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DNA-based assays and biosensors for the detection of toxic microalgae and viruses in the marine environment

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Doctoral Thesis 2019

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I STATE that the present study, entitled **"DNA-based assays and biosensors for the detection of toxic microalgae and viruses in the marine environment"**, presented by **Anna Toldrà Filella** for the award of the degree of Doctor, has been carried out under my supervision at the Department Chemical Engineering of this university, and that it fulfils all the requirements to be eligible for the International Doctorate Award.

Tarragona, 5th July 2019

Dr. Mònica Campàs **Dr. Ciara K. O'Sullivan**

Even stars collide,

and out of their crashing

new worlds are born

Charles Chaplin

In: *As I began to love myself* poem

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TABLE OF CONTENTS

SUMMARY

The presence of biological hazards such as toxic microalgae and viruses in the marine environment has become a serious concern due to their direct impact on marine life, human health and economic-related activities (e.g. aquaculture and tourism). Nonetheless, the application of monitoring programs can help to prevent and mitigate their impacts. Microscopy and traditional molecular biology techniques are currently used to assess the presence of these hazards. However, these techniques are extremely time consuming, expensive and/or inaccurate.

The main goal of this doctoral thesis is to contribute to the development and applicability of DNA-based assays and biosensors for the detection of toxic marine microalgae and viruses, as well as to other steps in the environmental analysis process, such as sample pre-treatment and DNA extraction. More generally, this thesis aims at demonstrating the opportunities that nucleic acids offer to environmental analytical chemistry by providing new approaches that will pave the way towards the implementation of rapid, specific, simple, low-cost and *in situ* analysis tools for the improvement of monitoring activities.

In order to achieve this objective, this thesis describes colorimetric assays and electrochemical biosensors that exploit isothermal recombinase polymerase amplification (RPA), which is more rapid and simple than traditional thermal amplification, as well as being more amenable to *in situ* analysis. Nevertheless, the development of qPCR assays is also achieved. Primer design for both microalgae and viruses and optimisation of RPA and qPCR parameters are explored. Magnetic beads (MBs) are exploited as immobilisation supports in the development of electrochemical biosensors, as well as in capture and concentration strategy for the detection of viruses. Finally, this thesis describes the application of the developed DNA-based tools to the analysis of environmental samples, and the subsequent comparison of with well-established techniques.

This thesis has the following structure:

Chapter 1 contains a general introduction, which includes a brief description of the toxic marine microalgae and viruses, which are the targets for detection. It describes how relevant the development of new methods is and provides the state of the art of DNA-based tools for their detection, with a focus on isothermal amplification techniques. Moreover, an overview of the key factors in the development and implementation of DNA-based tools is provided.

Chapter 2 includes the general and specific objectives of this thesis. A list of the scientific publications achieved through this thesis, together with my personal contribution, is provided.

Regarding the experimental part, the thesis has been divided into three different sections according to the target analyte:

Chapter 3 is focused on the development of qPCR and colorimetric assays to detect the microalgae *Karlodinium*. Chapter 3A describes the development of a qPCR assay to detect and discriminate between *K. veneficum* and *K. armiger,* and its subsequent application to environmental planktonic samples. The establishment of a new DNA extraction method is also addressed. In Chapter 3B, the development of a colorimetric assay exploiting RPA to detect and discriminate between *K. veneficum* and *K. armiger* is pursued. The use of different approaches (synthetic DNA, genomic DNA or cells) to construct the calibration curves is explored.

Chapter 4 describes the development of colorimetric assays and electrochemical biosensors, both exploiting RPA, to detect the microalgae *Ostreopsis.* Chapter 4A is focused on the development of a colorimetric assay to detect and discriminate between *O*. cf. *ovata* and *O*. cf. *siamensis*. Primer design for RPA and elimination of the cleaning step following amplification is examined. Moreover, a predictive model is proposed to evaluate the relationship between this system and microscopy counts in the analysis of environmental planktonic and benthic samples. In Chapter 4B, the development and application of a magnetic bead (MB)-based electrochemical biosensor for *O*. cf. *ovata* is described.

Chapter 5 is focused on the development of electrochemical biosensors and MB-based capture strategies for ostreid herpesvirus-1. Chapter 5A describes the development of an electrochemical biosensor exploiting RPA and its application to the analysis of oysters. In this case, gold electrodes are used as DNA immobilisation supports. In Chapter 5B, the use of MBs able to capture and concentrate viable viral particles from seawater and oyster tissue homogenate is presented. Experimental oyster infections as well as DNA and RNA analysis by qPCR are covered.

In **Chapter 6**, a general discussion of the findings elucidated in this thesis is provided.

Finally, **Chapter 7** summarises the general conclusions of the thesis and the future perspectives and applications of this research.

CHAPTER 1

General Introduction

General Introduction

1. Quality of the marine environment

Oceans and seas cover 72% of the Earth's surface and contain 97% of the planet's water. The quality of the marine environment is influenced by a wide diversity of anthropogenic and natural hazards, which may adversely affect human health, living resources and the ecosystems (Violante *et al.* 2018; Zielinski *et al.* 2009). In addition to physical hazards (e.g. extreme climatic events such as tsunamis) and chemical contaminants derived from human activities (e.g. metals, polyaromatic hydrocarbons (PAHs), polychlorinated biphenyls (PCBs), microplastics and pharmaceuticals), which are not addressed in this thesis, an important group that has to be considered regarding marine environmental quality are natural compounds, including those produced by organisms (e.g. marine toxins) or biological hazards (e.g. toxic microalgae and pathogens: bacteria, viruses and protozoa). Although biological hazards arise from natural sources, their presence and abundance is modulated by human activities such as sewage inflow, eutrophication, ship ballast water or climate change, among others (**Fig. 1**). This thesis is focused on two types of microorganisms: toxic microalgae and viruses.

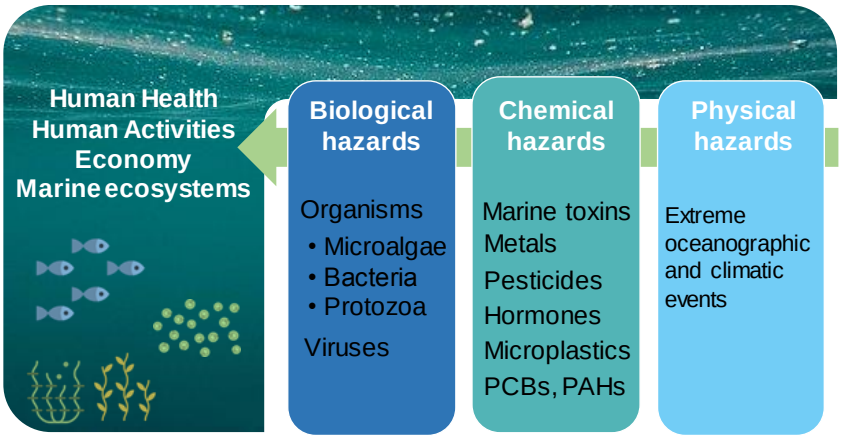

Fig. 1. Classification of selected marine hazards.

1.1. Marine microalgae

Microalgae are single-celled microorganisms (from 5 µm to typically less than 1 mm) that support healthy aquatic ecosystems by forming the base of the food web, fixing carbon and producing oxygen. Under certain circumstances, some species can proliferate and cause negative impacts on marine ecosystems, human health and/or socioeconomic related activities (e.g. aquaculture and recreational activities) (Steidinger *et al.* 2008). Collectively, these organisms and events are referred to as

"harmful algal blooms (HABs)" that, to a large extent, can be associated either with aquatic organisms' mortalities or human health risks (Reguera *et al.* 2016). The first group includes non-toxic microalgae that, after reaching high concentrations, cause fish and invertebrates' mortalities through oxygen depletion. It also includes ichthyotoxic microalgae that produce haemolytic toxins or oxidising substances (reactive oxygen species – ROS) that are released into the environment and can kill fish. The second group includes microalgae that produce toxins that bioaccumulate in fish or shellfish causing foodborne diseases in humans, as well as microalgae associated with respiratory and skin irritations. It should be noted that some HABs have multiple adverse effects and thus they can belong to different groups. **Table 1** classifies microalgae species based on the harm they cause.

Table 1. Different types of microalgae forming HABs.

GROUP 1: species associated with organisms' mortalities	**Examples**
Species that reach high concentrations	*Noctiluca*, *Scripsiella*
Ichthyotoxic species	*Karlodinium*, *Prymnesium*
GROUP 2: species associated with human health risks	**Examples**
Species associated with human foodborne illnesses:	
• Diarrhetic shellfish poisoning (DSP)	*Dinophysis*, *Prorocentrum*
• Paralytic shellfish poisoning (PSP)	*Alexandrium*
• Amnesic shellfish poisoning (ASP)	*Pseudo-nitzschia*
• Azaspiracid shellfish poisoning (AZP)	*Azadinium*
• Neurotoxic shellfish poisoning (NSP)	*Karenia brevis*
• Ciguatera fish poisoning (CFP)	*Gambierdisucs*
Species associated with respiratory/skin irritations	*Ostreopsis*

An example of the first group is *Karlodinium*, a planktonic (i.e. grows in the water column) dinoflagellate genus involved in numerous fish mortality events due to the production of haemolytic toxins. It proliferates in calm and low-turbulence areas all around the world such as Alfacs Bay (NW Mediterranean Sea), where winter blooms of theses species have been reported. Different *Karlodinium* species (i.e. *K. veneficum* and *K. armiger*) produce different toxins and show different levels of ichthyotoxicity, posing different risks to ecosystems and aquaculture (Garcés *et al.* 2006; Place *et al.* 2012). *Ostreopsis* represents an example of the second group. It is a benthic (i.e. grows associated with macroalgae) dinoflagellate genus that proliferates during the summer period in tropical and temperate regions, such as the Mediterranean Sea. *Ostreopsis* cells and/or their toxins (i.e. palytoxin (PLTX)-like compounds) are present in marine aerosols that irritate swimmers' respiratory tract and skin. Additionally, these toxins have been associated with mortalities of marine invertebrates and have been found

in seafood. Similarly, different *Ostreopsis* species (i.e. *O.* cf. *ovata* and *O.* cf. *siamensis*) produce different PLTX-like compounds, showing different levels of toxicity to humans and ecosystems (Accoroni and Totti 2016; Berdalet *et al.* 2017; Vila *et al.* 2016).

1.2. Marine viruses

Viruses are small particles, usually about 20 – 200 nm long, consisting of genetic material (DNA or RNA, single or double stranded) surrounded by a protein coat and, sometimes, by an additional lipidic envelope. Like toxic microalgae, viruses can be associated to seafood-borne illnesses. Bivalve species, in the process of filter feeding, concentrate and retain human viruses that are excreted in faeces and can be transmitted, resulting in infection. An example of this is the hepatitis virus (HAV) and norovirus (NoV), which cause acute hepatitis and gastroenteritis, respectively, following shellfish consumption (Girones *et al.* 2008). On the other hand, some viruses can infect marine organisms resulting in their death. This is the case of ostreid herpesvirus-1 (OsHV-1), one of the major threats to shellfish aquaculture, particularly to the production of Pacific oysters (*Crassostrea gigas*). Recurrent mass mortality outbreaks of *C. gigas* have been reported worldwide. These mortalities mostly take place during the spring period, when the seawater temperature reaches 16 °C, causing major economic losses to oyster farmers (EFSA 2015).

2. Environmental monitoring: current and emerging methods

Bearing in mind the existence of these biological hazards, the need to regularly monitor their presence in marine waters and/or in seafood is of utmost importance to understand and manage risks associated with human health and ecosystems.

The implementation of monitoring programs in fish and shellfish aquaculture areas aimed at preventing cases of seafood poisoning due to some marine toxins is mandatory in the European Union (Rodgers and Diogène 2016). Such monitoring programs concentrate most efforts on determining the level of toxins in seafood and describing the species of microalgae present in the environment (EC854/2004). Although monitoring of other microalgae apart from those associated with food-borne diseases is not obligatory within the EU, many countries or regions that are regularly affected by specific blooms perform monitoring activities in order to, for instance, satisfy the sanitary requirements for bathing waters (e.g. *Ostreopsis*) or reduce economic loses in aquaculture (e.g. *Karlodinium*). Whether mandatory or not, official maximum abundance levels have not yet been established for microalgae. Nevertheless, some regions have defined its own thresholds based on risk assessment. For example, a level of 200,000 cells/L for *Karlodinium* spp. has been proposed as a warning level in Alfacs Bay (Fernández-Tejedor *et al.* 2004), and a level of 10,000 -

30,000 cells/L for *Ostreopsis* spp. has been defined in Italy and Spain (Vassalli *et al.* 2018).

As for microbiological hazards, although current regulation uses faecal indicators (e.g. *Escherichia coli*) to indicate or infer the presence of enteric viruses such as HAV and NoV, several studies have shown that no evident relationship exists between faecal indicators and viruses (Rodgers and Diogène 2016). Therefore, the current criterion is insufficient to protect against viral foodborne infections in general, and detection of viral contamination is likely to be included in mandatory testing. Moreover, surveillance of viral pathogens in water and/or shellfish is an essential task in aquaculture to reduce economic losses.

Current monitoring programs for the detection and quantification of toxic microalgae in the environment routinely use light microscopy. However, this technique is time consuming, requires a high level of taxonomic expertise and is based on morphological characteristics, which in some cases are insufficient to identify organisms at the genus or species level (Edler and Elbrächter 2010). Similarly, and because of the lack of classical culture techniques for most viruses infecting shellfish, detection of these pathogens was originally based on the observation of damaged or infected tissue trough microscopy-based techniques such as histology or immunohistochemistry, respectively (Girones *et al.* 2008). As a result, there is an expanding need for new bioanalytical tools to assess the presence of such biological hazards in marine waters and/or in seafood easier and faster, thus facilitating the implementation of appropriate preventative measures and eventually minimising their impact.

Biochemical assays and biosensors appear well suited as complementary or alternative tools to conventional methods, since they allow the detection of the analytes of interest in a rapid, cost-effective and user-friendly manner. Biochemical assays are based on the interaction of the analyte with a biorecognition molecule, which can be an enzyme, an antibody or nucleic acid. These methods can be classified into solution-based or surface-based assays, depending on whether the biorecognition event takes place in solution or on a solid support, respectively. The immobilisation of the biorecognition element in intimate contact with a physicochemical transducer leads to the development of biosensors (Kroger *et al.* 2002). While the bioreceptor recognises the analyte, the transducer converts the biological interaction into a measurable signal, which can be electrochemical, optical, piezoelectric or thermal, and is proportional to the analyte concentration. Regarding electrochemical biosensors, their inherent high sensitivity, low cost of both electrodes and potentiostats, possibility of miniaturisation and compatibility with microfluidic systems make them attractive for *in situ* monitoring (Ronkainen *et al.* 2010).

