Detection of *Ostreopsis* cf. *ovata* in environmental samples using an electrochemical DNA-based biosensor

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Abstract

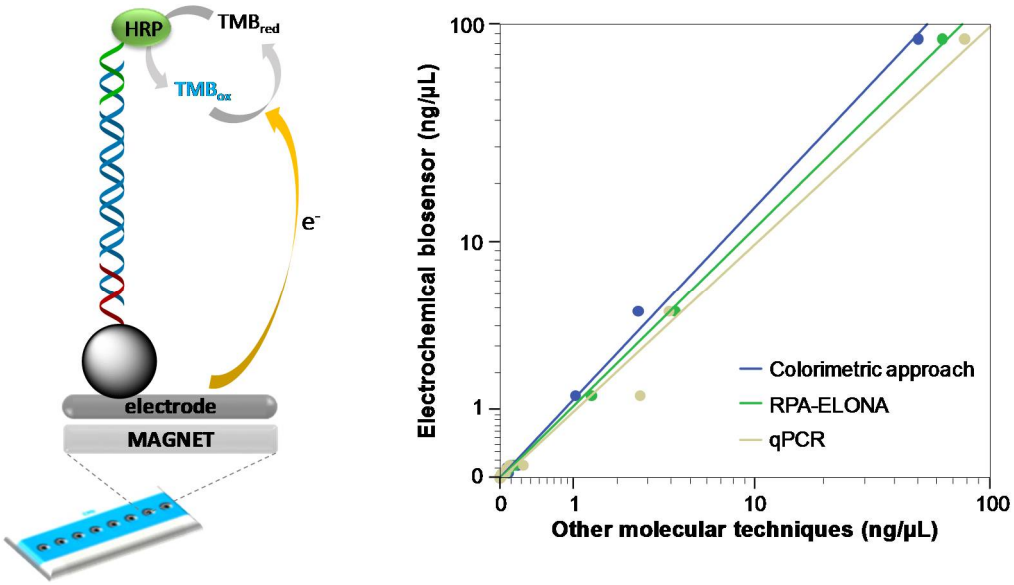
Given the increasing spread of toxic marine microalgae around the world, rapid, simple and low-cost detection tools are essential to protect human health and ecosystems. Herein, an electrochemical biosensor for the detection of *Ostreopsis* cf. *ovata*, a benthic microalgae known to produce palytoxins (PlTXs), is described. The detection strategy involves isothermal recombinase polymerase amplification (RPA) of the target using tailed primers and a sandwich hybridisation assay on maleimide-coated magnetic beads immobilised on electrode arrays. The biosensor attained a limit of detection of 9 pg/µL of *O*. cf. *ovata* DNA (which corresponds to ~ 640 cells/L), with no interferences from two non-target *Ostreopsis* species (*O*. cf. *siamensis* and O. *fattorussoi*). The biosensor was applied to the analysis of planktonic and benthic environmental samples. Electrochemical *O*. cf. *ovata* DNA quantifications demonstrated an excellent correlation with other molecular methods and allowed the construction of a predictive regression model to estimate *O*. cf. *ovata* cell abundances. The performance of the biosensor demonstrates its potential implementation as an early warning tool in marine monitoring programs.

Keywords: marine microalgae; *Ostreopsis* cf. *ovata*; recombinase polymerase amplification (RPA); maleimide-coated magnetic bead (MB); electrochemical biosensor.

Highlights:

- First electrochemical biosensor for the detection of *Ostreopsis* cf. *ovata*
- The biosensor combines isothermal amplification with magnetic beads as supports
- The application of the biosensor to environmental samples is successfully proved
- Excellent correlation with other molecular techniques and light microscopy
- A rapid, simple and inexpensive alternative tool for marine monitoring is provided

Graphical abstract:



1. Introduction

Harmful algal blooms (HABs) include *Ostreopsis* species, a benthic microalgae known to produce palytoxin (PlTX)-like compounds. While initially reported in tropical and subtropical regions, *Ostreopsis* spp. have been reported in more temperate regions such as the Mediterranean Sea (Mangialajo et al., 2011; Rhodes, 2011). In this area, periodic summer blooms have been increasing during the last decade, where different *Ostreopsis* species (*O*. cf. *ovata*, *O*. cf. *siamensis* and O. *fattorussoi*) have been described (Accoroni et al., 2016; Penna et al., 2005), with *O*. cf. *ovata* being both the most toxic and the most widely distributed (Ciminiello et al., 2010). Proliferations of *O*. cf. *ovata* have been involved in respiratory and skin distress in bathers, as well as in mass mortalities of invertebrates (Berdalet et al., 2017; Mangialajo et al., 2011; Vila et al., 2016). Additionally, PlTX-like compounds have been found in seafood (Aligizaki et al., 2008; Amzil et al., 2012), thus representing a potential risk to consumers.

HABs are natural phenomena that, realistically, cannot be eliminated. Nevertheless, monitoring programs can significantly contribute to prevent and mitigate their impacts. In this sense, *Ostreopsis* spp. monitoring is implemented in countries that regularly experience their negative effects, and is commonly performed using light microscopy (Giussani et al., 2017; Vassalli et al., 2018). However, this method is time consuming and based on morphology, which hampers correct microalgae identification, especially among *Ostreopsis* species. Emerging molecular methods have demonstrated to provide faster and more accurate identification and quantification of HABs than microscopy. PCR and quantitative PCR (qPCR) have been extensively applied to several toxic microalgae and, specifically for *Ostreopsis* species, qPCR/PCR assays have been described 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). Although qPCR is a good strategy for the detection of HAB species, alternative user-friendly and *in-situ* molecular methods able to provide even shorter analysis times and lower cost are highly desired.

Biosensors could address the needs of monitoring programs and, among them, electrochemical biosensors stand out for several reasons: high sensitivity, short analysis times, simple and inexpensive instrumentation, ease of handling, and compatibility with microfluidic systems and miniaturisation (Ronkainen et al., 2010). The combination of these outstanding properties with the inherent nucleic acid specificity positions electrochemical nucleic acid biosensors as attractive candidates for microalgae monitoring. However, there are very few reports detailing electrochemical nucleic acid biosensors for microalgae detection. Such biosensors commonly take advantage of a sandwich hybridisation format, where the target ribosomal RNA (Diercks-Horn et al., 2011; Metfies et al., 2005) or DNA is sandwiched between an immobilised capture probe and a labelled reporter probe. When targeting DNA, an amplification step is required prior to the electrochemical detection. In this respect, a biosensor combining PCR amplification and electrochemical

detection was reported for microalgae detection (LaGier et al., 2007). However, PCR methodology relies on thermal cycling, which hinders its implementation in miniaturised devices for *in-situ* testing.

