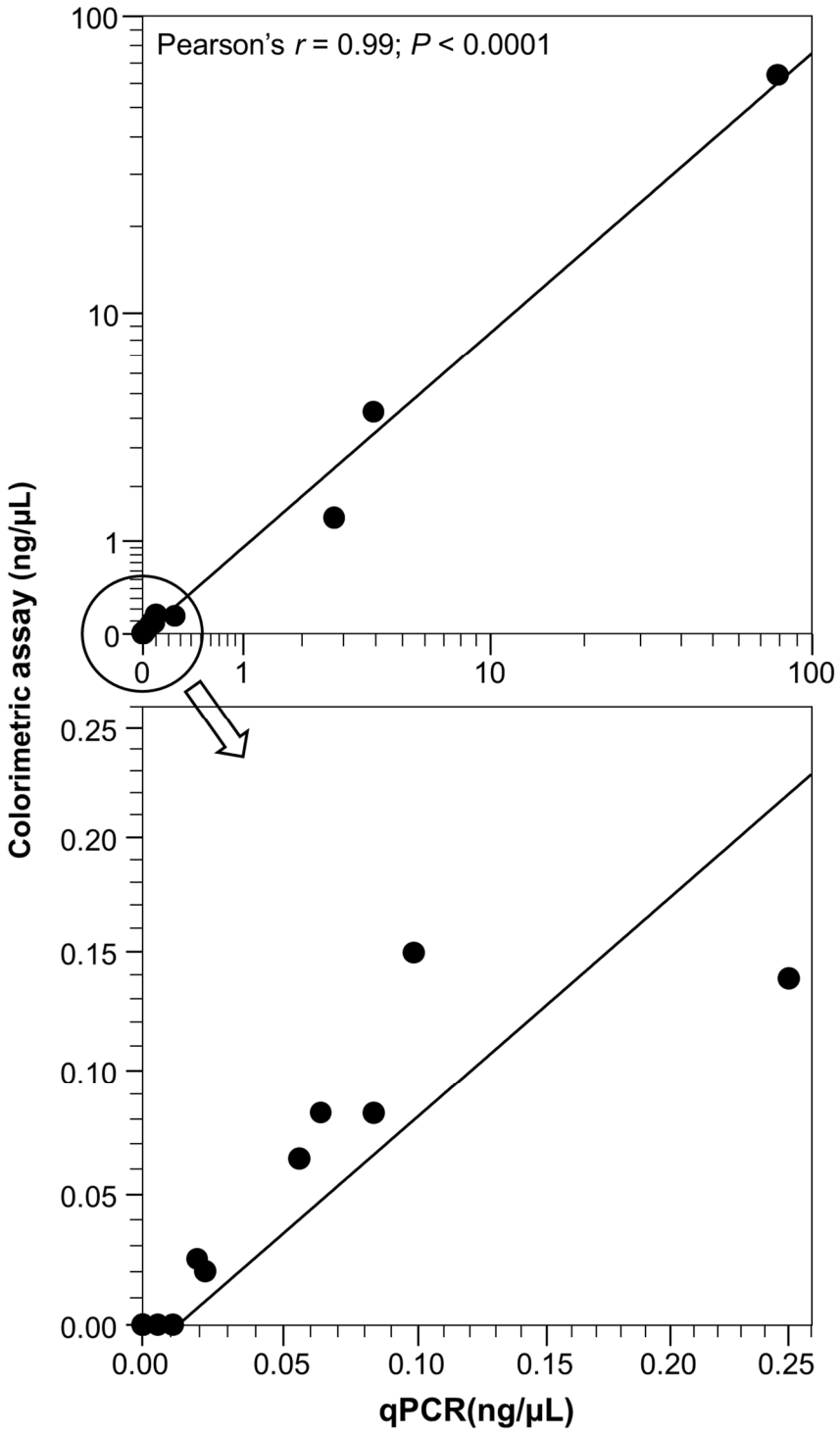
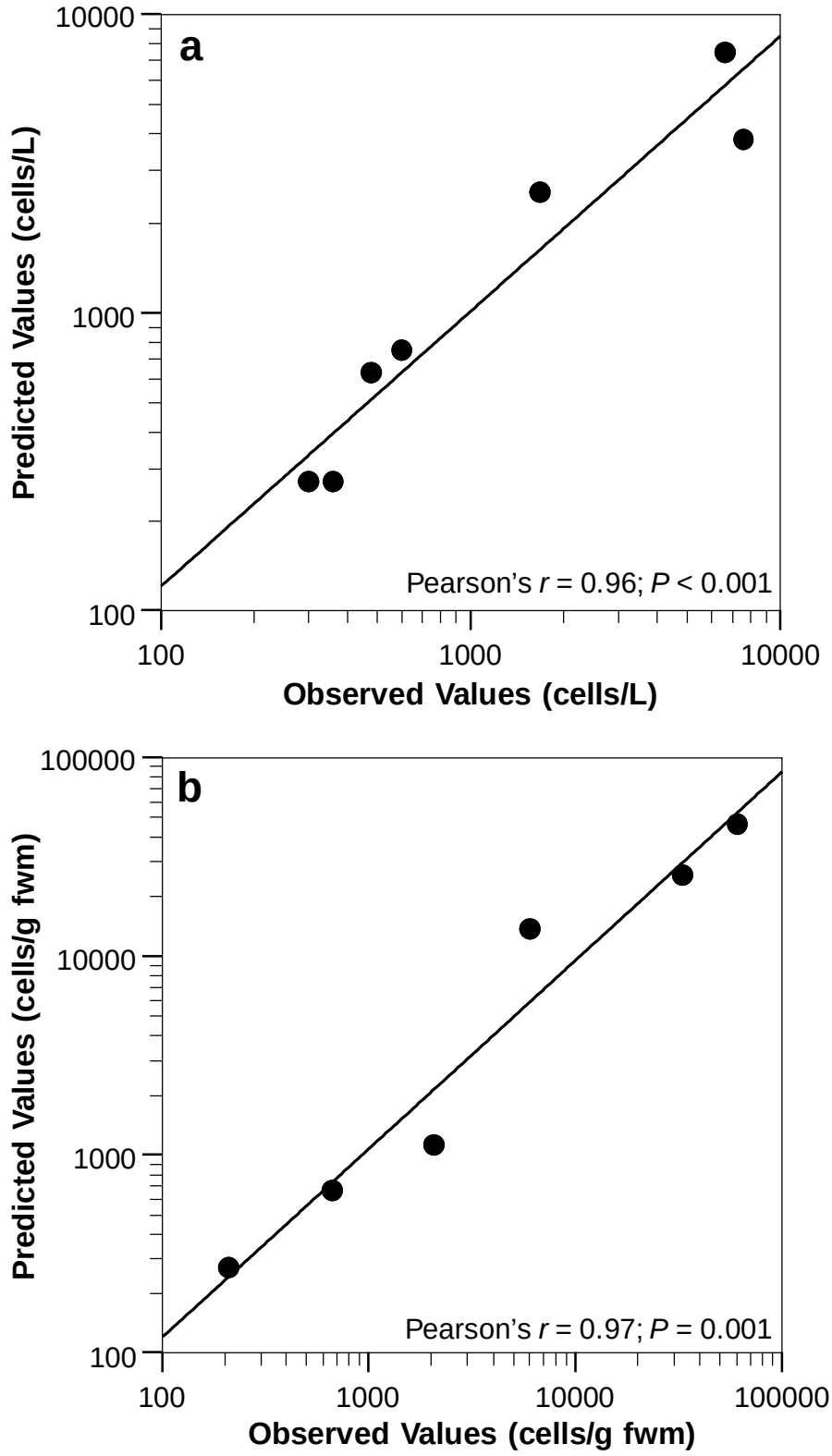