3. Nucleic acid-based methods

Nucleic acids are found in all cells and also in viruses. Beyond their hereditary role in nature, their chemical structure offers incredible opportunities in analytical chemistry. Among the different types of biorecognition molecules in biochemical assays and biosensors, nucleic acids are particularly interesting due to their inherent high affinity and specificity, facile synthesis and easy modification with functional groups.

Molecular (nucleic acid) assays are based on the hybridisation between a nucleic acid target (DNA or RNA) and its complementary primer/probe, which is present either in solution and/or on a solid support. Molecular methods have recently been developed and applied in environmental quality monitoring. Most of them have their origin in medical diagnostics and, during the last decades, these techniques have been tested, modified and refined for their application in, for instance, microalgae and virus detection and quantification.

Among the different traditional molecular methods, quantitative real-time PCR (qPCR) has been designed for a variety of toxic microalgae including *Alexandrium minutum* (Galluzzi *et al.* 2004), *Karenia mikimotoi* (Yuan *et al.* 2012) and *Prorocentrum donghaiense* (Zhang *et al.* 2016). In addition, qPCR methods have been designed to differentiate species within the same genus: *Gambierdiscus* (Nishimura *et al.* 2016) and *Pseudo-nitzschia* (Andree *et al.* 2011). Moreover, qPCR protocols have been developed for some viruses such as OsHV-1, and are currently routinely used for screening in aquaculture facilities (Pepin *et al.* 2008).

In a qPCR assay, amplification of DNA by PCR is monitored in solution and in real time. Exponential amplification of the target occurs when the double-stranded DNA (dsDNA) target denatures into single stranded DNA (ssDNA) by heating, allowing primers to bind to their complementary strand, facilitating DNA polymerase-mediated extension. Quantification of the amplified DNA relies on fluorophore-based detection, either using molecular beacons, TaqMan probes or SYBR green intercalating dyes. Alternatively, RNA targets can be detected following reverse transcription from RNA to DNA. When addressing the identification of microalgae and viruses, qPCR technique has some outstanding advantages as compared to traditional microscopy techniques: (i) it is faster and enables high throughput analysis; (ii) it usually provides lower limits of detection; (iii) it is more accurate and allows the identification of morphologically similar species or genera in the case of microalgae; and (iv) fewer hours of training are required to attain a level of expertise sufficient for routine screening (Penna and Galluzzi 2013).

Although qPCR has been proved successful in detecting microalgae and viruses, PCR-based methods inherently require a power supply and a precise temperature control to perform the thermal cycling, hindering its integration in easy-to-use miniaturised devices for *in situ* testing. Hence, qPCR still requires samples to be sent to centralised laboratories, resulting in a time lag between sample procurement and analysis. Alternative user-friendly and *in situ* molecular methods are of great importance to better anticipate the presence of these biological hazards. Accordingly, there is an increasing demand for portable and autonomous systems able to perform accurate and rapid analysis in the field.

Isothermal amplification methods can certainly address these requirements. Unlike typical thermal cycling amplification, some isothermal techniques are carried out at a constant temperature, which reduces the power needed and makes them more compatible for integration within miniaturised systems (Deng and Gao 2015). An alternative strategy is to avoid amplification by targeting ribosomal RNA genes, as they exist in a high copy number. This approach has been exploited to detect toxic microalgae in assays and biosensors including microarrays with fluorescence detection (Metfies *et al.* 2005) and sandwich hybridisation systems followed by colorimetric (Diercks *et al.* 2008a) or electrochemical detection (Diercks *et al.* 2008b). However, RNA is inherently unstable (i.e. it is susceptible to ribonucleases), which may compromise the reliability of these assays.

4. Isothermal amplification-based methods

Isothermal amplification techniques have garnered huge attention and impressive progress has been made in the past 20 years. Such techniques offer solutions to the drawbacks of their PCR-based amplification counterparts due to their isothermal nature, simple protocols and excellent amplification power. In recent years, several isothermal techniques have been described, including: loop-mediated isothermal amplification (LAMP), rolling circle amplification (RCA), nucleic acid sequence-based amplification (NASBA), recombinase polymerase amplification (RPA), strand displacement amplification (SDA) and helicase-dependent amplification (HDA) (Deng and Gao 2015). Such techniques differ from each other in terms of their operating conditions, amplification mechanism, strengths and weaknesses.

Although widely used in clinical diagnostics, the application of isothermal amplification-based analytical methods (including nucleic acid amplification and detection steps) to detect microalgae and marine viruses is still in its infancy. Herein, we only describe the state of the art of isothermal amplification-based assays and biosensors developed for microalgae because of scarce literature for marine viruses.

4.1. Existing isothermal analytical methods for microalgae

Among all isothermal techniques described to date, LAMP, RCA and NASBA, coupled with different detection strategies (e.g. hybridisation in solution or in a solid support, end-point or real-time) have been reported in the literature to detect microalgae (**Table 2**).

Table 2. Isothermal amplification-based assays for microalgae detection.

Isothermal method	Detection format	Microalgae species
LAMP	Fluorescence	*Alexandrium species, Karenia mikimotoi, Prorocentrum minimum*
	Lateral Flow	*Karlodinium veneficum*, *Prymnesium parvum*, *Amphidinium carterae*
RCA	Fluorescence	*Heterosigma akashiwo, Amphidinium carterae*
	Lateral Flow	*Karlodinium veneficum*
	Dot blot	Multiplex detection of various microalgae species
NASBA	Fluorescence	*Karenia brevis*

Approximately, half of the publications describing isothermal methods for microalgae exploit LAMP. In LAMP, 4-6 primers are used to amplify the target double-stranded DNA (dsDNA) at a constant temperature of 60-65 °C for 60 min. Single-stranded DNA (ssDNA) LAMP products are a mixture of different lengths with multiple loops and several repeats of the target sequence. LAMP products are generally visualised by a colour change upon addition of a fluorescent intercalating dye, as reported for the detection of *Prorocentrum minimum* (Zhang *et al.* 2014), *Karenia mikimotoi* (Zhang *et al.* 2009) and *Alexandrium* species (Nagai *et al.* 2016; Zhang *et al.* 2012). Another strategy to detect LAMP products relies on lateral flow (LF) dipsticks, recently described for *Karlodinium veneficum* (Huang *et al.* 2017), *Prymnesium parvum* (Zhu *et al.* 2019) and *Amphidinium carterae* (Wang *et al.* 2019). In these dipsticks, the use of a biotin-labelled primer results in biotinylated LAMP products that hybridise with a FITC-labelled probe, and the addition of gold-labelled anti-FITC antibodies generates the characteristic red band (**Fig. 2a**). Such LAMP strategies achieve around 10-fold improvement in sensitivity as compared to standard PCR.

Because of its excellent amplification power, RCA strategies usually show better sensitivities than PCR and LAMP. RCA starts with a linear ssDNA sequence (padlock probe, PLP) consisting of two terminal fragments complementary to the target DNA and two universal primers. The PLP hybridises to the target, circularises and

amplification occurs. As with LAMP, RCA products have been detected with fluorescent dyes for *Heterosigma akashiwo* (Zhang *et al.* 2018b) and *Amphidinium carterae* (Chen *et al.* 2015), and LF for *K. veneficum* (Liu *et al.* 2019). Additionally, a dot blot DNA array has been developed to simultaneously detect multiple microalgae species (including *Chatonella marina*, *Heterosigma akashiwo*, *Prorocentrum lima*, among others) (**Fig. 2b**) by using different capture probes immobilised on a nylon membrane and an enzyme-streptavidin reporter conjugate (Zhang *et al.* 2018a). Although amplification is performed at 65 °C for 15 min, ligation and exonuclease steps (37-65 °C for ~2h) are required. Additionally, two denaturation steps at 95 °C are needed to generate ssDNA from the target dsDNA and from the RCA product to enable hybridisation with the PLP and the probe, respectively, hindering its true application at the point-of-need, particularly in resource limited settings.

NASBA has found extensive applications in the amplification and detection of RNAs owing to the combination of reverse transcription with the amplification process. Two primers and three enzymes (i.e. reverse transcriptase, RNase and RNA polymerase) are involved in NASBA, which amplifies target RNA at 41 °C for 90 min. NASBA has been used to detect *Karenia brevis* (Casper *et al.* 2004; Loukas *et al.* 2017). In contrast to the previous strategies, which are end-point, NASBA products from *K. brevis* were monitored in real time using molecular beacons and a fluorescence reader (**Fig. 2c**), in a format similar to qPCR.

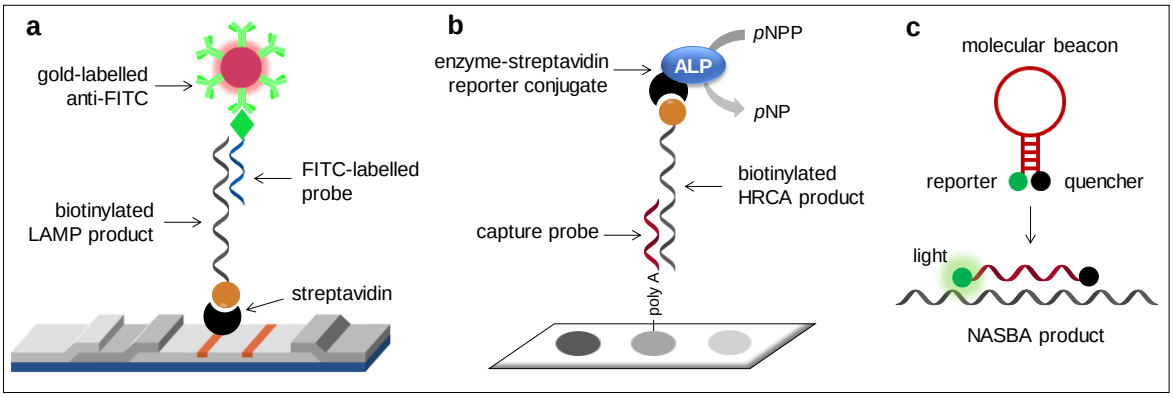

Fig 2. Examples of isothermal amplification-based assays for microalgae detection: (**a**) LAMP followed by a colorimetric lateral flow assay, (**b**) RCA followed by a colorimetric dot blot assay and (**c**) NASBA with a fluorescent read-out.

4.2. Recombinase polymerase amplification

Among the isothermal techniques developed to date, RPA is particularly attractive since it offers outstanding advantages. RPA is one of the most rapid amplification techniques, often completed in just 20 min. It operates at a low and constant temperature of about 37-42 °C, without the need for an initial melting step to generate ssDNA from the dsDNA target. Additionally, in contrast to the majority of isothermal

techniques, it only requires the use of two primers (forward and reverse), which greatly simplifies the experimental design (Deng and Gao 2015).

RPA technology was developed by Piepenburg *et al.* in 2006 (Piepenburg *et al.* 2006) using proteins that are present in the living cells and are essential for the repair and maintenance of DNA. Currently, RPA kits are exclusively commercialised under the license of Abbott (Illinois, US). RPA employs a mixture of three core proteins to achieve amplification: a recombinase, a single-stranded DNA-binding (SSB) protein and a strand-displacing DNA polymerase. The RPA process starts when the T4 uvsX (recombinase) binds to primers in the presence of ATP and a crowding agent (a high molecular poly(ethyleneglycol)), forming a recombinase-primer complex. This complex scans the dsDNA target for homologous sequences. When the homologous sequence is found, the recombinase proteins facilitates a strand-displacement process and the formation of a D-loop, thus primers are able to hybridise. To prevent re-hybridisation of the initial dsDNA template and, therefore, the ejection of the inserted primers, the displaced DNA strand is stabilised by SSB proteins. Finally, the recombinase disassembles and the strand-displacing *Bsu* polymerase binds to the 3' end of the primer to elongate it. Cyclic repetition of this process results in the achievement of exponential amplification (**Fig. 3**).

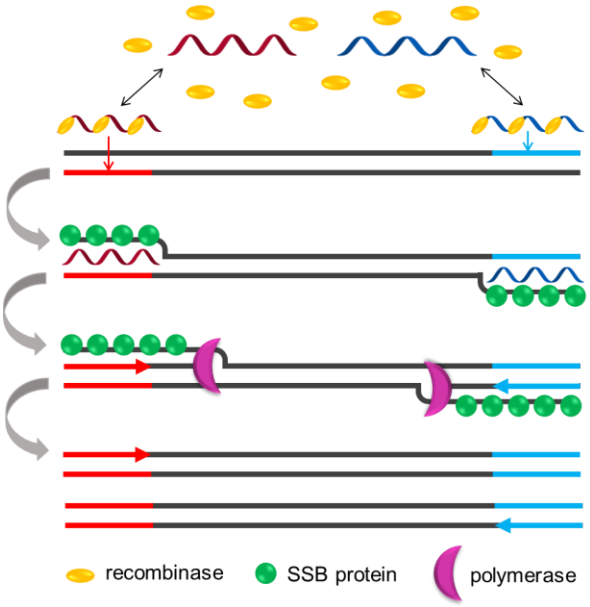

Fig. 3. Schematic representation of the RPA process.

There are several important experimental parameters in each RPA assay that have to be taken into account including reaction time, temperature and primer design. Initially, RPA primers were designed to be 30-35 bases long with a GC content between 40-60% for the amplification of relatively short amplicons (100-200 bp) (Appendix Twist Dx) but traditional PCR primers have also been successfully implemented.

In recent years, RPA has attracted immerse interest in the scientific world and it has been used for the detection of a wide number of targets, including bacteria, virus, protozoa, animals and plants. Both end-point and real-time detection methodologies have been described, with the majority of the detection methods being based on lateral flow assays, real-time fluorescence, electrochemistry or colorimetry (Lobato and O'Sullivan, 2018).

5. Key points in developing DNA-based assays and biosensors

Several different aspects have to be carefully controlled during the development of DNA-based assays and biosensors. Herein, we specifically refer to assays and biosensors that include an amplification step and subsequent detection of the amplified product through its hybridisation with a complementary probe immobilised on a solid support. These factors are discussed in the following sections.

5.1. Target DNA

In contrast to the analysis of toxins, whose standards are commercially available, this is not the case of DNA-based methods. Different approaches have been reported in the literature to construct standard curves for quantitative purposes: (i) using dilutions of the synthetic target sequence (ssDNA or dsDNA); (ii) using dilutions of genomic DNA extracted from a pool of cells/particles; and (iii) using genomic DNA extracted from dilutions of cells/particles.

Whilst working with synthetic DNA is the simplest approach since it can be easily synthesised, obtaining genomic DNA implies culturing the strains of microalgae at specific growth conditions (e.g. temperature, salinity and light), followed by DNA extraction. However, obtaining pure genomic DNA from viruses is much more difficult. Indeed, this is the case for OsHV-1. Because no stable bivalve cell lines are available, the virus cannot be replicated and can only be obtained from infected bivalves. Additionally, subsequent virus isolation and purification involves high-speed ultracentrifugation, which is time consuming and requires expensive equipment. The use of simple and rapid isolation/purification methods such as the use of magnetic beads (MB) able to capture viruses would be of great interest.