Methods to isothermally amplify DNA have become increasingly popular due to the benefits of reducing instrumental requirements and power consumption, thus being more suitable for *in-situ* analysis. Among isothermal amplification methods, nucleic acid sequence-based amplification (NASBA) (Casper et al., 2004; Loukas et al., 2017), loop-mediated isothermal amplification (LAMP) (Huang et al., 2017; Zhang et al., 2014), rolling circle amplification (RCA) (Chen et al., 2015; Liu et al., 2019) and recombinase polymerase amplification (RPA) (Toldrà et al., 2019; Toldrà et al., 2018) have been applied to microalgae detection. RPA is particularly attractive due to its simplicity, high sensitivity, rapid amplification (~20-30 min), easy primer design as well as its operation at low and constant temperature (~37-42 °C) (Lobato and O'Sullivan, 2018). To date, isothermal amplification techniques have been successfully coupled with different detection techniques, including lateral flow, fluorescence, turbidity and colorimetric readout. However, the combination of isothermal amplification techniques with an electrochemical biosensor for the detection has never been reported for microalgae.

In our previous work, the combination of RPA with tailed primers for the colorimetric detection of *O*. cf *ovata* was described (Toldrà et al., 2019). Tailed primers consist of a single-stranded DNA (ssDNA) sequence (tail) that is added to the species-specific primer using a C3 spacer, which prevents the formation of phosphodiester bonds and further elongation, resulting in a double-stranded DNA (dsDNA) product flanked with ssDNA tails. This facilitates the subsequent detection through a sandwich-type format assay using complementary oligonucleotide probes: a thiolated capture probe and a labelled reporter probe. In this work, with the aim to moving towards miniaturised and compact devices, we report an electrochemical biosensor for the detection of *O*. cf. *ovata* that exploits RPA, tailed primers and maleimide-activated magnetic beads (MBs). Oligocomplexes consisting of the RPA amplicon hybridised to a capture probe-functionalised MBs were immobilised on a screen-printed carbon electrode array by magnetic capture and the resulting reduction current was measured by amperometry (Fig. 1). A colorimetric approach was tested to demonstrate the feasibility of the strategy prior to the biosensor development. The attainable LOD was determined using *O*. cf. *ovata* genomic DNA and the specificity of the biosensor was evaluated using non-target *Ostreopsis* species (*O*. cf. *siamensis* and *O*. *fattorussoi*). Additionally, the reusability of the electrodes and the stability of the capture probe-functionalised MBs was studied. The biosensor was applied to the analysis of environmental samples, and *O*. cf. *ovata* quantifications were compared with those obtained by qPCR, RPA-ELONA and light microscopy.

2. Materials and Methods

2.1. Reagents

The TwistAmp Basic kit containing all enzymes and reagents necessary for the DNA amplification was obtained from TwistDx Ltd. (Cambridge, UK). Custom DNA oligonucleotides were purchased from Biomers (Ulm, Germany). PureCube maleimide-activated MagBeads (25 µm in diameter) were supplied by Cube Biotech (Monheim, Germany). Tween-20, 3,3',5,5'-tetramethylbenzidine (TMB) liquid substrate, 6-mercapto-1-hexanol, skimmed milk powder, bovine serum albumin (BSA) and all other reagents were acquired from Sigma-Aldrich (Tres Cantos, Spain).

2.2. Equipment

Disruption of microalgae cells for subsequent DNA extraction was carried out using a BeadBeater-8 (BioSpec, Bartlesville, USA). RPA reactions were performed in a Nexus Gradient Thermal Cycler (Eppendorf Ibérica, San Sebastián de los Reyes, Spain). A NanoDrop 2000 spectrophotometer (Thermo Fisher Scientific, Madrid, Spain) was used to quantitatively and qualitatively check extracted genomic DNA. Colorimetric measurements were performed with a Microplate Reader KC4 (BIO-TEK Instruments, Inc., Winooski, USA) using Gen5 software to collect data. Arrays of eight screen-printed electrodes (DRP-8x110) and a magnetic support (DRP-MAGNET8X) were provided by Dropsens S.L. (Oviedo, Spain), and consist of 8 carbon working electrodes (2.5 mm in diameter), each with its own carbon counter electrode and Ag/AgCl reference electrode. Electrochemical measurements were performed using an 8-channel multiplexer PalmSens potentiostat (PalmSens BV, Houten, The Netherlands) controlled by PalmSens PC software. A MagneSphere Technology Magnetic Separation Stand (Promega Corporation, Madison, USA) was used for the magnetic separations.

2.3. *Ostreopsis* cultures and environmental samples

The present study employed genomic DNA extracted from: a) *Ostreopsis* cultures to perform the calibration curves and specificity tests, and b) environmental samples to evaluate the applicability of the method. Strains of *O*. cf. *ovata* (IRTA-SMM-16-133, MH790463), *O*. cf. *siamensis* (IRTA-SMM-16-84, MH790464) and *O. fattorussoi* (IRTA-SMM-16-135: MH790465) were selected: *O*. cf. *ovata* as a positive control and *O*. cf. *siamensis* and *O. fattorussoi* as negative controls. Additionally, 16 environmental samples (9 planktonic and 7 benthic samples) (Table S1) were collected in August 2017 at 9 stations along the Catalan coast (NW Mediterranean Sea) and counted as described in Toldrà et al., 2019. Pellets from both *Ostreopsis* cultures and 50 mL-environmental samples were prepared by centrifugation (4500 rpm, 25 min) and stored at -20 °C. Subsequent extraction of genomic DNA was carried out using a bead-beating system and the

phenol/chloroform/isoamylalcohol method. Extracted DNA samples (50 µL) were quantified using a NanoDrop and stored at -20 °C until RPA reaction.