Figure 7 Relationship between the observed and predicted cell abundance values by the regression analysis: (**a**) Relationship between the observed and predicted cells/L in in planktonic samples and (**b**) Relationship between the observed and predicted cells/g fwm in benthic samples. Pearson's correlation coefficient (*r*) is shown.



SUPLEMENTARY INFORMATION

Table 1. Location and geographical coordinates of the sampling stations in the Catalan coast.

Station number	Locality	Geographic coordinates
1	Palamós, La Fosca	N 41°51'20.71'' E 3°8'32.01''
2	Palamós, La Fosca	N 41°51'28.18'' E 3°8'39.84''
3	Sant Andreu de Llavaneres	N 41°33'7.69'' E 2°29'31.66''
4	Sant Andreu de Llavaneres	N 41°33'12.25'' E 2°29'45.20''
5	Sant Andreu de Llavaneres	N 41°33'17.06'' E 2°29'54.47''
6	L'Ametlla de Mar	N 40°52'28.35'' E 0°47'43.67''
7	L'Ametlla de Mar	N 40°50'47.90'' E 0°45'44.04''
8	Les Cases d'Alcanar	N 40°32'1.00'' E 0°31'7.24''
9	Les Cases d'Alcanar	N 40°33'15.71'' E 0°31'58.71''

Table 2. Microalgae cell abundances (cells/L) determined by light microscopy in planktonic and benthic samples collected at different stations along the Catalan coast.