5.2. Primer design

Primers used for the amplification step are designed from sequences available in molecular databases (e.g. GeneBank). Whilst the genome of most viruses is sequenced, this is not the case for microalgae species. Primers for microalgae are commonly designed within the ribosomal DNA (rDNA) genes, since they have been widely used in phylogenetic analyses, and significant sequences are accessible (Penna

et al. 2014). Different regions of the rDNA can be employed, including the small subunit (SSU), the large subunit (LSU), the two internal transcribed spacers (ITS1 and ITS2), and the external transcribed spacer (ETS). The different degrees of sequence variability within these regions may be exploited to target microalgae genera or species. For instance, ITS sequences are generally more useful for designing primers to discriminate species of the same genus due to their higher variability (Shao *et al.* 2004) . If strains within the same species or species within the same genus show differences in specific nucleotides, primers often incorporate degenerate bases, which are equimolar mixtures of two or more different bases (e.g. Y, R, W and S).

Primers can be designed for single or multiplex amplification, to measure a single target or more than one target, respectively. The design of primers to amplify multiple targets in a single reaction is not straightforward, since cross-reactivity among primers is likely to occur (Mayboroda *et al.* 2018). To this end, most multiplex amplifications are actually parallelised single reactions that do not require mixing several sets of primers, although they have the advantage of still being performed using the same experimental conditions. Parallelised multiplexing is usually a prior step to develop real "one-spot" multiplexing. Moreover, a way to simplify primer design for multiplexing consists of minimising the number of required primers by using common primers among targets. For instance, instead of using 4 primers to specifically detect *K. veneficum* and *K. armiger* species, the design of one common primer for both species results in the use of only three primers, thus reducing the possibility of primer-dimer formation.

The design of specific primers for the sequence of interest without interferences from other non-target sequences is undoubtedly a key factor in the development of molecular methods, since it determines their specificity. Environmental samples contain a wide range of microorganisms which, indeed, show a rich molecular diversity. Because probe specificity is based *in silico* and limited sequences of marine microorganisms are currently available, the design of reliable primers for the environmental field still remains a challenge.

5.3. Probe design

Once amplified, products are subsequently hybridised with a complementary short sequence called capture probe in order to be detected. Among DNA hybridisation assays, sandwich hybridisation formats have attracted particular interest due to their enhanced specificity as a consequence of the two hybridisation events (capture and reporter). Prior to hybridisation, amplified products are generally purified to remove possible interfering molecules (e.g. enzymes, unincorporated primers and nucleotides). Moreover, DNA hybridisation assays and biosensors (when the capture

probe is immobilised onto a transducer surface) inherently require the amplified product to be single stranded. In order to avoid a previous denaturation step of the amplified products, and to simplify assay cost and time, an innovative approach was proposed in our research group (O'Sullivan *et al.* 2013).

The approach is based on the "tailed primer" concept. A tailed primer consists of a ssDNA sequence (“tail”) that is added to its 5’-end. Between the primer and the tail a stopper consisting of a multi-carbon alkyl chain spacer is located to prevent the polymerase from further elongation. Following amplification, this results in dsDNA products flanked by ssDNA tails, which can be easily detected in a sandwich-type format by using oligonucleotide probes complementary to the tails: a capture probe and a reporter probe (**Fig. 4**).

Tails (and probes) consist of short synthetic oligonucleotide sequences (typically between 15 and 20 bp) without any biological function. Although the ones used in this thesis were originally designed not to bind to the human genome, they were also suitable for the detection of microalgae and viruses. Prior to their use, tails and probes need to be checked *in silico* to ensure they do not bind to any part of the genome (or, alternatively, available sequences) of the target organism as well as to the primers.

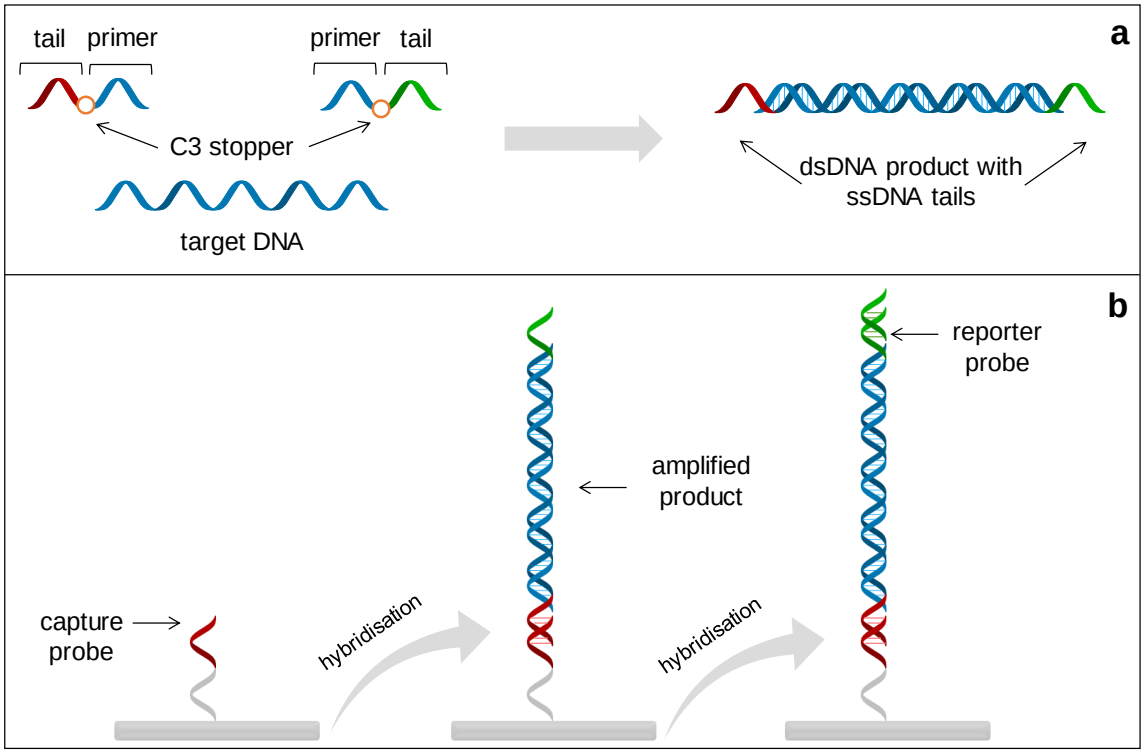

Fig. 4. Schematic representation of the “tailed primer" concept: (**a**) amplification; (**b**) sandwich hybridisation assay.

5.4. Probe immobilisation

The immobilisation step is also of crucial importance in the development of DNA-based assays and biosensors since it affects a wide range of parameters including sensitivity and stability. Hence, some general aspects must be taken into account when attaching the capture probe on a solid support. The most successful immobilisation strategies are covalent attachment (e.g. amino, hydroxy and carboxylic-modified probes), affinity interaction (e.g. streptavidin-biotin) and self-assembly (e.g. thiol-modified probes). These immobilisation strategies are able to preserve the biorecognition capability of the capture probe because: (i) they lead to well oriented probes, (ii) they allow control of the interchain space through modulation of the surface coverage, thus limiting steric impediments between the target and the probe, and (iii) they prevent leakage of the probes (Palchetti *et al.* 2011). Particularly in the case of electrochemical biosensors, the immobilisation procedure should also ensure both a close proximity between the reporter signal and the transducer and spacing of the immobilised probes, in order to obtain an efficient electron transfer.

Compared to covalent binding and affinity interactions, self-assembling is more widely used due to the simplicity of the protocol. It is used to immobilise thiolated-modified probes onto metal surfaces through the formation of dative covalent bonds between the sulphur and the metal atoms. Although thiols chemisorbs onto different metals including silver, platinum or copper, gold is usually the substrate of choice because of its inert properties and the formation of well-defined self-assembled monolayers (Pensa *et al.* 2012). In fact, a plethora of biosensors have been developed using thiol-modified DNA probes. Additionally, thiolated probes can be covalently immobilised to maleimide-modified surfaces. To control probe spacing and block unreacted groups, a second thiolated molecule (e.g. mercaptohexanol) can be anchored on the surface simultaneously or subsequently to the thiolated probe immobilisation (Henry *et al.* 2010).

Although the choice of the most appropriate immobilisation strategy depends on the characteristics of the transducer material (e.g. metal, glass and carbon), a wide range of supports (e.g. plates, electrodes and MBs) can be used to tailor the DNA-based assays and biosensors according to the final application. Microtitre plates, usually made of polystyrene, are the gold standard in the development of conventional colorimetric assays. Pre-activated plates are also available for surface-tethering of the biomolecule, such as plates coated with streptavidin or modified with maleimide groups.

A wide variety of electrodes of different material, size and geometry are available. Lithography and screen-printing are the primary technologies used to manufacture

electrodes (Kim *et al.* 2017). Whilst photolithographic techniques are quite expensive, thus increasing the cost per device, screen-printing techniques can significantly lower the fabrication costs while producing devices at large scale with appropriate precision and accuracy. Nonetheless, while screen-printed electrodes are designed for single use, thin-film electrodes are reusable after applying cleaning procedures. These procedures are usually chemical methods based on Piranha solution or electrochemical methods such as cyclic voltammetry between two defined potentials in the presence of sulphuric acid. Among the different materials, carbon and gold electrodes are extensively used because of their electrical conductivity as well as low cost and ease of modification with thiol groups, respectively.

In the past few years, the use of MBs as immobilisation supports has gained popularity. Nowadays, MBs of a variety of materials, sizes and modified with different functional groups are commercially available. MBs offer attractive properties since they can be functionalised with the capture probe and easily manipulated by an external magnetic field. Moreover, their combination with electrochemical biosensing strategies has been demonstrated to improve the analytical performance. Instead of direct surface modification of the electrode, both the hybridisation and washing steps can be performed on MBs, thus reducing matrix effects and improving assay kinetics. Following modification, MBs are easily captured by applying a magnetic field at the bottom of the electrode surface (Leonardo *et al.* 2017; Pinacho *et al.* 2014).

5.5. Detection

Detection of hybridised products can be divided into label-free and label-based formats. Label-free approaches translate the biorecognition event into a readable signal by means of physicochemical changes in the transducer microenvironment subsequent to probe-target hybridisation. By contrast, when electroactive compounds, nanoparticles or redox enzymes are bound to the reporter probe, the approaches are label-based (Palchetti *et al.* 2011). Among the most commonly used enzymes is horseradish peroxidase (HRP), which can catalyse the oxidation of 3,3',5,5'-tetramethylbenzidine (TMB) in the presence of H_2O_2, and the substrate can be then detected either by colorimetric or electrochemical methods. Other enzymes such as alkaline phosphatase are also common.

Although label-based techniques are frequently employed due to the higher sensitivities attained, the concept of direct detection offers simplicity. The choice of the detection strategy depends on the detection technique used. For instance, biosensors based on impedimetric electrochemical techniques or on scattering optical techniques are able to operate under a label-free format. On the other hand, colorimetric assays and amperometric electrochemical biosensors are usually label-based methods.

6. Towards implementing molecular tools in environmental monitoring

The detection of microalgae and viruses is highly desired to avoid economic losses in the shellfish industry and to protect human health and marine ecosystems. Monitoring programs aim at surveying the presence of such hazards in order to enable appropriate management activities. However, detection of these toxic events are still very difficult to anticipate, thereby alternative rapid, low-cost and reliable early warning molecular tools are required.

To make molecular tools suitable for monitoring activities and also in-field applications, other steps besides detection itself need to be taken into consideration, especially in the analysis of environmental samples. Herein, a description of these steps is provided, together with approaches currently used and challenges that still need to be tackled.

6.1. Sampling

Monitoring activities require the design and implementation of sampling strategies, which include the proper selection of the sampling points, sample techniques and sample types. Regarding the latter, legislated monitoring activities for toxic microalgae focus on planktonic microalgae samples from the water column. In contrast, different approaches (i.e. water column or macroalgae substrate) has been described for benthic microalgae (e.g. *Ostreopsis* and *Gambierdiscus*) neither for their sampling technique (Jauzein *et al.* 2018). On the other hand, although monitoring of virus presence is performed in shellfish tissue samples, there is increasing interest to evaluate the presence of viruses in their transmission vehicle: seawater.

6.2. Sample concentration

A concentration step is necessary in environmental monitoring because the microorganism of interest is normally present at low concentration in water samples. For this reason, some detection methods that work for clinical samples cannot be directly transferred to environmental samples.

Microalgae and viruses differ markedly in size. For example, the size of OsHV-1 is 120 nm, whereas the size of *O*. cf. *ovata* is 30-50 µm. Two traditional approaches are commonly used to concentrate microalgae: filtration and conventional centrifugation (Karlson *et al.* 2010). Although filtration is a good option for large sample volumes (>500 mL), centrifugation is more suitable for smaller volumes (<500mL) since it is generally more efficient and rapid. On the other hand, concentrating viruses inherently requires extremely time-consuming and expensive ultracentrifugation (Goodwin and Litaker 2008). Accordingly, faster and simpler technologies such as the use of MBs (see section 5.1) are highly desirable.

6.3. DNA extraction

Another crucial pre-treatment step is DNA extraction, especially from microalgae. Due to the large amount of polysaccharides and polyphenols in microalgae, the isolation of high-purity DNA is not straightforward (Greco *et al.* 2014). At present, DNA extraction methods are mostly based on phenol/chloroform or standard spin kits, and a bead-beat protocol is normally implemented to disrupt the cells. Unfortunately, these methods are time consuming and require many reagents and instrumentation, limiting the speed of the whole assay and their use for *in situ* testing, respectively. Additionally, the wide microalgae diversity (e.g. naked or thecate microalgae) has hampered the implementation of universal protocols (Yuan *et al.* 2015). Therefore, rapid, reliable and standardised methods to extract DNA from microalgae and from viruses are highly desired.

6.4. Applicability and validation

In the past few years, most of the advances have been made on the development of molecular systems but not on their application into the analysis of environmental samples and their subsequent validation, which involves the comparison of results with current reference analysis methods.

When estimating microalgae abundances, good correlation is not always obtained between molecular methods and light microscopy counts. As stated before (section 5.2), most molecular methods that detect microalgae target the rDNA. However, since rDNA copy numbers per microalgae cell may vary depending on the species, strain, growth phase and/or environmental conditions, it is challenging to determine cell abundancy in environmental samples using molecular methods (Galluzzi *et al.* 2010). This issue should be considered when studying the applicability of these molecular assays.

Moreover, little information regarding maximum permitted levels is available for microalgae species and viruses. The availability of new bioanalytical assays for their detection could favour further investigations for risk assessment purposes, to ultimately define appropriate thresholds.