2.4. RPA reaction

The primers used in this study include: one forward primer specific for *Ostreopsis* genus and one reverse primer specific for *O.* cf. *ovata*. Primers amplified a fragment of 148 bp of the ITS1-5.8S ribosomal DNA gene and were modified with oligonucleotide tails. Primers and probe sequences are listed in Table S2. RPA reaction was performed at 37 °C for 30 min. Briefly, the RPA mixture was prepared by mixing 2.4 µL of 10 µM tailed primers, 14.75 µL of rehydration buffer, 22.95 µL of molecular biology-grade water and 5 µL of genomic DNA extracted from: a) cultures of *O.* cf. *ovata* for the calibration curves (4-fold serial dilutions: from 10 to 0.002 ng/µL); b) cultures of *O.* cf. *siamensis* and *O. fattorussoi* for the the specificity study (1 ng/µL); and c) environmental samples. 1/2 of lyophilised pellet was then added and the reaction was finally triggered by addition of 2.5 µL of 480 mM magnesium acetate to a final volume of 50 µL. Positive controls and blanks (NTC = no template control) were always included.

2.5. Colorimetric and electrochemical detections

Magnetic oligocomplexes were prepared as follows: 1) 10 µL of maleimide-activated magnetic beads were transferred to a tube; 2) 100 µL of of 500 nM thiolated capture probe in binding buffer (100 mM phosphate, 150 mM NaCl, pH 7.4) were added; 3) 100 µL of 100 µM 6-mercapto-1-hexanol in binding buffer were added to block any non-functionalised maleimide groups; 4) 100 µL of 5% w/v skimmed milk in binding buffer were added to avoid non-specific adsorption and, finally, conjugates were re-suspended in 10 µL of washing buffer. When the amounts of MB varied, volumes were adjusted proportionally. Once the capture probe-MB conjugates were prepared: 5) 4.5 µL of conjugate were added to a new tube and placed on a magnetic stand to remove the supernatant; 6) 45 µL of RPA product and 45 µL of binding buffer were incubated; 7) 90 µL of 10 nM HRP-labelled reporter probe in 1% w/v BSA-washing buffer were added and 8) finally, conjugates were re-suspended in 45 µL of washing buffer. All steps were performed with agitation for 30 min at room temperature, except for the capture probe conjugation step, which was incubated at 4 °C overnight. After each step, conjugates were rinsed three times with 100 mM potassium phosphate, 150 mM NaCl, 0.05% v/v Tween-20, pH 7.4, by placing the tube on the magnetic separation stand and removing the washing buffer.

For the colorimetric approach (to evaluate the feasibility of the strategy): 9) 10 µL of the magnetic oligocomplex suspension (equivalent to 10 µL of RPA product) were placed into a new tube and the supernatant was removed; 10) 125 µL of TMB/H_2O_2 were incubated for 3 min; 11) tubes were placed on a magnetic separator stand and 100 µL were collected to read the absorbance at 620 nm.

For the electrochemical biosensor: 9) 10 µL of the magnetic oligocomplex suspension were placed on each working electrode of the 8-electrode array with a magnetic support on the reverse side; the magnetic oligocomplex was captured, and the supernatant was removed; 10) 10 µL of TMB/H_2O_2 were added and incubated for 3 min; 11) the reduction current was measured by amperometry after applying -0.2 V vs Ag/AgCl for 4 s.

For the regeneration of the electrodes, the magnet was removed and the 8-electrode arrays were rinsed with distilled water and air-dried. Fresh magnetic oligocomplexes were placed on the electrodes and the current was again recorded. The process was repeated consecutively 10 times. To evaluate the storage stability, the capture probe-coated MBs were prepared as described above, washed and aliquots were kept at 4 and -20 °C. Electrochemical signals were measured at day 0 (reference value) and at 7 and 17 days. Magnetic oligicompmexed were obtained using 1 ng/µL of *O*. cf. *ovata* genomic DNA in the RPA to both test the reusability of electrodes and the stability storage of the MBs.

2.6. Data analysis

Colorimetric and electrochemical calibration curves were fitted to the sigmoidal logistic four-parameter equation:

$$y = y_0 + \frac{a}{1 + \left(\frac{x}{x_0}\right)^b}$$

where a and y_0 are the asymptotic maximum and minimum values, respectively, x_0 is the genomic concentration (x) at the inflection point and b is the slope at the inflection point. The LOD was defined as the blank plus three times its standard deviation (SD). All measurements were performed in triplicate. Curve fittings were performed with SigmaPlot 12.0 (Systat Software Inc., California, USA).

Quantifications of 50-mL environmental samples obtained by the two approaches were expressed as ng/µL of *O*. cf. *ovata* in 50 µL of extracted DNA. Quantifications determined with the MB-based electrochemical biosensor were compared with those obtained by the MB-based colorimetric method, as well as with those obtained by RPA-ELONA and qPCR (Toldrà et al., 2019). Correlations were then analysed using Pearson's correlation coefficient (r). To assess the relationship between electrochemical results and light microscopy cell abundances, a quadratic polynomial regression model was developed for both benthic (cells/g fwm) and planktonic (cells/L) samples. The correlation between predicted cell abundances from the electrochemical tests and measured cell abundances from light microscopy counts was evaluated using Pearson's correlation coefficient (r). IBM SPSS Statistics 23.0 (IBM Corp., New York, USA) was used for statistical analyses.

3. Results and discussion

3.1. Colorimetric assay

To demonstrate the feasibility of the strategy, the system was primarily tested using colorimetric detection. After the sandwich-type assay on MBs, the analytical signal is proportional to the amount of HRP-labelled reporter probe and, consequently, to the RPA amplicon concentration. In the colorimetric approach, the enzymatic reaction between the HRP-labelled reporter probe and the enzymatic substrate (TMB/H_2O_2) was performed in solution and the absorbance of the resulting product was measured.

The colorimetric calibration curve using different concentrations of *O*. cf. *ovata* genomic DNA was fitted to the sigmoidal logistic four-parameter equation (R^2 = 0.998) (Fig. 2a). Relative standard deviations (RSD) were below 14% (n=3), which indicated good assay reproducibility. An LOD of 10 pg/µL was attained, which corresponds to 100 pg/sample (considering 50-mL samples and taking into account the DNA extraction, RPA and detection protocols). Taking into account the amount of DNA per *O*. cf. *ovata* cell (Toldrà et al., 2019), the LOD corresponds to ~800 cells/L.