Class	Genus/Species	1, planktonic	1, benthic	2, planktonic	2, benthic	3, planktonic	3, benthic	4, planktonic	5, planktonic	6, planktonic	6, benthic	7, planktonic	7, benthic	8, planktonic	8, benthic	9, planktonic	9, benthic
	Pennales	3672	11356800	1377	42019824	28917	25102752	15606	3213	n.d.	41474112	3213	22556096	4590	10383360	n.d.	2683084
	Guinardia striata (Stolterfoth) Hasle	n.d.	n.d.	3672	n.d.	7803	n.d.	20655	9180	2754	n.d.	120	336	580	n.d.	1377	n.d.
	Chaetoceros Ehrenberg	4590	14294	2754	n.d.	4131	n.d.	9639	3672	918	n.d.	1377	n.d.	2754	n.d.	4131	n.d.
	Cylindrotheca closterium (Ehrenberg) Reimann & J.C.Lewin	1377	n.d.	459	n.d.	3672	n.d.	5049	4131	2295	13738	4131	n.d.	2295	n.d.	n.d.	n.d.
	Licmophora C.Agardh	2295	1554800	n.d.	473961	918	508306	1377	459	n.d.	219808	1377	164856	1836	249124	1377	535782
	Cerataulina pelagica (Cleve) Hendey	3672	n.d.	5967	n.d.	2754	n.d.	n.d.	1377	n.d.	n.d.	n.d.	n.d.	40	n.d.	n.d.	n.d.
	Coscinodiscus Ehrenberg	180	473744	160	1432494	2754	1296066	3213	20	3213	226677	n.d.	n.d.	1377	973440	20	336
	Pseudo-nitzschia H.Peragallo	1836	n.d.	1377	n.d.	459	n.d.	n.d.	1377	1377	n.d.	918	n.d.	20	n.d.	4131	n.d.
Bacillariophyceae Haeckel	*Asterionellopsis glacialis* (Castracane) Round	n.d.	n.d.	n.d.	n.d.	1377	n.d.	4131	4590	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
	Leptocylindrus minimus Gran	n.d.	n.d.	n.d.	n.d.	1377	n.d.	n.d.	1836	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
	Thalassionema nitzschioides (Grunow) Mereschkowsky	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	2295	n.d.	20	n.d.
	Proboscia alata (Brightwell) Sundström	20	n.d.	1377	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
	Striatella unipunctata (Lyngbye) C.Agardh	n.d.	n.d.	459	672	459	n.d.	n.d.	n.d.	n.d.	13738	n.d.	672	20	800	n.d.	10752
	Pleurosigma W.Smith	n.d.	n.d.	n.d.	336	n.d.	n.d.	459	n.d.	20	n.d.	n.d.	n.d.	60	500	80	336
	Guinardia flaccida (Castracane) H.Peragallo	40	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	60	n.d.
	Hemiaulus hauckii Grunowex Van Heurck	20	n.d.	60	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
	Rhizosolenia imbricata Brightwell	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	40	n.d.
	Entomoneis Ehrenberg	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	8168	n.d.	336
	Ostreopsis Johs.Schmidt	2840	4258800	360	16800	7600	1682612	6620	600	1680	52080	480	142464	300	620768	n.d.	n.d.
	Gymnodinium F.Stein	3672	49008	7803	48083	1836	0	1836	4590	2754	n.d.	2295	20607	459	n.d.	2754	n.d.
	Scrippsiella acuminata (Ehrenberg) Kretschmann, Elbrächter, Zinssmeister, S.Soehner, Kirsch, Kusber & Gottschling	n.d.	n.d.	3213	n.d.	n.d.	n.d.	1836	n.d.	918	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
	Heterocapsa F.Stein	n.d.	n.d.	n.d.	6869	n.d.	n.d.	n.d.	n.d.	459	n.d.	40	n.d.	918	n.d.	459	n.d.
	Protoperidinium Bergh	80	n.d.	40	n.d.	n.d.	n.d.	n.d.	1377	n.d.	n.d.	20	n.d.	n.d.	n.d.	n.d.	n.d.
	Gyrodinium Kofoid & Swezy	918	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	20	n.d.
Dinophyceae Fritsch	*Tripos furca* (Ehrenberg) F.Gómez	40	n.d.	n.d.	n.d.	20	n.d.	n.d.	n.d.	n.d.	n.d.	20	n.d.	n.d.	n.d.	n.d.	n.d.
	Prorocentrum lima (Ehrenberg) F.Stein	n.d.	8168	n.d.	144249	40	54952	n.d.	n.d.	n.d.	20607	n.d.	1344	n.d.	28588	n.d.	336
	Torodinium teredo (Pouchet) Kofoid & Swezy	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	40	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
	Phalacroma oxytoxoides (Kofoid) F.Gomez, P.Lopez-Garcia & D.Moreira	20	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
	Tripos fusus (Ehrenberg) F.Gómez	n.d.	n.d.	n.d.	n.d.	20	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
	Tripos trichoceros (Ehrenberg) Gómez	20	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
	Coolia A.Meunier	n.d.	n.d.	n.d.	151118	n.d.	54952	n.d.	n.d.	n.d.	144249	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
	Amphidinium Claperède & Lachmann	n.d.	n.d.	n.d.	n.d.	n.d.	61821	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
***Euglenophyceae* Schoenichen**	*Eutreptiella* A.M.da Cunha	918	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	1377	n.d.	n.d.	n.d.	n.d.	60	n.d.	918	n.d.
***Thecofilosea* Cavalier-Smith**	*Ebria tripartita* (J.Schumann) Lemmermann	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	459	n.d.	459	n.d.
***Litostomatea* Small & Lynn**	*Mesodinium rubrum* Lohmann	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	20	n.d.	n.d.	n.d.	459	n.d.	n.d.	n.d.

n.d.: not detected

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