6.5. *In situ* analysis

To move molecular diagnostics from the laboratory to the field, not only do the amplification steps need to be compatible with miniaturisation, but also other steps such as detection have to be improved. In general, optical systems require the use of bulky optical detection equipment. In contrast, electrochemical detection systems do

not require complex instrumentation and can be easily integrated into microfluidic platforms for subsequent automation. Nevertheless, to date, the only microfluidic platform that exploits an isothermal amplification technique for the detection of microalgae uses a portable fluorescence reader (Loukas *et al.* 2017). Future improvements in microfluidics and nanotechnology could lead to micro total analysis systems (μTAS) or lab-on a-chip devices that integrate sampling, pre-treatment, DNA extraction, amplification and detection in a single chip, thus being deployable for on-site analysis.

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Appendix Twist Dx Appendix to the TwistAmp reaction kit manuals. https://www.twistdx.co.uk/docs/default-source/RPA-assaydesign/newappendix(2).pdf

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CHAPTER 2

Objectives and scientific publications

Objectives

The **general objective** of this thesis is the development of DNA-based assays and biosensors for the detection of marine toxic microalgae and viruses, and their application to the analysis of environmental samples. Moreover, this thesis aims to contribute to other steps in the environmental analysis process, including sample pre-treatment and DNA extraction.

To achieve this goal, the following **specific objectives** have been defined:

- To establish a rapid and simple DNA extraction method for microalgae.
- To develop qPCR assays to detect microalgae.
- To exploit isothermal recombinase polymerase amplification and sandwich hybridisation formats to develop colorimetric assays and electrochemical biosensors for microalgae and viruses.
- To evaluate the applicability of the DNA-based assays and biosensors to the analysis of environmental (planktonic, benthic and spiked) and oyster samples.
- To develop a MB-based strategy to capture and concentrate viral particles.

Overall, this thesis aims to demonstrate the opportunities that nucleic acids offer to environmental analytical chemistry by providing new approaches that will pave the way towards the implementation of rapid, specific, simple, low-cost and *in situ* analysis tools to improve environmental monitoring.

Scientific publications and own contribution

The development of this thesis has led to the publication of twelve scientific publications, seven of them as a first author and one of them currently under review. To achieve this, different institutions and researchers have collaborated. The aim of this section is to provide a list of these publications and a statement of my personal contribution to each of them.

The thesis includes six publications, which are found in Chapters 3, 4 and 5:

1. Toldrà A, Andree KB, Fernández-Tejedor M, Diogène J, Campàs M (2018) **Dual quantitative PCR assay for identification and enumeration of *Karlodinium veneficum* and *Karlodinium armiger* combined with a simple and rapid DNA extraction method**. Journal of Applied Phycology 30:2435-2445 *(Chapter 3A)*

   Personal contribution:
   - Microalgae culture, harvesting, counting, DNA extraction and molecular identification.
   - Development, optimisation and characterisation of the qPCR assay for two *Karlodinium* species.
   - Application and optimisation of the new DNA extraction method to microalgae.
   - Pre-treatment and DNA extraction of environmental seawater samples.
   - Analysis of seawater samples by qPCR.

2. Toldrà A, Jauset-Rubio M, Andree KB, Fernández-Tejedor M, Diogène J, Katakis I, O'Sullivan CK, Campàs M (2018) **Detection and quantification of the toxic marine microalgae *Karlodinium veneficum* and *Karlodinium armiger* using recombinase polymerase amplification and enzyme-linked oligonucleotide assay**. Analytica Chimica Acta 1039:140-148 *(Chapter 3B)*

   Personal contribution:
   - Microalgae culture, harvesting, counting, DNA extraction and molecular identification.
   - Primer modification with tails.
   - Development, optimisation and characterisation of the colorimetric assay for two *Karlodinium* species.
   - Preparation, pre-treatment and DNA extraction of spiked samples.
   - Analysis of spiked samples by the colorimetric assay.

3. Toldrà A, Alcaraz C, Andree KB, Fernández-Tejedor M, Diogène J, Katakis I, O'Sullivan CK, Campàs M (2019) **Colorimetric DNA-based assay for the specific detection and quantification of *Ostreopsis* cf. *ovata* and *Ostreopsis* cf. *siamensis* in the marine environment**. Harmful Algae 89:27-35 *(Chapter 4A)*

   Personal contribution:
   - Sampling of environmental planktonic and benthic samples and isolation of *Ostreopsis* species.
   - Microalgae culture, harvesting, counting, single-cell DNA extraction and molecular identification.
   - *Ostreopsis* primer design for RPA, and further modification with tails.
   - Development, optimisation and characterisation of the colorimetric assay for two *Ostreopsis* species.
   - Establishment of qPCR calibration curves for two *Ostreopsis* species.
   - Pre-treatment and DNA extraction of environmental samples.
   - Analysis of environmental samples by the colorimetric assay and qPCR.
   - Development of a predictive model to estimate environmental cell abundances.

4. Toldrà A, Alcaraz C, Diogène J, O'Sullivan CK, Campàs M (2019) **Detection of *Ostreopsis* cf. *ovata* in environmental samples using an electrochemical DNA-based biosensor.** Science of the Total Environment 689:655-661 *(Chapter 4B)*

   Personal contribution:
   - Sampling of environmental planktonic and benthic samples and isolation of *Ostreopsis* species.
   - Microalgae culture, harvesting, counting, single-cell DNA extraction and molecular identification.
   - *Ostreopsis* primer design for RPA, and further modification with tails.
   - Development, optimisation and characterisation of the MB-based electrochemical biosensor for *O*. cf. *ovata*.
   - Study of the storage stability of the functionalised-MB conjugates.
   - Pre-treatment and DNA extraction of environmental samples.
   - Analysis of spiked samples by the electrochemical biosensor, qPCR and colorimetric assay.
   - Development of a predictive model to estimate environmental cell abundances.

5. Toldrà A, Furones MD, O'Sullivan CK, Campàs M (2020) **Detection of isothermally amplified ostreid herpesvirus 1 DNA in Pacific oyster (*Crassostrea gigas*) using a miniaturised electrochemical biosensor.** Talanta, In Press *(Chapter 5A)*

   Personal contribution:
   - Primer modification with tails.
   - Development, optimisation and characterisation of the colorimetric assay and the electrochemical biosensor for ostreid herpesvirus-1 (OsHV-1).
   - Study of the storage stability of the functionalised-MB gold electrodes.
   - Analysis of oysters by the electrochemical biosensor and qPCR.

6. Toldrà A, Andree KB, Bertomeu E, Roque A, Carrasco N, Gairín I, Furones MD, Campàs M (2018) **Rapid capture and detection of ostreid herpesvirus-1 from Pacific oyster *Crassostrea gigas* and seawater using magnetic beads**. PLoS One 13(10)15 *(Chapter 5B)*

   Personal contribution:
   - Preparation of two types of OsHV-1-contaminated matrices: seawater and oyster tissue homogenate.
   - Capture of OsHV-1 to MBs and analysis of the conjugates using qPCR.
   - Characterisation of the OsHV-1-MB conjugates.
   - Experimental infections of oysters and mortality monitoring.
   - RNA analysis of infected oysters.
   - Pre-concentration experiment using MBs.

Moreover, six publications (including one review and one book chapter) have been achieved. Four of these publications are presented as annexes and maintain the format of the journal where they have been published.

7. Toldrà A, O'Sullivan CK, Campàs M **Detecting harmful algal blooms with isothermal molecular strategies.** Trends in Biotechnology, In Press *(Annex 1)*

   Personal contribution:
   - Manuscript writing.

8. Leonardo S, Toldrà A, Campàs M (2017) **Trends and Prospects on Electrochemical Biosensors for the Detection of Marine Toxins**. Recent Advances in the Analysis of Marine Toxins. Comprehensive Analytical Chemistry 74:303-342 *(Annex 2)*

   Personal contribution:
   - Aptasensor section writing.

9. Leonardo S, Toldrà A, Rambla-Alegre M, Fernández-Tejedor M, Andree KB, Ferreres L, Campbell K, Elliot CT, O'Sullivan CK, Pazos Y, Diogène J, Campàs M (2017) **Self-assembled monolayer-based immunoassays for okadaic acid detection in seawater as monitoring tools**. Marine Environmental Research 133:6-14 *(Annex 3)*

   Personal contribution:
   - Single-cell DNA extraction and molecular identification of *Dinophysis* species.

10. Reverté L, Toldrà A, Andree KB, Fraga S, de Falco G, Campàs M, Diogène J (2018) **Assessment of cytotoxicity in ten strains of *Gambierdiscus australes* from Macaronesian Islands by Neuro-2a cell-based assays**. Journal of Applied Phycology 30:2447-2461 *(Annex 4)*

    Personal contribution:
    - Microalgae culture, harvesting, counting, DNA extraction and molecular identification of *Gambierdiscus* species.

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

    Personal contribution:
    - Single-cell DNA extraction and molecular identification of *Gambierdiscus* species, jointly with the first author.

12. Magriñá I, Toldrà A, Campàs M, Ortiz M, Simonova A, Katakis I, Hocek M, O'Sullivan CK (2019) **Electrochemical genosensor for the direct detection of tailed PCR amplicons incorporating ferrocene labelled dATP**. Biosensors and Bioelectronics 134:76-82

    Personal contribution:
    - Preparation, pre-treatment and DNA extraction of spiked samples.
    - Analysis of spiked samples by qPCR jointly with the first author.

CHAPTER 3

Karlodinium: qPCR and colorimetric assays

Chapter 3A

Dual quantitative PCR assay for identification and enumeration of *Karlodinium veneficum* and *Karlodinium armiger* combined with a simple and rapid DNA extraction method

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Abstract

Karlodinium is a dinoflagellate genus responsible for massive fish mortality events worldwide. It is commonly found in Alfacs Bay (NW Mediterranean Sea), where the presence of two *Karlodinium* species (*K. veneficum* and *K. armiger*), with different toxicity, has been reported. Microscopy analysis is not able to differentiate between these two species. Therefore, new and rapid methods that accurately and specifically detect and differentiate these two species are crucial to facilitate routine monitoring, to provide early warnings and to study population dynamics. In this work, a quantitative real-time PCR (qPCR) method to detect and enumerate *K. veneficum* and *K. armiger* is presented. The ITS1 region of the ribosomal DNA was used to design species-specific primers. The specificity of the primers together with the melting curve profile provided a reliable qualitative identification and discrimination between the two *Karlodinium* species. Additionally, a simple and rapid DNA extraction method was used. Standard curves were constructed from 10-fold dilutions of cultured microalgae cells. Finally, the applicability of the assay was tested with field samples collected from Alfacs Bay. Results showed a significant correlation between qPCR determinations and light microscopy counts (y = 2.838 x + 564; R^2 = 0.936). Overall, the qPCR method developed herein is specific, rapid, accurate and promising for the detection of these two *Karlodinium* species in environmental samples.

1. Introduction

Harmful algal blooms (HABs) of dinoflagellates of the genus *Karlodinium* (initially classified as *Gymnodinium* or *Gyrodinium* before the erection of the new genus by Daugbjerg *et al.* (2000)) have been implicated in numerous fish-killing events around the word, including Europe, Southwest Africa, North America, Australia and East Asia (Garcés *et al.* 2006; Place *et al.* 2012). In Alfacs Bay (Ebro Delta, NW Mediterranean Sea) winter blooms of *Karlodinium* spp. have been periodically reported since 1994, causing mortality of fish in aquaculture ponds, raft cultures of mussels as well as local wild fauna. Among the 11 *Karlodinium* species reported to date (AlgaeBase), two *Karlodinium* species, identified by morphological and genetic analyses as *K. veneficum* and *K. armiger*, have been described and have become well established in this region (Garcés *et al.* 2006). Both species are known to be mixotrophic, combining photosynthesis with prey feeding (phagotrophy), and produce haemolytic toxins. These toxins function as prey immobilisation agents before ingestion (Berge *et al.* 2012). Recently, karlotoxins from *K. veneficum* (Van Wagoner *et al.* 2008) and karmitoxins from *K. armiger* (Rasmussen *et al.* 2017) have been isolated and chemically characterised. It seems that *K. armiger* has a higher level of ichthyotoxicity in comparison to *K. veneficum* (Garcés *et al.* 2006), which may be attributed to the higher toxicity of karmitoxins (Berge *et al.* 2012).

In Alfacs Bay, *Karlodinium* spp. outbreaks tend to concentrate in calm and low turbulence areas and can attain very high densities (above 4,000,000 cells/L). Toxicity studies with *Karlodinium* spp. populations have set 366,000 cells/L as the No Observed Effect Concentration (NOEC) in a fish mortality assay (Fernández-Tejedor *et al.* 2004). Accordingly, a level of 200,000 cells/L has been established as a warning level for *Karlodinium* spp. in this geographic area. The implementation of a monitoring program that provides adequate early warnings of possible imminent blooms is essential to mitigate adverse economic, health and environmental effects caused by HABs. Current monitoring programs use light microscopy to detect and enumerate toxic microalgae. However, this method is time-consuming, requires a great deal of taxonomic expertise and is based on morphological characteristics, which in some cases are insufficient to identify at genus or species level. Identification of cells of the genera *Karlodinium* is particularly difficult because their unarmoured morphology is poor in distinct features (Shao *et al.* 2004), and thus they can be easily misidentified with other genera like *Gymnodinium*, *Karenia, Heterocapsa* and *Ansanella* (Bergholtz *et al.* 2006; Garcés *et al.* 2006; Jeong *et al.* 2014). Moreover, *Karlodinum* species cannot be differentiated using light microscopy (Bergholtz *et al.* 2006).

To address these challenges, molecular methods are being developed and employed to study HAB species. Molecular tools have advantages with respect to traditional

microscopy techniques: (i) they are faster, which makes possible their use as early warning tools; (ii) they are more accurate, allowing the identification of morphologically similar species or genera; and (iii) fewer hours of training are required to attain a level of expertise sufficient for routine laboratory screening. Among the different molecular methods for microalgae detection, quantitative real-time PCR (qPCR) has been used to identify and quantify HAB species in marine environmental samples (Penna *et al.* 2013). Several qPCR methods have been reported for different microalgae species including *Alexandrium minutum* (Galluzzi *et al.* 2004), *Karenia mikimotoi* (Yuan *et al.* 2012), *Karlodinium veneficum* (Berge *et al.* 2012; Eckford-Soper and Daugbjerg 2015b; Park *et al.* 2009; Place *et al.* 2012), *Prymnesium parvum* (Eckford-Soper and Daugbjerg 2015b; Galluzzi *et al.* 2008) and *Prorocentrum donghaiense* (Zhang *et al.* 2016). In addition, various qPCR methods have been designed to differentiate between two species within the same genus: *Ostreopsis* (*O.* cf. *ovata* and *O.* cf. *siamensis*) (Battocchi *et al.* 2010; Perini *et al.* 2011), *Pseudochattonella* (*P. farcimen* and *P. erruculosa*) (Eckford-Soper and Daugbjerg 2016), *Alexandrium* (*A. catanella* and *A. taylori; A. catanella and A. tamarense*) (Galluzzi *et al.* 2010; Hosoi-Tanabe and Sako 2005) and *Dinophysis* (*D. acuta* and *D. acuminata*) (Kavanagh *et al.* 2010). Even more challenging is targeting more than two species, as described for *Gambierdiscus* (Nishimura *et al.* 2016; Vandersea *et al.* 2012) and *Pseudo-nitzschia* (Andree *et al.* 2011).