3.2. Electrochemical biosensor

Once the strategy had been successfully demonstrated using the colorimetric assay, the resulting magnetic oligocomplexes were integrated on an 8-electrode array to develop the biosensor. The enzymatic reaction between the HRP-labelled reporter probe and the enzymatic substrate (TMB/H_2O_2) was carried out on the electrode surface, and the TMB reduction current was subsequently measured using amperometry.

The electrochemical calibration curve achieved with the biosensor (R^2 = 0.998) is shown in Fig. 2b. The LOD attained with the electrochemical biosensor was 9 pg/µL (90 pg/sample, ~640 cells/L), very similar to the one achieved in the colorimetric approach and below the alarm thresholds established for *Ostreopsis* abundances. In addition, the RSD of the biosensor was below 8.4% (n=3), demonstrating the high reproducibility of the measurements. Surprisingly, high electrochemical signals (~3000 nA of NTC-subtracted maximum current intensity, Fig. 2b) were obtained when the magnetic oligocomplexes were anchored on the electrode surface, whereas lower current intensities (~1000 nA, *data not shown*) were recorded when performing the enzymatic reaction in suspension and transferring the resulting oxidised TMB product (without MBs) to the electrodes. To understand this difference in the signal, the electrochemical behaviour of bare MBs on the electrode surface was studied using cyclic voltammetry. As reported by other authors (Baldrich et al., 2011), the presence of MBs on the electrode showed a decrease in electron transfer (demonstrated by higher peak-to peak separation, lower peak currents, and lower charge) when compared to bare electrodes (Fig. S1). As a consequence, the higher intensities registered with the biosensor are not due to the intrinsic properties of the

MBs but could be explained by the confinement of the oligocomplexes on the electrodes, which brings the generation of oxidised TMB be closer to the transducer surface and its electrochemical detection less diffusion-dependent.

The LODs provided by the MB-based colorimetric and electrochemical approaches are similar to those reported for the colorimetric RPA-ELONA, where the sandwich assay is performed on maleimide plates instead of on MBs. It is important to highlight that the methods described herein use approximately 5-fold less RPA product as compared with the RPA-ELONA. If instead of using 10 µL of oligocomplex, the whole amount from the RPA reaction was used (45 µL), amplified signals and improved LODs could be obtained. Although compared to qPCR the LOD of the biosensor is almost 10-fold higher, the biosensor enables quantifications of *O*. cf. *ovata* DNA below the current alarm thresholds. Moreover, it allows measurements to be performed in a rapid and simple manner, paving the way towards its integration in a compact device and its true application in the field, something more difficult to envisage with qPCR or colorimetric assays.

3.3. Specificity study

The final objective of the work is to apply the electrochemical biosensor to the analysis of environmental samples. During the DNA extraction protocol of environmental samples, not only target DNA is extracted, but also DNA from all organisms present in the sample. Consequently, it is necessary to ensure that the presence of non-target microalgae species will not interfere in the biosensor performance causing false positive results. With this aim, the specificity of the electrochemical biosensor for *O*. cf. *ovata* was evaluated using non-target *Ostreopsis* species present in the Mediterranean (*O*. cf. *siamensis* and *O*. *fattorussoi*, which are taxonomically close to *O*. cf. *ovata*) and comparing the current intensities with those obtained in the absence of target DNA (NTC). No significant responses were obtained from the non-target *Ostreopsis* species (Fig. S2), indicating the high specificity of the biosensor, which derives from the specificity of both the primers and the assay configuration. Detection of *O*. cf. *ovata* without interferences from other taxonomically similar *Ostreopsis* species present in the Mediterranean suggests therefore that the method is species-specific and should not detect any other microalgae genus.

3.4. Electrode array regeneration

Screen-printed carbon electrodes are extensively used due to their low cost. Despite being originally designed for single use, the possibility to re-use them and consequently reduce the biosensor cost was investigated. In our strategy, oligonucleotides are not directly immobilised on the electrode surface as in most DNA-based biosensors, but on MBs. The use of a magnetic field for immobilisation facilitates detachment of the magnetic oligocomplexes from the electrode surface by simple magnet separation and subsequent facile removal of the oligocomplexes from the electrode surface. Additionally, since all steps (immobilisation, blocking and

hybridisation), with the exception of electrochemical transduction are performed in solution, electrode fouling is not likely to occur. With this purpose in mind, the possibility of electrode re-utilisation was evaluated.

Following the first electrochemical measurement, the magnet was removed and the electrodes were washed with distilled water to remove the magnetic oligocomplexes. Subsequent cycles of magnetic immobilisation/electrochemical measurement/cleaning resulted in responses close to 100 % (Fig. 3), indicating not only that the magnetic oligocomplexes had been effectively removed from the electrodes, but also that the electrodes had not suffered any damage. As expected, these results clearly demonstrate the re-usability of the electrodes for at least 10 consecutive measurements.

3.5. Stability of the functionalised MBs

To investigate the possibility of shortening the protocol time, the storage stability of capture probe-functionalised MBs at 4 and -20 °C was tested over 17 days. Electrochemical signals were constant at both temperatures, demonstrating the real-time stability of the MBs with immobilized capture probes up to at least 17 days (Fig. S3). Additionally, such stability can be used to predict shelf life of DNA-coated MBs using the Q Rule method (Anderson and Scott, 1991) according to the equation:

$$predicted\ stability\ (time) = \frac{real\ stability\ (time)}{(Q10)^n}$$

where *n* is the temperature change divided by 10, and the value of Q10 is typically set at 2, 3, or 4, which correspond to reasonable activation energies. In this work, taking into account that the functionalized MBs are stable for at least 17 days, an *n* value of 2.4 and a conservative Q10 value of 2, the predicted stability of the product at -20 °C is at least 3 months. This long-term stability of functionalised MBs significantly reduces the assay time, as large amount of MBs can be prepared on the same day and stored until use.

3.6. Analysis of environmental samples

To demonstrate the applicability of the electrochemical biosensor, 16 environmental samples collected along the Catalan coast were analysed. Sampling was performed in the summer period, when sea temperature exceeds 24 °C and *Ostreopsis* proliferates, and included 4 locations (Table S1) where *Ostreopsis* blooms have previously been reported: 2 locations in the south of the Catalan coast (Carnicer et al., 2015) and 2 locations in the north of the Catalan coast (Vila et al., 2001). Specifically for the latter, *Ostreopsis* blooms have been periodically associated with respiratory problems and skin irritations in humans. Environmental samples included 9 seawater samples (planktonic samples) and 7 macroalgae samples (benthic samples). Although *Ostreopsis* is a benthic genus that grow attached to macroalgae, *Ostreopsis* cells can be easily re-suspended in the water column by mechanical action or hydrodynamic processes (Giussani et al., 2017). Consequently, monitoring *Ostreopsis* cell abundances in water and on macroalgae is essential.