Most molecular assays use primers that are designed to hybridise to ribosomal DNA (rDNA) genes, since they are phylogenetically informative and tandemly repeated in high copy number, and significant databases of homologous sequences exist for interspecies comparisons. Different regions of the rDNA can be employed, including the small subunit (SSU), the large subunit (LSU), the two internal transcribed spacers (ITS1 and ITS2) and the external transcribed spacer (ETS). The different degrees of sequence variability within these regions may be exploited to target genus or species. For instance, ITS sequences are generally more useful for designing primers to distinguish among species of the same genus due to its higher content in non-conserved loci (Andree *et al.* 2011; Shao *et al.* 2004). Other molecular markers such as protein-encoding genes have also been exploited although to a lower extent (Penna *et al.* 2013). With reference to *Karlodinium* spp., primers previously developed for *K. veneficum* are based on the LSU rDNA region (Eckford-Soper and Daugbjerg 2015b), the ITS2 rDNA region (Park *et al.* 2009; Zhang *et al.* 2008) and the ferredoxin gene (Zhang *et al.* 2008).

Although quantitative molecular techniques have been increasingly used for microalgae, there is still a lack of a standardised, efficient and simple method for extracting high-quality DNA from phytoplankton (Yuan *et al.* 2015). Due to the large

amount of polysaccharides and polyphenolics in microalgae (Greco *et al.* 2014), the isolation of high-purity DNA, free from PCR inhibitors, is not straightforward. At present, DNA extraction/purification methods for microalgae are mostly based on cetyltrimethylammonium bromide (CTAB), phenol/chloroform or column-based commercial kits, and normally a bead-beating step is introduced to disrupt the cells (Erdner *et al.* 2010; Kamikawa *et al.* 2007; Yuan *et al.* 2015; Zhang *et al.* 2016). These methods require many reagents and instrumentation, and reproducibility and DNA yield are sometimes poor (Zhang *et al.* 2016). Additionally, these methods are time-consuming, limiting the speed of the whole assay. Although the use of crude extracts (without DNA purification) has been proposed to overcome these limitations, the protocol is tedious (Galluzzi *et al.* 2004; Park *et al.* 2009). Therefore, rapid and reliable methods to extract DNA from microalgae are highly desired.

The objective of this study was to develop a qPCR assay for the detection and enumeration of two *Karlodinium* species (*K. veneficum* and *K. armiger*) commonly found in Alfacs Bay, combined with the evaluation of a new, simple and rapid DNA extraction method. Species-specific primer sets were designed within the ITS1 region, and their specificity was tested. The performance of the assay was assessed by testing environmental samples and results were correlated with light microscopy determinations.

2. Materials and methods

2.1. Algal cultures

Clonal cultures of *K. veneficum* (strain IRTA-SMM-00-01) and *K. armiger* (strain K-0668) isolated from Alfacs Bay were obtained from IRTA Culture Collection of Algae (Sant Carles de la Ràpita, Spain) and the Scandinavian Culture Collection of Algae and Protozoa (Copenhagen, Denmark), respectively. *K. veneficum* and *K. armiger* clonal cultures were grown in f/2 medium (Guillard 1975; Guillard and Ryther 1962) and L1 + Urea medium (Guillard and Hargraves 1993), respectively, at a practical salinity of 36. Cultures were maintained at 18 ± 2 °C under a light intensity of 110 µmol photons m^{-2} s^{-1} on a standard 12:12 h light:dark cycle. Additionally, three microalgae commonly found in the Mediterranean Sea were used as control: *Ostreopsis* cf. *ovata, Pseudo-nitzschia fraudulenta* and *Ansanella granifera*. The ITS1-5.8S-ITS2 region of *Karlodinium* species and control non-target microalgae was PCR-amplified using primers described in Table 1 and bi-directionally sequenced (Sistemas Genómicos, LLC, Valencia, Spain) to identify species. Sequences were manually edited and aligned using BioEdit v7.0.5.2 (Hall 1999) and deposited in GenBank (**Table 1**).

Culture samples were fixed with Lugol's iodine (Throndsen 1978) and cells were counted under an inverted light microscope (Leica DMIL) following the Uthermöl

method (Utermöhl 1958). Cultures were collected at the exponential phase and harvested by centrifugation (3,700 g; 25 min). For all microalgae, pellets containing 10^4 cells were prepared. Additionally, for *K. veneficum* and *K. armiger*, 10-fold serial dilutions from 10^2 to 10^5/10^6 cells were prepared. Cell pellets were stored at -20 °C until extraction of genomic DNA.

Table 1. Microalgae species used in this study.

Species	Location and year	Source	Strain	GenBank number	Primers
Karlodinium veneficum	Alfacs Bay, Spain, 2000	Fernández-Tejedor	IRTA-SMM-00-01	MG642757	MicroSSU/ DinoE
Karlodinium armiger	Alfacs Bay, Spain, 2000	Fernández-Tejedor	K-0668	MG642758	MicroSSU/ DinoE
Ostreopsis cf. *ovata*	Fangar Bay, Spain, 2015	This study	IRTA-SMM-15-13	MG551865	ITSA/ITSB
Pseudo-nitzschia fraudulenta	Vilanova, Spain, 2016	This study	IRTA-SMM-16-02	MG551866	MicroSSU/ DinoE
Ansanella granifera	Alfacs Bay, Spain, 2015	This study	IRTA-SMM-16-43	MG551867	MicroSSU/ DinoE

MicroSSU/DinoE (Andree *et al.* 2011); ITSA/ITSB (Sato *et al.* 2011)

2.2. DNA extraction methods

2.2.1. Biomeme (BIM) method

The Biomeme Sample Prep Kit for DNA (provided with standard columns) was obtained from Biomeme Inc. (Philadelphia, USA). DNA extraction was carried out according to general recommendations provided by the manufacturer. Reagents and the sequence of steps to apply are provided, but no protocol exists for non-standard applications. To assess the applicability of the Biomeme (BIM) method to microalgae samples, results were compared with those obtained by the standard phenol/chloroform/isoamylalcohol (PCI) method (see below). The BIM method was first qualitatively tested by qPCR for *K. veneficum, O.* cf. *ovata* and *P. fraudulenta* using pellets of 10^4 cells, and the addition of a mechanical disruption step using a bead beater was evaluated. The primers used were: Karlo20/KaV160 (this study) for *K. veneficum*, OvataF/OstreopsisR (Battocchi *et al.* 2010) for *O.* cf. *ovata*, and 5.8S/QPfrau (Andree *et al.* 2011) for *P. fraudulenta.* The BIM method was quantitatively tested constructing qPCR standard curves of *K. veneficum* and *K. armiger* cell dilutions (10^2-10^6 cells) and comparing with the PCI method (10^2-10^5

cells). Additionally, 10-fold serial dilutions of the extracted DNA (1:10; 1:100; 1:1,000) were analysed to evaluate the potential presence of PCR inhibitors.

The protocol for the BIM method after optimisation was as follows: cell pellets were resuspended in 300 µL of lysis buffer (1 M NaCl, 70 mM Tris, 30 mM EDTA, pH 8.6), transferred to 2-mL screw-cap cryotubes containing ~50 µg of 0.5-mm diameter zirconium glass beads (Biospec, USA) and disrupted using a BeadBeater-8 (BioSpec, USA) pulsed for 45 s at full speed. The volume used for each buffer was: Biomeme Lysis Buffer (BLB, 500 µL), Biomeme Protein Wash (BPW, 500 µL), Biomeme Wash Buffer (BWB, 750 µL) and Biomeme Elution Buffer (BEB, 500 µL). The procedure consisted of mixing 250 µL of the homogenised samples with BLB and pumping the fluid through a syringe with an ion-exchange cartridge attached (10 pumps). This was followed by passing, first BPW, and second BWB, through the cartridge one time each by pumping the fluid with the syringe plunger. After the washing steps, the columns were dried (~50 pumps, passing only air through). Finally, purified DNA was eluted in BEB (5 pumps). Genomic DNA was quantified and checked for its purity by reading the absorbance at 260/280 using a NanoDrop 2000 spectrophotometer (Thermo Fisher Scientific, Spain), and stored at -20 °C until qPCR analysis.

2.2.2. Phenol/chloroform/isoamylalcohol (PCI) method

The PCI method was used for qPCR specificity tests and as a reference to evaluate the performance of the BIM method. The PCI method, in brief, included resuspension of cells in 200 µL of lysis buffer, which were then transferred to 2-mL screw-cap cryotubes containing ~50 µg of 0.5-mm diameter zirconium glass beads. Then, 25 µL of 10% dodecyltrimethylammonium bromide (DTAB) and 200 µL of chloroform were added to the cryotubes, and the mixture disrupted using a BeadBeater-8 pulsed for 45 s at full speed. After centrifugation (2,300 g; 5 min), the aqueous phase (300 µL) was transferred to a fresh tube and the standard phenol/chloroform/isoamylalcohol (25/24/1, v/v/v) procedure was applied thereafter (Sambrook, 1989). Precipitation of the DNA from the final aqueous solution (240 µL) was achieved by the addition of 2 volumes (480 µL) of absolute ethanol and 0.1 volume (24 µL) of 3 M sodium acetate (pH 8.0). The DNA pellet was then rinsed with 70% ethanol and dissolved in 500 µL of molecular biology-grade water. Genomic DNA was quantified as stated in section 2.2.1.

2.3. Primer design and specificity verification

Primers for *K. veneficum* and *K. armiger* were designed with the aid of the software Amplify4 (Bill Engels, University of Wisconsin, USA). The ITS1-5.8S-ITS2 rDNA sequences obtained for *K. veneficum* and *K. armiger* were aligned with 33 other *Karlodinium* species sequences (**Table S1**) in a multiple sequence alignment using

ClustalW implemented in the program BioEdit v7.0.5.2. Variable regions were manually identified from the alignments. One genus-specific (*Karlodinium*) primer was designed at the beginning of the ITS1 region, and two species-specific (*K. veneficum* and *K. armiger*) primers were designed downstream in a more highly variable region within the ITS1 region (**Table 2**). Primer sets were designed to amplify products of approximately the same size to ensure that both assays had similar amplification efficiencies. Oligonucleotides were purchased from Sigma-Aldrich (Spain). Primer specificity was checked *in silico* by BLAST analysis and using Amplify4, as well as experimentally assessed by qPCR and electrophoretic analysis with 4 ng of DNA from target and non-target microalgae. Additionally, cross-reactivity experiments for the two *Karlodinium* species in both single and mixed DNA samples were conducted.

Table 2. Oligonucleotide primers targeting the ITS1 rDNA region of genus and species of *Karlodinium*.

Species and/or genus	Primer	Sequence (5'-3')	Amplicon size (bp)	Primer location
Karlodinium spp.	Karlo20	acatccaaccatytcactgtgaac	136/149	ITS1 (sense)
K. veneficum	KaV160	atagcttcgcagacaaaggtgaatc	135	ITS1 (antisense)
K. armiger	KaA160	atagcttcacagcagaggttacaac	149	ITS1 (antisense)

2.4. Quantitative real-time PCR (qPCR) assay

Optimisation of the qPCR protocol was investigated by testing different reagent concentrations and thermocycling conditions. The qPCR assays were performed with an ABI 7300 thermocycler (Applied Biosystems, Thermo Fisher Scientific, Spain) using the following two-step cycling protocol: 95 °C for 10 min followed by 45 cycles at 95 °C for 20 s and 58 °C for 30 s. Each 20-µL reaction mixture contained 10 µL 2X SYBR Green dye (Applied Biosystems, Thermo Fisher Scientific, Spain), primers (final concentration 0.5 µM) and 2 µL of DNA extracts from cultured cells (see section 2.1) or field samples (see section 2.5) in triplicate. At the end of each run, a dissociation step was included to evaluate melting curve profiles for the absence of primer dimers or non-specific products. The thermal profile for melting curve determination consists of 1 min at 60 °C with a gradual increase of temperature (1° C/15 s). In each qPCR experimental plate, a non-template control containing molecular biology-grade water was included as well as a positive control for each target.

2.5. Field samples analysis

Seawater samples from Alfacs Bay were collected in March 2017 at 4 different stations (**Fig. 1**). At each station, samples were collected at the surface (S) and at a depth of 5.5 m (B) (Table 3) using polyethylene bottles (1 L). Seawater samples were fixed with Lugol's iodine solution. For light microscopy counting, a volume of 50 mL was settled in sedimentation chambers for 24 h and counted following the Utermöhl method. For qPCR analysis, 50-mL aliquots were centrifuged (3,700 g; 25 min) and maintained at -20 °C until DNA extraction by the BIM method. The total number of *Karlodinium* cells obtained from the two counting methods (optical microscopy for genus-level detection and the sum of two qPCR assays for species-level) was compared by linear regression analysis using SigmaStat software 3.1 (Systat Software Inc., California, USA).

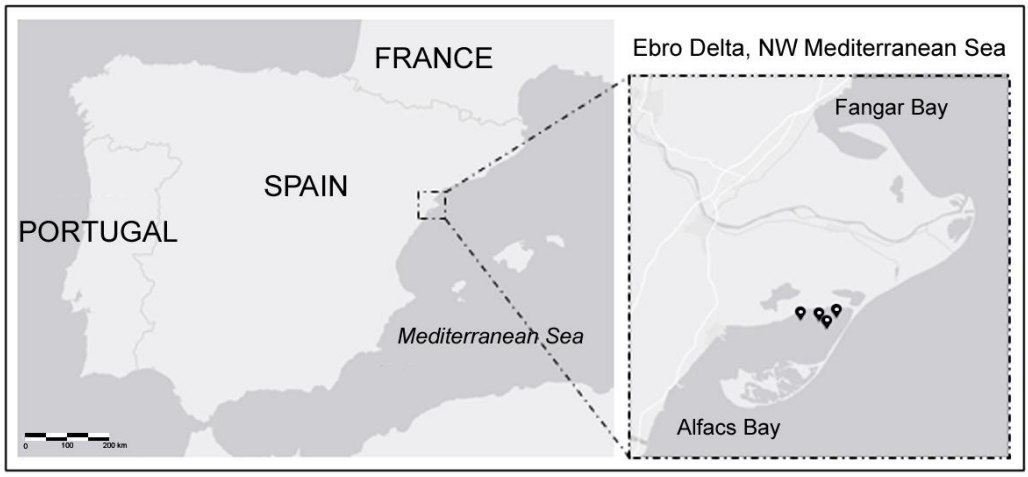

Fig. 1. Locations of the sampling in Alfacs Bay, NW Mediterranean Sea.