Ostreopsis cf. *ovata* DNA quantifications provided using the electrochemical biosensor were compared with those provided by the colorimetric method, and previous results obtained by RPA-ELONA and qPCR (Table 1). The samples contained a wide range of *O*. cf. *ovata* DNA concentrations, from undetected to 85.57 ng/µL. From a qualitative point of view, most samples that provided negative results using the electrochemical biosensor also gave negative results using other techniques, with the only exception being samples 1 and 13, which were deemed positive using the more sensitive method of qPCR. As shown in Fig. 4, excellent correlations were obtained when quantitatively comparing results of the techniques using RPA, both between the electrochemical and colorimetric approaches (Pearson's r = 0.998; P < 0.001) and between the electrochemical biosensor and RPA-ELONA (r = 0.999; P < 0.001). Similarly, good agreement was achieved when comparing quantifications provided by the electrochemical biosensor and qPCR (r = 0.993; P < 0.001).

In order to evaluate the relationship between *O*. cf. *ovata* DNA quantification using the electrochemical biosensor and light microscopy cell abundances, a quadratic polynomial regression model was constructed. Although cells counted by light microscopy include all *Ostreopsis* species, they were identified as *O*. cf. *ovata* after species-specific analysis using molecular methods. Additionally, environmental samples contained a broad range of other microalgae genera at high abundances (Toldrà et al., 2019). When constructing the model, samples that resulted negative for both electrochemistry and light microscopy (samples 15 and 16) were not included, nor sample 1, which was considered as an outlier. The regression model was used to predict cell abundances in the environmental samples from the biosensor DNA quantifications. The relationship between the model-predicted and observed cell abundances was highly significant for both planktonic (Pearson's r = 0.932; P < 0.01) and benthic (Pearson's r = 0.975; P = 0.001) samples (Fig. 5). This result indicates that it is possible to correctly estimate *O*. cf. *ovata* cell concentrations from the developed biosensor in a range below the alarm thresholds proposed for *Ostreopsis* cells (10000-30000 cells/L and 100000 cells/g fwm for planktonic and benthic samples, respectively (Giussani et al., 2017; Vassalli et al., 2018)). Additionally, this excellent correlation demonstrates the high specificity of the method, detecting *O*. cf. *ovata* without interferences from other microalgae species present in the samples, even at much higher cell abundances.

4. Conclusions

In this work, the combination of the isothermal RPA technique using tailed primers with MBs as immobilisation supports for the electrochemical detection of *O*. cf. *ovata* is described. Firstly, the use of RPA allows the amplification of *O*. cf. *ovata* DNA without the need for thermal cycling, thus reducing power requirements. Secondly, the use of tailed primers allows the detection of the amplified RPA product *via* a sandwich hybridisation configuration. Finally, the use of maleimide-coated MBs as supports improves the assay kinetics and enables reutilisation of the electrodes. Additionally, the stability of the capture probe immobilization provides ready-to-use MBs, shortening the assay time.

Given the excellent analytical performance in terms of sensitivity, specificity, storage stability and good correlation with other molecular methods as well as light microscopy, the implementation of this biosensor as a quantitative and/or screening tool in routine microalgae monitoring programs is feasible. Additionally, it offers great potential for subsequent integration in miniaturised devices, bringing it closer to in-field deployment. This work thus constitutes a breakthrough in the development of rapid, simple, cost-effective and easy-to-use analysis tools for the detection of toxic marine microalgae.

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408

Table 1 *O.* cf. *ovata* DNA quantifications (ng/µL) of 16 environmental samples (planktonic and benthic) provided by the electrochemical MB-based biosensor and the colorimetric MB-based assay (mean ± SD, *n* = 3). Quantifications obtained by RPA-ELONA and qPCR are shown (Toldrà et al., 2019).

Sample number	*O.* cf. *ovata* DNA (ng/µL)			
	Electrochemical	**Colorimetric**	**RPA-ELONA**	**qPCR**
Sample 1	nd	nd	nd	0.010 ± 0.002
Sample 2	85.573 ± 8.968	50.516 ± 1.870	63.721 ± 11.896	78.781 ± 6.367
Sample 3	nd	nd	nd	nd
Sample 4	nd	nd	nd	nd
Sample 5	0.086 ± 0.008	0.081 ± 0.011	0.083 ± 0.033	0.063 ± 0.021
Sample 6	1.299 ± 0.420	1.045 ± 0.218	1.369 ± 0.185	2.748 ± 0.248
Sample 7	0.132 ± 0.037	0.110 ± 0.006	0.149 ± 0.069	0.098 ± 0.016
Sample 8	0.039 ± 0.005	0.031 ± 0.008	0.025 ± 0.011	0.019 ± 0.001
Sample 9	0.047 ± 0.016	0.082 ± 0.040	0.064 ± 0.024	0.056 ± 0.022
Sample 10	0.102 ± 0.031	0.071 ± 0.002	0.082 ± 0.016	0.083 ± 0.021
Sample 11	0.035 ± 0.015	0.038 ± 0.012	0.020 ± 0.005	0.022 ± 5E-05
Sample 12	0.132 ± 0.033	0.185 ± 0.045	0.139 ± 0.042	0.250 ± 0.009
Sample 13	nd	nd	nd	0.005 ± 2E-04
Sample 14	4.403 ± 1.042	2.686 ± 0.321	4.220 ± 0.855	3.918 ± 0.257
Sample 15	nd	nd	nd	nd
Sample 16	nd	nd	nd	nd

nd: not detected

Figure 1 Schematic illustration of the electrochemical biosensor.