3. Results

3.1. Primers and qPCR specificity

BLAST analysis against the dataset of GenBank showed that primers for *K. veneficum* and *K. armiger* matched only with their respective ITS1 sequences. Analysis by qPCR showed DNA amplification for target species, while amplification products were not detected using control non-target microalgae. Moreover, *K. veneficum* and *K. armiger* specific primers did not show cross-reactivity neither in single nor in mixed DNA samples. Electrophoresis of the qPCR products using target DNA showed bands at the expected molecular weight, and no other bands were visible. Differences in the melting temperature (Tm) and shape of the melting curve profile were evident from the melting curve analysis of *K. veneficum* and *K. armiger*, with Tm of 83.5 °C and 84.1 °C, respectively (**Fig. S1**). Additionally, DNA from another *K. veneficum* strain (strain CCMP 415, taxonomic synonym formerly identified as *K. micrum*) (**Table S1**) that had

a somewhat different genotype in a region encompassed by the *K. veneficum* primers was tested. This region was also amplified and showed a slightly different melting curve profile and Tm of 84.3 °C (**Fig. S1**).

3.2. Biomeme (BIM) method evaluation

To assess the performance of the new DNA extraction method, results were compared with those obtained by the PCI method. After reading the absorbance at 260/280 nm, good yield and purity were obtained using both methods. However, a small peak at 230 nm was observed using the BIM method, which was also observed in the BWB solution, and thus certainly due to a component of the buffer. Both methods were tested using three microalgae with different cell morphology and structural composition. DNA extracted by PCI was detected for all microalgae by qPCR (**Fig. 2**). When the BIM method was applied without bead beating, DNA was extracted from *O.* cf. *ovata* and *P. fraudulenta*, but no DNA was extracted from *K. veneficum.* Nevertheless, DNA yields increased (Ct values decreased) for all microalgae when a bead-beating step was incorporated in the protocol.

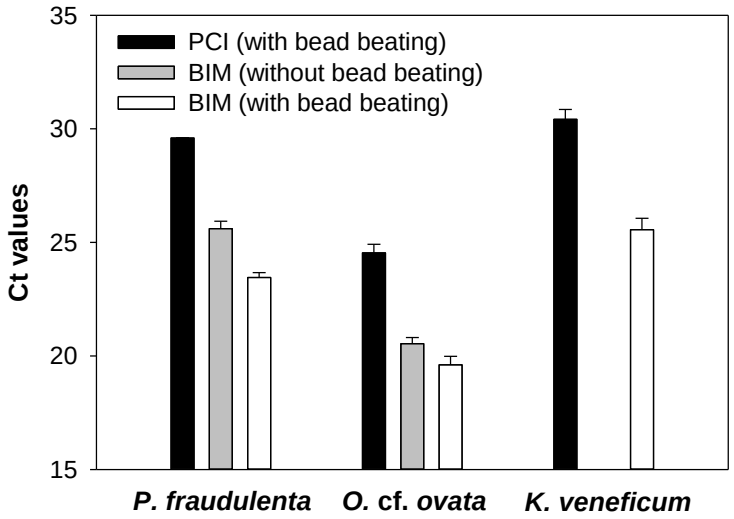

Fig. 2. Comparison of different methods for DNA extraction. Extraction efficiency was assessed by qPCR using 10^4 cells. All results are means of triplicates samples. Error bars represent standard deviation of the Ct values.

3.3. Standard curves for qPCR assays

Calibration curves for *K. veneficum* and *K. armiger* were constructed using DNA extracted from serial dilutions of cultured cells. The correlation between Ct values and number of cells is shown in **Fig. 3**. Using the PCI method, calibration curves showed a slope of -3.261 (R^2 = 0.962) and -3.442 (R^2 = 0.973) for *K. veneficum* and *K. armiger*, respectively. The efficiency of the reaction was calculated using the formula $E = (10^{(-1/m)} - 1)$, where *m* is the slope of the linear regression from the calibration curves, and

was 102.6% and 95.2%, respectively. Using the BIM method, slopes were -4.334 (R^2 = 0.994) for *K. veneficum* and -4.389 (R^2 = 0.982) for *K. armiger*, which correspond to efficiencies of 70.1% and 69.0%, respectively. To check if the lower efficiency of the BIM method was due to the potential presence of inhibitors (the above-mentioned component of the BWB), 10-fold serial dilutions of the extracted DNA were tested, but the efficiency did not improve. Although using the BIM method the efficiency of the qPCR assays was lower, a high linearity over 5 orders of magnitude was obtained with this method ($R^2 > 0.98$), which indicated that it is reliable in the quantification of target cells. On the other hand, using the PCI method high linearity ($R^2 > 0.96$) was only achieved over 4 orders of magnitude.

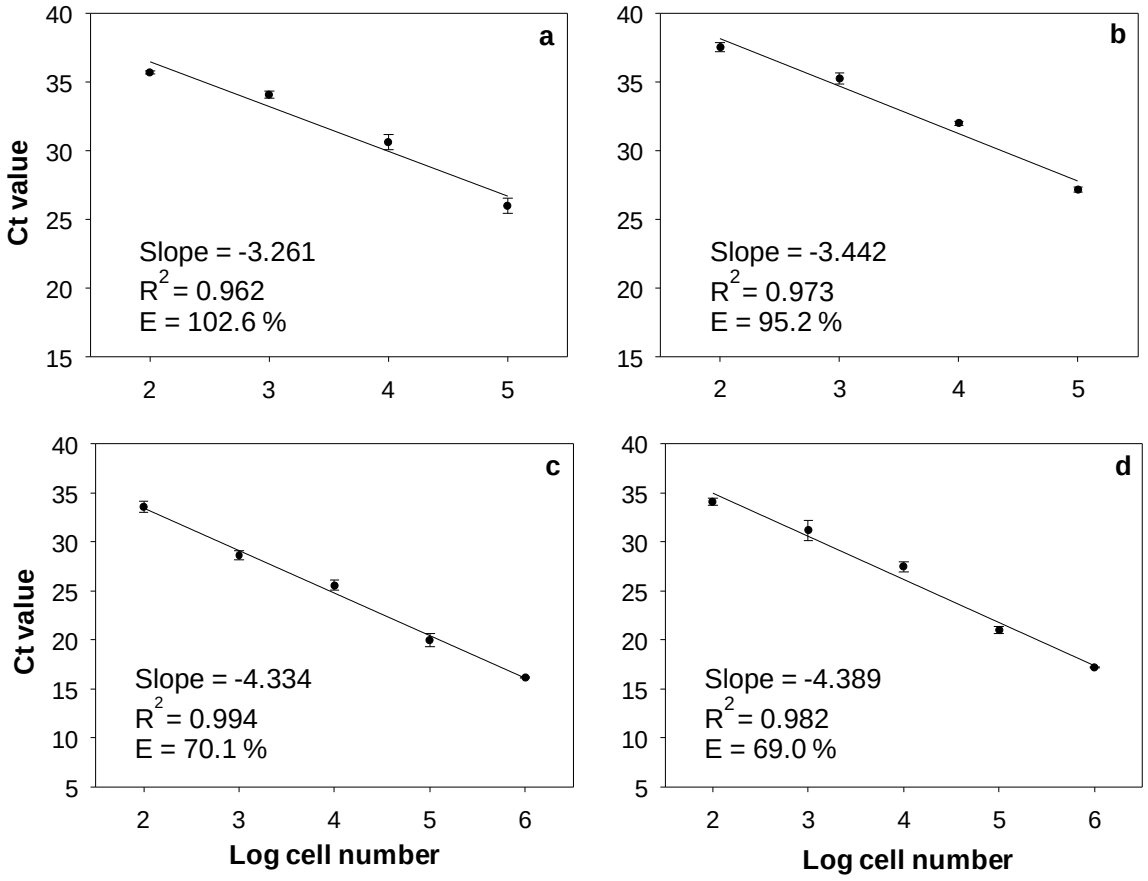

Fig. 3. Standard curves obtained by the correlation between cell numbers in the pellet and Ct values. Standard curves were obtained for each species-specific qPCR assay (**a**, **c**: *K. veneficum*; **b**, **d**: *K. armiger*) and each extraction method (**a**, **b**: PCI method; **c**, **d**: BIM method). All results are means of triplicates samples. Error bars represent the standard deviation of the Ct values.

3.4. Field sample analysis

To assess the ability of the qPCR assay to detect *Karlodinium* species from the environment, 8 seawater samples were collected from Alfacs Bay. All samples were analysed by light microscopy and qPCR. Cells of *Karlodinium* spp. were observed by

light microscopy in all samples, although with low abundances (from 1,000 cells/L to 18,549 cells/L), cell densities being higher at the surface than at the bottom (Table 3). Based on light microscopy analysis, all samples also contained significant abundances of other toxic species, *A. minutum* (maximum of 5,038 cells/L), and potentially toxic species of *Pseudo-nitzschia* (maximum of 106,029 cells/L) (**Table S2**). The qPCR assay detected the presence of both *Karlodinium* species in 7 out of 8 samples, *K. veneficum* being always more abundant than *K. armiger* (**Table 3**). In general, total cell densities of *Karlodinium* species determined by qPCR were higher than cell densities of *Karlodinium* spp. estimated by light microscopy. Nonetheless, a significant correlation (R^2 = 0.936; P < 0.0001) of 1 to 2.8 ratio (y = 2.838 x + 564) was found between techniques (**Fig. 4**). Only one sample displayed a different result: microscopy analysis revealed the presence of 1,000 cells/L in sample Station 1 (B), while the same sample analysed by qPCR did not show presence of *Karlodinium* species.

Table 3. Seawater samples (surface and bottom) collected at different stations in Alfacs Bay and results obtained using qPCR and optical microscopy. *Karlodinium* mean abundance was determined in triplicates ± standard deviation.

Sample	Geographic coordinates	Total cell number for the two *Karlodinium* species (cells/L)		Number of cells counted using each species-specific assay (cells/L)	
		Microscopy	qPCR	*K. veneficum*	*K. armiger*
Station 1 (S)	40°37'22.9"N 0°42'25.5"E	10,305	35,314	29,505 ± 847	5,809 ± 767
Station 1 (B)	40°37'22.9"N 0°42'25.5"E	1,000	n.d.	n.d.	n.d.
Station 2 (S)	40°37'34.2"N 0°43'31.4"E	8,772	23,647	11,919 ± 623	11,729 ± 2,536
Station 2 (B)	40°37'34.2"N 0°43'31.4"E	2,977	9,582	7,282 ± 1,008	2,301 ± 41
Station 3 (S)	40°36'59.9"N 0°42'55.6"E	8,473	20,881	19,184 ± 3,030	1,697 ± 448
Station 3 (B)	40°36'59.9"N 0°42'55.6"E	2,220	14,574	11,292 ± 1,460	3,282 ± 265
Station 4 (S)	40°37'28.9"N 0°41'07.7"E	18,549	52,390	26,859 ± 4,701	25,532 ± 4,014
Station 4 (B)	40°37'28.9"N 0°41'07.7"E	2,320	3,144	2,415 ± 2,226	729 ± 117

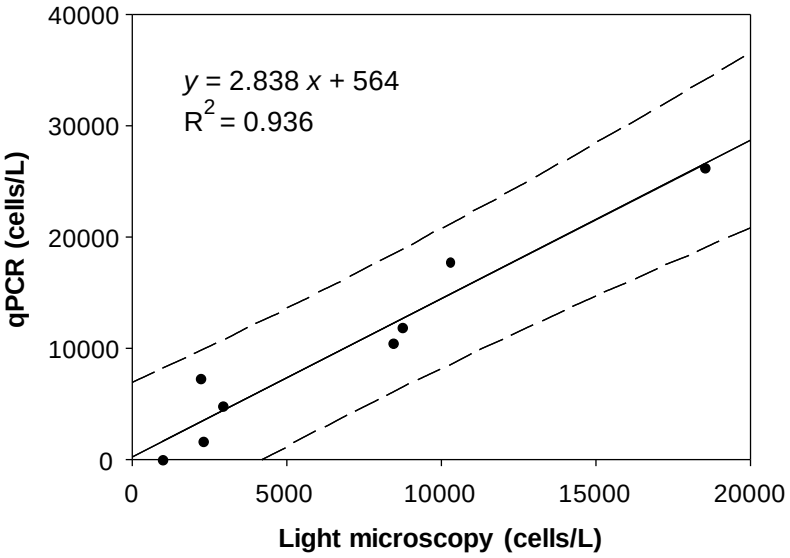

Fig. 4. Comparison of qPCR and light microscopy results for abundances of *Karlodinium* spp. in natural seawater samples. Dashed lines represent the prediction intervals of 95%.

4. Discussion

K. veneficum and *K. armiger* have been reported to co-occur forming blooms in Alfacs Bay (Garcés *et al.* 2006). Given that they show different levels of toxicity to marine organisms, the need for discrimination between these two species has become a serious concern within the framework of the local monitoring program. Current toxic microalgae monitoring is performed via light microscopy using the Uthermöl cell-counting method, which is time consuming and does not enable differentiation among *Karlodinium* species. In this study, a qPCR assay was developed for *K. veneficum* and *K. armiger* discrimination. Further, we have used a novel method that requires no centrifugation equipment or long incubations in thermal blocks to extract DNA quickly and with sufficient purity to perform qPCR assays.

Primers were designed within the ITS1 because it has been demonstrated to be a good region for species-specific detection due to its high variability (Shao *et al.* 2004). The design aimed at minimising the number of required primers: one genus-specific (*Karlodinium*) and two species-specific (*K. veneficum* and *K. armiger*) primers. A similar strategy has been described for quantifying *Pseudo-nitzschia* species (Andree *et al.* 2011) and total microalgae biomass (Godhe *et al.* 2008). Besides rDNA genes, mitochondrial genes (mtDNA) have also been evaluated for species-level discrimination. Although mtDNA was found to be a suitable marker for *Dinophysis* species (Raho *et al.* 2013), this is not the case for other microalgae since it is too conserved at an inter-species level (Penna *et al.* 2014). Hence, rDNA genes remain the target of choice to differentiate microalgae at the species level.

Screening of the primers against a large dinoflagellate rDNA gene database confirmed the specificity of the primers. Specificity tests demonstrated that the qPCR assay was specific for target species and was not affected by non-target microalgae that are sympatric in Alfacs Bay (and elsewhere): *O.* cf. *ovata* and *P. fraudulenta*, as well as *A. granifera*, a species morphologically similar to *Karlodinium* spp. Additionally, melting curve analyses were conducted to ensure that amplification derives from the intended product rather than from non-specific amplifications. *K. veneficum* was identified by a peak Tm at 83.5 °C, while *K. armiger* cells presented a peak Tm at 84.1 °C. Moreover, it was possible to identify the two species by a change in the melting curve profile, since melting curve shapes are a function of GC content, length and sequence of the amplicon (Ririe *et al.* 1997). The specificity of the primers together with the melting curve profile offer a reliable qualitative discrimination between the two *Karlodinium* species of this work.

Phytoplankton comprise a wide range of microalgae, some of them without a cell wall (e.g. naked dinoflagellates) and some others with fortified cell walls (e.g. diatoms with silica frustules or thecate dinoflagellates). Although different DNA extraction methods have been applied to microalgae (Kamikawa *et al.* 2007; Nishimura *et al.* 2016; Zhang *et al.* 2016), the wide microalgae diversity has hampered the implementation of a standardised, rapid and simple method. In fact, using an appropriate DNA extraction method is decisive in terms of sensitivity and rapidity of the whole assay. Therefore, in this study, the BIM method was tested for subsequent qPCR assays. The BIM method was tested with three microalgae representing different cell morphology and structural composition: *K. veneficum* (as a model of a small unarmoured dinoflagellate), *O.* cf. *ovata* (as a model of a medium thecate dinoflagellate) and *P. fraudulenta* (as a model diatom, possessing a silica frustule). Before DNA extraction, a mechanical disruption step with a bead beater is commonly used to break microalgae (Eckford-Soper and Daugbjerg 2015a; Erdner *et al.* 2010; Fawley and Fawley 2004; Yuan *et al.* 2015). The BIM method was qualitatively tested with and without a bead-beating step, showing that cells of some species (*O.* cf. *ovata* and *P. fraudulenta*) were easily disrupted with the lysis buffer before any mechanical homogenisation. However, DNA yield substantially increased after bead beating, indicating that some cells had not been broken with only the lysis buffer. On the other hand, DNA from *K. veneficum* was successfully extracted only when the BIM method was used in conjunction with bead beating. It is unclear why *K. veneficum* was so difficult to lyse and this issue should be further investigated. Our results concluded that the BIM method, with bead beating included, provided a higher recovery yield over the traditional PCI method. To assess the feasibility of applying the BIM method in downstream quantitative applications, calibration curves for *K. veneficum* and *K. armiger* were constructed and compared with those obtained with the PCI method.

The efficiencies obtained reflect not only the quality of the DNA extraction, but also the performance of the primers. Although efficiencies obtained using the PCI method (~100%) were higher than the BIM method (~70%), the latter provided a strong linear regression over a wide dynamic range of 5 orders of magnitude. Similar efficiencies were observed with *P. fraudulenta* and *O.* cf. *ovata* using the BIM method (data not shown). Additionally, similar slopes have been reported for *K. veneficum* (Eckford-Soper and Daugbjerg 2015a). As previously mentioned, DNA extracted by the BIM method showed an absorbance peak at 230 nm. This peak could be due to the presence of some slight amount of carry-over from a component of the BWB solution and could affect downstream qPCR assays. In order to assess the potential presence of inhibitors, DNA dilutions were tested. However, efficiency did not increase with increasing dilutions. Despite this limitation, there are several advantages of the BIM method over traditional DNA extraction methods: (i) ease and simplicity (it requires fewer steps), (ii) versatility (it has been successfully applied to different microalgae, and it is likely to work with many other kinds of microalgae), (iii) low exposure to hazardous materials, (iv) no specialised equipment (the exception being a bead beater, although small hand-held versions of such devices do exist), (v) low cost and time requirement (it takes less than 4 min), and (vi) applicability for accurate quantifications.

An objective of this study was to develop a qPCR method for application to environmental samples. Seawater samples collected at four sites of Alfacs Bay during the winter of 2017 were analysed by light microscopy and the qPCR assay. Light microscopy examination of the samples revealed a phytoplankton community with a high species diversity including important toxic or potentially toxic species, dominated by *Pseudo-nitzschia* spp., *A. minutum* and *Karlodinium* spp., in decreasing order of abundance (Table S2). The occurrence of *Karlodinium* spp. was detected in all seawater samples, but at a relatively lower concentration (<20,000 cells/L) than in blooms, and well below the warning level (200,000 cells/L). The last *Karlodinium* spp. bloom episode reported by the local monitoring program took place in the winter of 2000 and reached 13,000,000 cells/L (Fernández-Tejedor *et al.* 2004), although high densities (> 1,000,000 cells/L) were also reported in 2003 and 2007 (ICES-IOC 2004; ICES-IOC 2007). Higher abundances have been regularly found at the bottom layers of the bay, probably due to the adaptation of *Karlodinium* spp. to low-light conditions and/or sedimentation processes (Garcés *et al.* 1999). However, in this work, abundances were higher at surface waters than at 5.5 m depth, which could be explained by the strong wind reported days before the sampling, which favoured water mixing. Based on species-specific qPCR analysis, co-existence of both species was found in all samples where *Karlodinium* spp. was detected. These results agree

with those reported by Garcés *et al.* (2006), who documented that both *Karlodinium* species co-occurred in a single bloom in Alfacs Bay.

When comparing the two analytical techniques, although qPCR results were 2.8-fold higher than the Uthermöl counts, a strong correlation (R^2 = 0.936; P < 0.0001) was observed, which indicated the capacity of the qPCR to detect and quantify *Karlodinium* species in environmental samples. Only one sample displayed a significant difference in the cell densities, which coincided with the lowest abundance value detected by light microscopy. Discrepancies between light microscopy and qPCR techniques to enumerate microalgae have been widely reported in the literature and several explanations have been proposed, the most documented being the rDNA gene copy number variation (Casabianca *et al.* 2014; Eckford-Soper and Daugbjerg 2016; Galluzzi *et al.* 2010; Nishimura *et al.* 2016; Vanderesa *et al.* 2012). The rDNA gene content has been observed to change throughout the growth phase of microalgae cultures (Eckford-Soper and Daugbjerg 2016; Galluzzi *et al.* 2010; Perini *et al.* 2011) and this phenomenon is also expected to happen in nature during the different stages of a phytoplankton bloom. Different approaches have been used to construct calibration curves for the detection of microalgae using molecular methods, such as the use of dilutions of plasmids containing the cloned target sequence or cells. Although it is easy to work with plasmids (Galluzzi *et al.* 2008; Galluzzi *et al.* 2004; Yuan *et al.* 2012; Zhang *et al.* 2016), the potential variation in the copy number of rDNA genes could hamper the reliability and accuracy of the quantification method (Galluzzi *et al.* 2010; Perini *et al.* 2011). On the other hand, some authors have suggested that the use of cell dilutions is more likely to represent an average copy number per cell, without relying on the determination of the exact copy number per genome (Andree *et al.* 2011). Although standard curves based on cell dilutions have sometimes provided better accuracy (Andree *et al.* 2011; Eckford-Soper and Daugbjerg 2015a), other works have pointed out some discordant results (Perini *et al.* 2011). Some environmental factors, such as the daily light cycle or the type of nutrition, are tightly coupled with the cell cycle and/or the type of reproduction (Garcés *et al.* 1999), and may lead to variation in rDNA content of natural samples when compared with cultured samples (Galluzzi et al. 2004; Perini et al. 2011). Moreover, differences in gene copy number among isolates/strains of the same species have been reported (Galluzzi *et al.* 2010; Perini *et al.* 2011). With all these factors taken into account, qPCR is likely to over- or under-estimate abundances depending on whether the population as a whole contains more or fewer copies of rDNA per cell than the cultures used to construct the standard curve. Perini *et al.* (2011) described the use of environmental samples to construct calibration curves (their 'gold standard') in order to normalise the variability of the rDNA copy number between natural populations and cultures. Although rDNA genes are the most reported markers for species-level differentiation (Shao *et al.* 2004), the

rDNA gene copy number variation has shown to complicate the development of a quantification method (Andree *et al.* 2011; Casabianca *et al.* 2014; Galluzzi *et al.* 2010). One way to overcome this problem could be to use the rDNA gene marker together with a control and invariant gene. However, although feasible, it implies longer and more tedious assay protocols than the one proposed herein.

Another explanation regarding discrepancies between light microscopy and qPCR concerns the presence of species morphologically similar to the target in the phytoplankton community, which can easily lead to misidentification using light microscopy (Galluzzi *et al.* 2004). Even for a highly trained taxonomist, *Karlodinium* spp. identification is a highly skilled task because it resembles other small dinoflagellates. In this study, *Gymnodinium* spp. and *Heterocapsa* spp. were found in natural samples by light microscopy, and an accurate *Karlodinium* spp. identification was extremely difficult. It is important to mention that ambiguous cells were not counted as *Karlodinium* spp. Therefore, this may have contributed to an underestimation of cell abundance using light microscopy. It is important to note that the presence of non-target microalgae species in the natural samples, even at high densities, did not interfere in the qPCR assay, proving again its high specificity.

Phytoplankton monitoring is regulated by the EU legislation (EC 854/2004), which states that monitoring programs must periodically sample for toxic phytoplankton at shellfish growing areas. The monitoring using microscopy includes the Utermöhl cell-counting method to monitor microalgae presence. The qPCR assay developed in this study offers advantages over the traditional microscopy examination. It provides useful information on the abundance of the individual *Karlodinium* species found in Alfacs Bay. Additionally, it is relatively inexpensive, fast (it takes less than 2 hours, including the DNA extraction) and allows high-throughput sample analysis. Finally, although it has a lower sensitivity (0.2 cells/reaction and 2,000 cells/L taking into account the DNA extraction step and the analysis of 50-mL samples) compared to the Utermöhl method (60 cells/L (EN 15204:2006) with an error higher than 100% (Edler and Elbrächter 2010)), it allows the quantification of *Karlodinium* species below the warning threshold of 200,000 cells/L. It is important to note that this quantification limit could be reduced by centrifuging a larger sample volume or reducing the volume of the elution buffer.

To confirm the applicability of the molecular assay developed herein, more data from field samples are necessary to properly assess the 2.8-fold correspondence between the two techniques. This overestimation is another area for further investigation to understand the effect of several factors present in natural samples that might contribute to gene content variability. Additionally, primer specificity against other recently reported *Karlodinium* species should be examined in detail to apply the assay

to areas where *Karlodinium* species other than *K. armiger* and *K. veneficum* may be present. Future work is also required to improve the efficiency of the BIM method. Nevertheless, this method meets the requirements for *in situ* sampling on a ship or on the shore. Combination of this method together with qPCR mobile devices, such as the one commercialised by Biomeme Inc., or biosensors is highly desirable, and will pave the way towards the deployment of in-field diagnostic tools for microalgae monitoring.

5. Conclusions

This study describes the first molecular assay for the identification, discrimination and quantification of multiple genotypes of the two *Karlodinium* species, *K. armiger* and *K. veneficum*, commonly co-occurring in Alfacs Bay. Accurate species-specific identification and quantification is important because these two species produce distinct toxins, and this poses different risks to marine organisms and the marine-based economy, and because they cannot be differentiated using light microscopy. Another significant finding of this study is the implementation of a rapid DNA extraction method, which considerably reduces the assay time. The qPCR assay developed in this study is a promising new tool for monitoring the cell abundance and dynamics of these two *Karlodinium* species in Alfacs Bay.

Acknowledgements

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Supplementary information

Fig. S1. Melting curves for *Karlodinium* species: (**a**) *K. veneficum* (strain IRTA-SMM-00-01); (**b**) *K. armiger* (strain K-0668); and (**c**) *K. veneficum* (strain CCMP 415, formerly *K. micrum*).

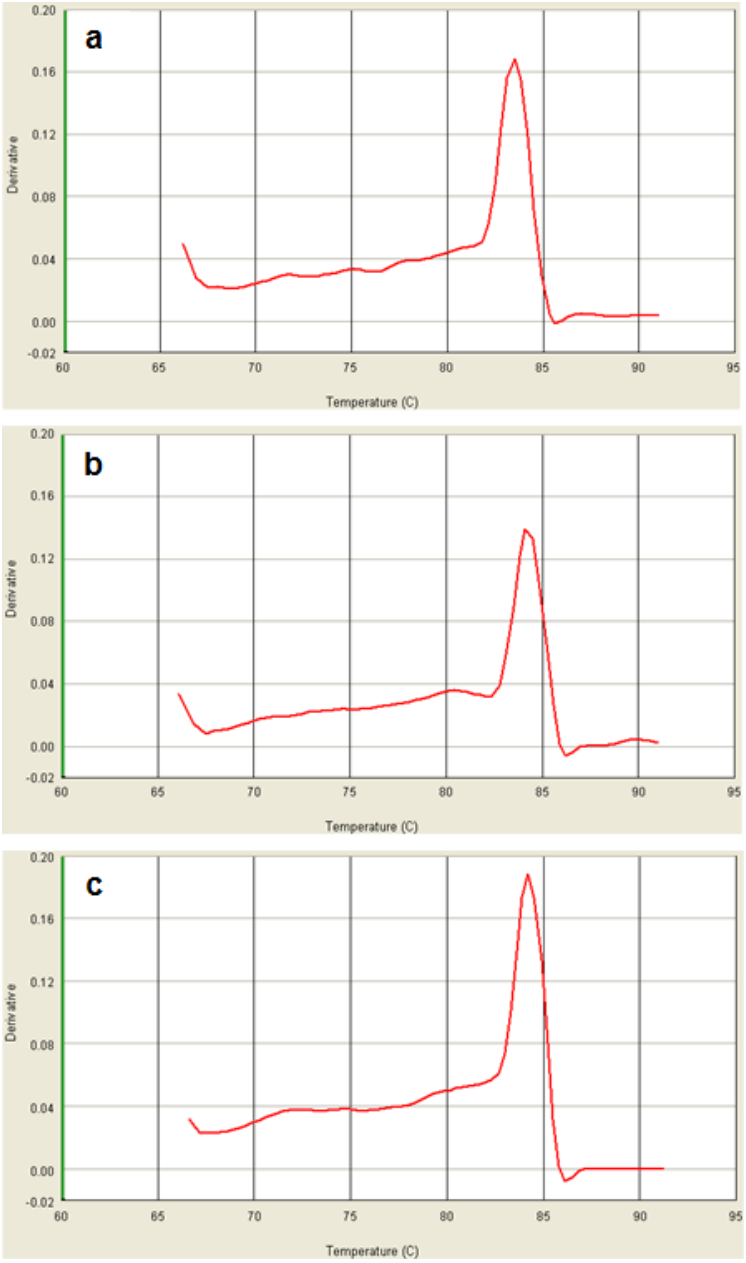

Table S1. ITS1-5.8S-ITS2 rDNA *Karlodinium* sequences used for primers design.