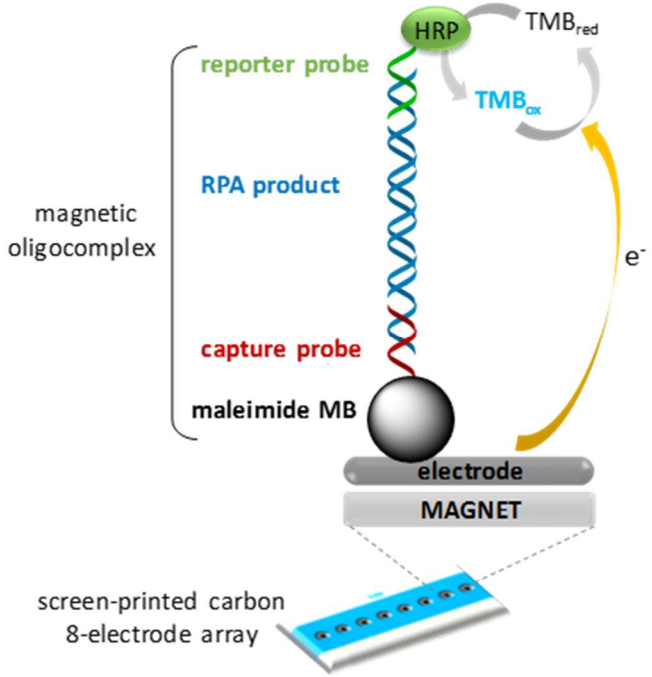


Figure 2 Calibration curves obtained using different concentrations of *O*. cf. *ovata* genomic DNA: (**a**) colorimetric assay and (**b**) electrochemical biosensor. Error bars are the standard deviation of the mean, $n = 3$.

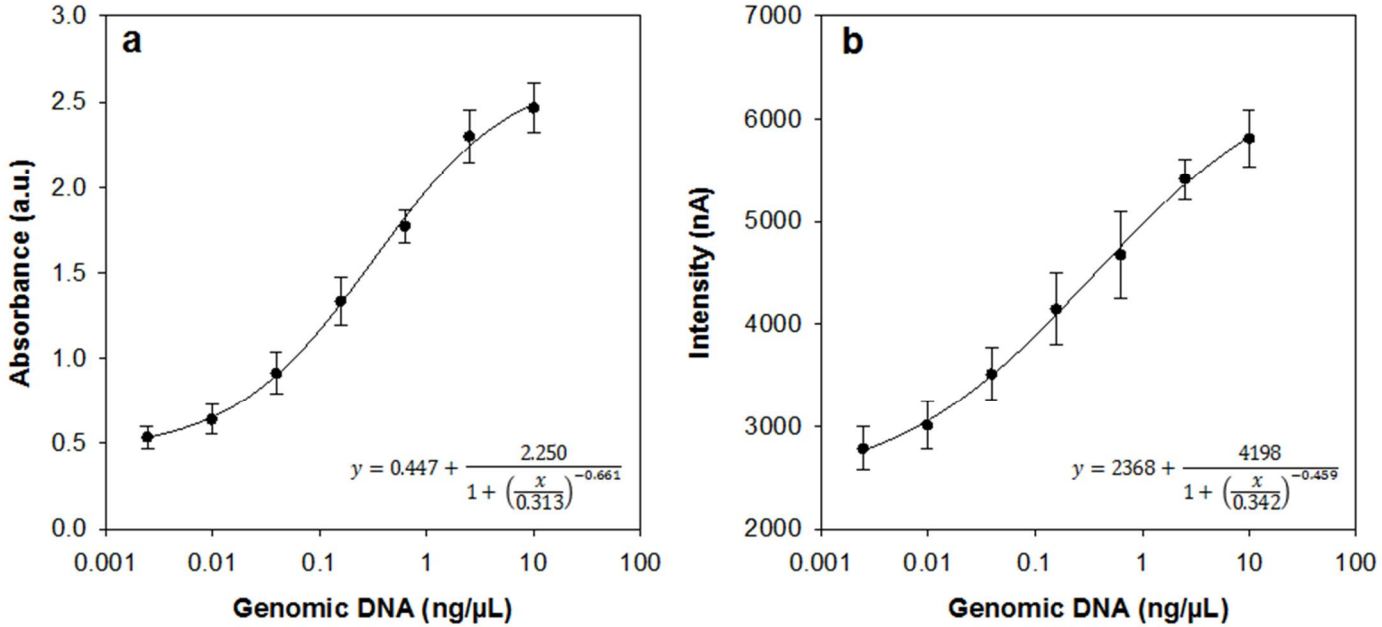


Figure 3 Electrochemical responses of the electrochemical biosensor after 10 cycles of magnetic immobilisation/electrochemical measurement/cleaning. Error bars are the standard deviation of the mean, *n* = 3.

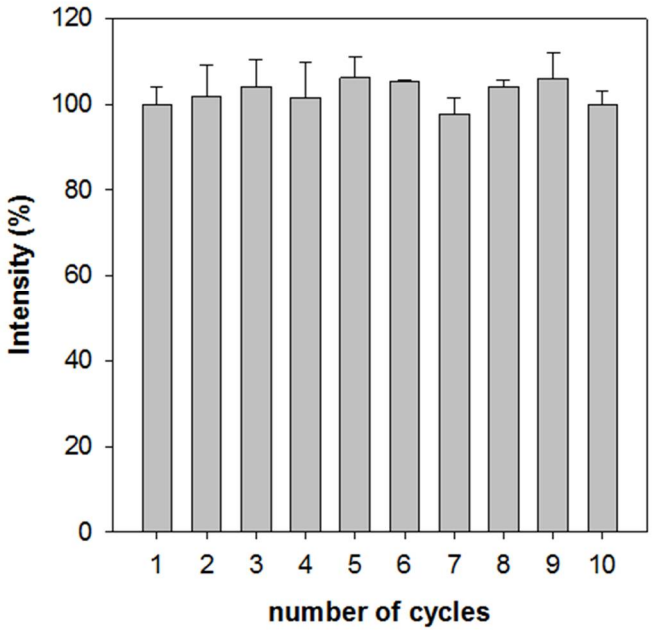


Figure 4 Correlation between *O.* cf. *ovata* DNA quantifications provided by the electrochemical biosensor and those obtained by the colorimetric assay, RPA-ELONA and qPCR in all examined environmental samples.

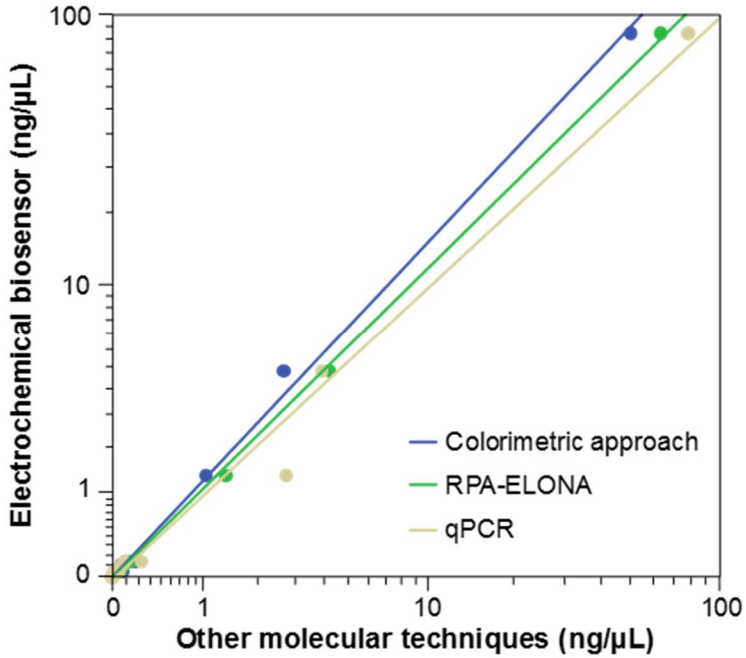


Figure 5 Relationship between predicted cell abundances provided by the regression model and those counted by light microscopy in: (**a**) planktonic samples (cells/L) and (**b**) benthic samples (cells/g fwm). Pearson's correlation coefficient is shown.

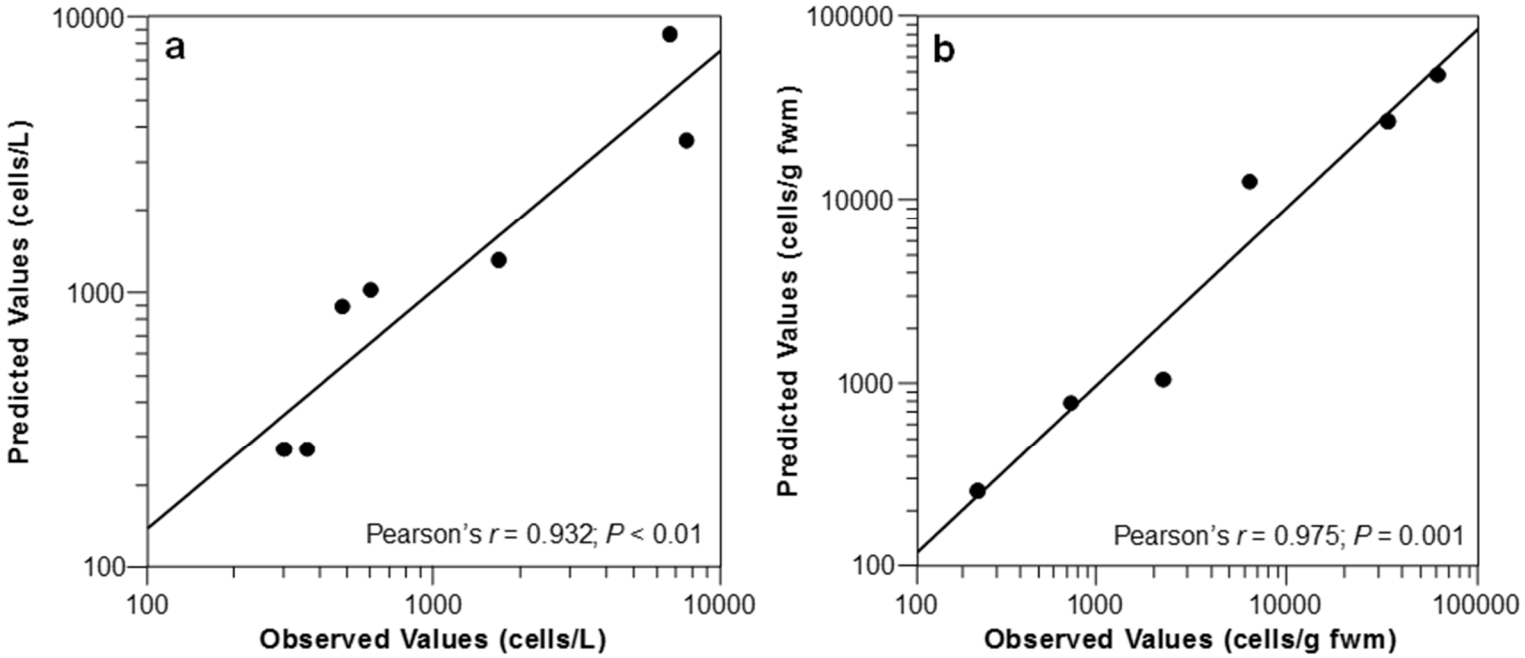


Table S1. Environmental samples collected in August 2017 along the Catalan cost. Location and geographical coordinates of the sampling stations as well as *Ostreopsis* spp. abundances obtained by light microscopy for planktonic (cells/L) and benthic (cells/g fwm) samples are shown. Data obtained from (*).

Sample number	Sample type	Locality	Station number	Geographical coordinates	*Ostreopsis* spp. cell abundances
Sample 1	planktonic	Palamós, La Fosca	1	N 41°51'20.71'' E 3°8'32.01''	2840
Sample 2	benthic	Palamós, La Fosca	1	N 41°51'20.71'' E 3°8'32.01''	60710
Sample 3	planktonic	Palamós, La Fosca	2	N 41°51'28.18'' E 3°8'39.84''	360
Sample 4	benthic	Palamós, La Fosca	2	N 41°51'28.18'' E 3°8'39.84''	210
Sample 5	planktonic	Sant Andreu de Llavaneres	3	N 41°33'7.69'' E 2°29'31.66''	7600
Sample 6	benthic	Sant Andreu de Llavaneres	3	N 41°33'7.69'' E 2°29'31.66''	32831
Sample 7	planktonic	Sant Andreu de Llavaneres	4	N 41°33'12.25'' E 2°29'45.20''	6620
Sample 8	planktonic	Sant Andreu de Llavaneres	5	N 41°33'17.06'' E 2°29'54.47''	600
Sample 9	planktonic	L'Ametlla de Mar	6	N 40°52'28.35'' E 0°47'43.67''	1680
Sample 10	benthic	L'Ametlla de Mar	6	N 40°52'28.35'' E 0°47'43.67''	667
Sample 11	planktonic	L'Ametlla de Mar	7	N 40°50'47.90'' E 0°45'44.04''	480
Sample 12	benthic	L'Ametlla de Mar	7	N 40°50'47.90'' E 0°45'44.04''	2071
Sample 13	planktonic	Les Cases d'Alcanar	8	N 40°32'1.00'' E 0°31'7.24''	300
Sample 14	benthic	Les Cases d'Alcanar	8	N 40°32'1.00'' E 0°31'7.24''	6015
Sample 15	planktonic	Les Cases d'Alcanar	9	N 40°33'15.71'' E 0°31'58.71''	nd
Sample 16	benthic	Les Cases d'Alcanar	9	N 40°33'15.71'' E 0°31'58.71''	nd

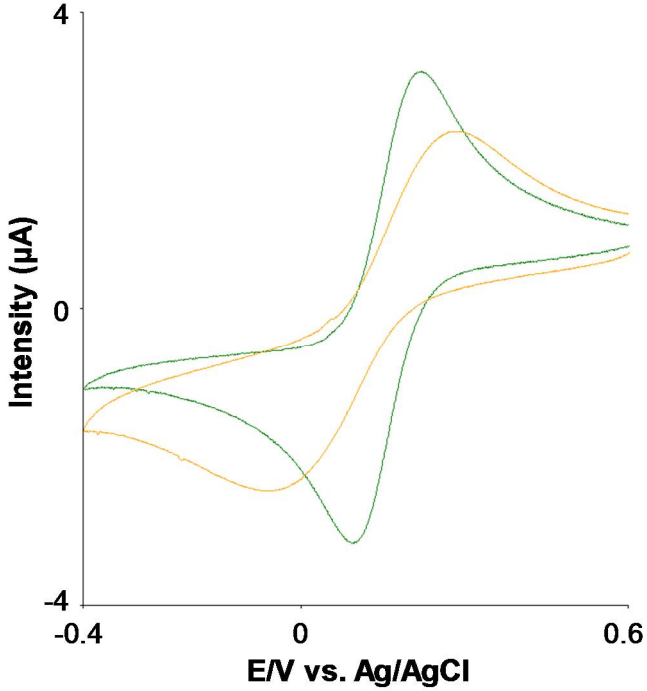
nd: not detected

(*) Toldrà A, Alcaraz C, Andree KB, Fernández-Tejedor M, Diogène J, Katakis I, et al. Colorimetric DNA-based assay for the specific detection and quantification of *Ostreopsis* cf. *ovata* and *Ostreopsis* cf. *siamensis* in the marine environment. Harmful Algae 2019; 84: 27-35.

Table S2. List of primers (underlined) and probes and their respective modifications.

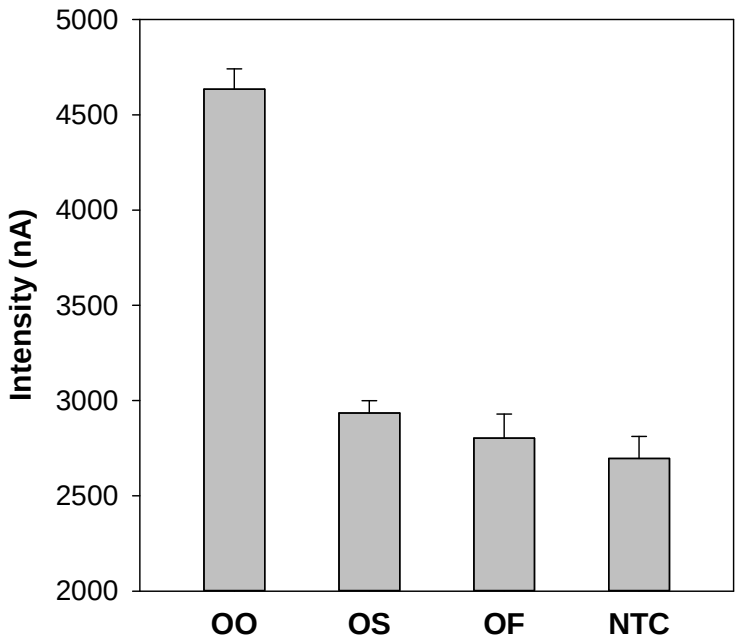
Name	Sequence (5'-3')
Forward *O.* cf. *ovata* primer with tail	gtt ttc cca gtc acg ac-C3-aca atg ctc atg cca atg atg ctt gg
Reverse *Ostreopsis* spp. primer with tail	tgt aaa acg acg gcc agt-C3-gca wtt ggc tgc act ctt cat aty gt
O. cf. *ovata* capture probe	gtc gtg act ggg aaa act ttt ttt ttt ttt tt-C3-SH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

Fig. S1. Cyclic voltammograms (CVs) and analytical parameters obtained using a bare screen-printed electrode (green line) and a MB-modified screen-printed electrode (orange line). CVs were performed using 10 µL of 1 mM $[Fe(CN)6]^{3-/4-}$ (in 0.1 M phosphate buffer solution with 0.1 M KCl, pH 7.2.) at a scan rate of 50 mV/s. 1 µL of malimide-activated MBs were used, which corresponds to 10 µL of oligocomplex.



	E_{ox} (V)	E_{red} (V)	ΔE (V)	Height E_{ox} (µA)	Height E_{red} (µA)	Charge E_{ox} (µC)	Charge E_{red} (µC)
Bare electrode	0.221	0.093	0.128	3.103	-3.180	1.170	-1.142
MB-modified electrode	0.291	-0.054	0.345	1.965	-1.752	0.928	0.920

Fig. S2. Electrochemical responses of the electrochemical biosensor using DNA from: *O.* cf. *ovata* (OO), *O.* cf. *siamensis* (OS), *O. fattorussoi* (OF) and no template control (NTC). Error bars are the mean standard deviation, *n* = 3.



Fig, S3, Electrochemical responses corresponding to the stability study of functionalized MBs stored at 4 °C (black bars) and at -20 °C (grey bars), respect to day 0 (white bar, reference value). Error bars are the standard deviation of the mean, *n* = 3.