Species	Source	Strain	GenBank number
K. veneficum	This study	IRTA-SMM-12-03	MG642759
K. veneficum	This study	IRTA-SMM-12-04	MG642760
K. veneficum	This study	IRTA-SMM-12-05	MG642761
K. veneficum	This study	IRTA-SMM-12-06	MG642762
K. veneficum	This study	IRTA-SMM-12-07	MG642763
K. veneficum	This study	IRTA-SMM-12-08	MG642764
K. veneficum	This study	IRTA-SMM-12-09	MG642765
K. veneficum	This study	IRTA-SMM-12-10	MG642766
K. veneficum	This study	IRTA-SMM-12-12	MG642767
K. veneficum	This study	IRTA-SMM-12-13	MG642768
K. veneficum	This study	IRTA-SMM-12-14	MG642769
K. veneficum	This study	IRTA-SMM-12-15	MG642770
K. veneficum	This study	IRTA-SMM-12-16	MG642771
K. veneficum	This study	IRTA-SMM-12-17	MG642772
K. veneficum	This study	IRTA-SMM-12-20	MG642773
K. veneficum	This study	IRTA-SMM-12-21	MG642774
K. veneficum	This study	IRTA-SMM-12-22	MG642775
K. veneficum	This study	IRTA-SMM-12-23	MG642776
K. veneficum	This study	IRTA-SMM-12-24	MG642777
K. veneficum	This study	IRTA-SMM-12-25	MG642778
K. veneficum	This study	IRTA-SMM-12-26	MG642779
K. veneficum	This study	IRTA-SMM-12-28	MG642780
K. veneficum	This study	IRTA-SMM-12-30	MG642781
K. veneficum	This study	IRTA-SMM-12-31	MG642782
K. veneficum	GenBank	GC-1	AJ534656
K. veneficum	GenBank	GC-5	AJ557028
K. veneficum	GenBank	GC-8	AJ557027
*K. veneficum**	This study	IRTA-SMM-12-36	MG642783
*K. veneficum**	GenBank	KM1 CSIC-1	AJ557025
*K. veneficum**	GenBank	CCMP 415	AJ557026
K. armiger	GenBank	GC-2	AM184204
K. armiger	GenBank	GC-3	AM184205
K. armiger	GenBank	GC-7	AJ557024

* Formerly *K. micrum*

Table S2. Microalgae cell abundances (determined by microscopy) of seawater samples (surface and bottom) collected at different stations in Alfacs Bay and results for toxic and potentially toxic species obtained using optical microscopy. Toxic species responsible for paralytic (PSP), diarrhetic (DSP) and amnesic (ASP) shellfish poisoning.

Sample	Geographic coordinates	PSP	DSP	ASP	*Karlodinium* spp. (cells/L)
		Alexandrium minutum (cells/L)	*Dinophysis sacculus* (cells/L)	*Pseudo-nitzchia* spp. (cells/L)	
Station 1 (S)	40°37'22.9"N 0°42'25.5"E	1,832	80	33,507	10,305
Station 1 (B)	40°37'22.9"N 0°42'25.5"E	2,061	20	18,360	2,061
Station 2 (S)	40°37'34.2"N 0°43'31.4"E	4,896	40	1,224	8,772
Station 2 (B)	40°37'34.2"N 0°43'31.4"E	5,038	n.d.	5,967	2,977
Station 3 (S)	40°36'59.9"N 0°42'55.6"E	4,122	n.d.	30,753	8,473
Station 3 (B)	40°36'59.9"N 0°42'55.6"E	1,374	40	11,475	1,374
Station 4 (S)	40°37'28.9"N 0°41'07.7"E	1,374	120	106,029	18,549
Station 4 (B)	40°37'28.9"N 0°41'07.7"E	1,145	80	81,702	1,374

n.d.: not detected

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Chapter 3B

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

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Abstract

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

1. Introduction

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

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

Due to the difficulties and limitations of techniques based on morphological identification, there is a demand for new tools to provide a more reliable early warning of HAB events in order to facilitate and implement appropriate preventive measures. In this regard, the use of molecular methods for microalgae identification are being increasingly explored because they are faster and more accurate than microscopic observations (Medlin and Orozco 2017; Penna and Galluzzi 2013). Most molecular techniques have their origin in medical diagnostics and, during the last three decades, these techniques have been tested, modified, and refined for their application in microalgae identification, detection and quantification (Karlson *et al.* 2010). Among the different molecular methods, quantitative PCR (qPCR) has been widely applied to

a variety of toxic microalgae, to detect one (Yuan *et al.* 2012), two (Eckford-Soper and Daugbjerg 2016) or more than two (Nishimura *et al.* 2016; Andree *et al.* 2011) species, mainly targeting ribosomal DNA (rDNA). Specifically for *Karlodinium* species, qPCR assays have been developed for *K. veneficum* (Eckford-Soper and Daugbjerg 2015; Park *et al.* 2009; Zhang *et al.* 2008) and more recently to discriminate between *K. veneficum* and *K. armiger* (Toldrà *et al.* 2018). PCR amplification has also been combined with biosensors for the electrochemical detection of *Karenia mikimotoi* (LaGier *et al.* 2007). However, although PCR is the gold standard amplification method, it has limitations, such as the requirement for thermal cycling equipment, which hampers the development of miniaturised and portable analysis systems for in-field application. A possible solution is the use of ribosomal RNA (rRNA) as a target, which may avoid DNA amplification (Orozco and Medlin 2013). This approach has been exploited in microarrays with fluorescence detection (Ahn *et al.* 2006; Taylor *et al.* 2013) and in sandwich hybridisation systems followed by colorimetric (Cai *et al.* 2006; Diercks *et al.* 2008a) or electrochemical (Diercks *et al.* 2008b; Diercks-Horn *et al.* 2011; Metfies *et al.* 2005) detection. However, the inherent rRNA instability and the high amount of rRNA required could compromise the reliability and sensitivity, respectively, of these RNA-based assays (Bruce *et al.* 2015; Metfies *et al.* 2005). Another possibility to avoid the need for cycling control and power sources is the use of isothermal nucleic acid amplification methods.

There are only a few reports detailing isothermal amplification for the detection of toxic microalgae, such as nucleic acid sequence-based amplification (NASBA) (Casper *et al.* 2004; Loukas *et al.* 2017) and loop-mediated isothermal amplification (LAMP). The detection of DNA isothermally amplified using LAMP has mainly been achieved by fluorescence or turbidity measurements (Chen *et al.* 2013; Zhang *et al.* 2009; Zhang *et al.* 2014). A lateral flow (LF) strip exploiting LAMP has also developed for the detection of *K. veneficum* (Huang *et al.* 2017). However, LAMP is highly dependent on extremely careful primer design and NASBA requires an initial DNA melting step (Mayboroda *et al.* 2018). Recombinase polymerase amplification (RPA) is a very attractive alternative that overcomes these drawbacks and it has been chosen in the present study for the detection of two *Karlodinium* species (*K. veneficum* and *K. armiger*). RPA is based on the use of a mixture of polymerases, recombinases and DNA binding proteins that are capable of pairing oligonucleotide primers with homologous sequences in single (ssDNA) and double stranded DNA (dsDNA), typically within 25 min and at a low and constant temperature (37-42 °C) (Piepenburg *et al.* 2006). Recent publications demonstrate that RPA technology has been successfully applied to the detection of viruses (Euler *et al.* 2013), protozoa (Crannell *et al.* 2016) and bacteria (Santiago-Felipe *et al.* 2014), but its application to microalgae has not yet been described. Our RPA strategy exploits the use of tailed primers that result in amplicons of dsDNA flanked

by ssDNA tails (**Fig. 1a**). This is accomplished by a C3 stopper located between the primer and the tail that prevents the polymerase from further elongation (Jauset-Rubio *et al.* 2016; Joda *et al.* 2015). Amplicons obtained after RPA are detected in a colorimetric sandwich enzyme-linked oligonucleotide assay (ELONA) using complementary oligonucleotides: a capture probe (specific for each *Karlodinium* species) immobilised through a thiol group on maleimide-coated microtiter plates and a reporter probe (common for the two species) conjugated to horseradish peroxidase (HRP) (**Fig. 1b**). Compared to other detection strategies, sandwich formats enhance the specificity of the assays because of the use of two hybridisation events (capture and reporter). Additionally, the use of tailed primers avoids the need for primer labelling and/or any post-amplification processing to generate ssDNA thus reducing complexity, time and cost of the assay.

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

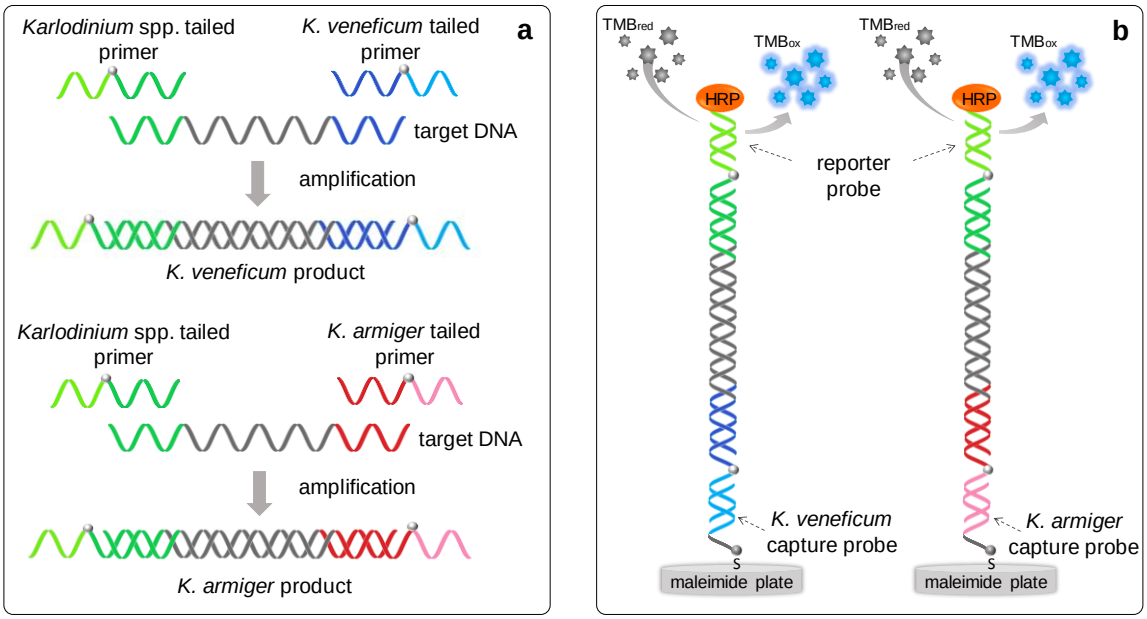

Fig. 1. Schematic representation of the RPA-ELONA method for *K. veneficum* and *K. armiger*: (**a**) RPA with tailed primers and (**b**) ELONA detection.

2. Materials and Methods

2.1. Reagents and materials

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

2.2. Microalgal cultures

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

2.3. Spiked environmental samples

Natural seawater (10 L) was collected in June 2017 from L'Ametlla de Mar (40°49'51.42"N; 0°45'6.90"E; Catalonia, Spain) and subsequently analysed using light microscopy to confirm the presence and absence of *Karlodinium* spp. as well as other phytoplankton species. For this purpose, a volume of 50 mL was settled in sedimentation chambers for 24 h and counted using the Utermöhl method after fixation in Lugol's iodine. Spiked samples of seawater (1 L) containing *Karlodinium*

species (previously counted as stated in section 2.2) were prepared at the warning level (200,000 cells/L) and at the fish mortality level (1,000,000 cells/L). Spiked concentrations were as follows: *K. veneficum* (200,000 cells/L and 1,000,000 cells/L), *K. armiger* (200,000 cells/L and 1,000,000 cells/L) and equal mixture of *K. veneficum* and *K. armiger* (400,000 cells/L and 2,000,000 cells/L). The spiked samples were fixed with Lugol's iodine solution. For light microscopy counts, a volume of 50 mL was settled in sedimentation chambers for 24 h and counted following the Utermöhl method. For RPA-ELONA analysis, 50-mL aliquots were centrifuged at 3,700 g for 25 min and maintained at -20 °C until DNA extraction.

2.4. DNA extraction

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

2.5. Primers and probes design and specificity

Primers used in this study were based on the two species-specific primers for *K. veneficum* and *K. armiger* previously designed within the ITS1 rDNA region for qPCR assay (Toldrà *et al.* 2018). The design aimed at minimising the number of required primers: one genus-specific (for *Karlodinium*) and two species-specific (for *K. veneficum* and *K. armiger*) primers. The genus-specific primer described by Toldrà *et*

al. (2018) was slightly modified 4 bp upstream in order to avoid primer-dimer formation. Primers for *K. veneficum* amplified a product of 139 bp, whereas primers for *K. armiger* amplified a product of 153 bp. Primers were modified by adding oligonucleotide tails, resulting in amplicons with one ssDNA tail at each end, which enable subsequent detection via sandwich ELONA through complementary capture and reporter probes (**Fig. 1**). All primers and probe sequences are shown in **Table 1**. Primer specificity was tested by electrophoresis of the RPA products in 2% w/v agarose gel.

Table 1. Summary of primers and primers used in this study. Tails are underlined.

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

2.6. Recombinase polymerase amplification (RPA) reaction

RPA was performed following the indications provided in the TwistAmp Basic kit with some minor modifications. The RPA conditions including reagent concentration (primers, rehydration buffer and enzyme pellet), reaction time and the requirement for a final purification step were systematically optimised. Following optimisation, each RPA reaction (50 µL) contained: 480 nM of each primer, 14 mM magnesium acetate, 0.5x rehydration buffer, 0.5x enzyme pellet and 5 µL of DNA, this DNA being: a) synthetic ssDNA and genomic DNA to check the specificity of the subsequent ELONA, b) synthetic ssDNA, genomic DNA and genomic DNA extracted from cell dilutions for the calibration curves, and c) genomic DNA extracted from spiked

samples. All reagents except the DNA and magnesium were prepared in a master mix, which was distributed into 0.2-mL reaction tubes. The DNA was then added into the tubes, and magnesium added to initiate the RPA reaction. The tubes were immediately placed into a Nexus Gradient Thermal Cycler (Eppendorf Ibérica, Madrid, Spain) at 37 ˚C for 30 min. Subsequently, RPA products were purified using GeneJET PCR purification kit following manufacturer instructions, with a final elution with 50 µL of TE buffer. RPA reactions were performed in triplicate and blanks (no DNA) were included in all cases.

2.7. Enzyme-linked oligonucleotide assay (ELONA) detection

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

2.8. Data analysis and statistics

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

3. Results and discussion

3.1. Primer specificity

Primer specificity is critical when detecting harmful algae because seawater samples commonly contain a wide range of microorganisms. Specific primer sets for *K. veneficum* and *K. armiger* were previously developed by Toldrà *et al.* (2018), and were demonstrated to be highly specific for the qPCR assay, and also deemed suitable for RPA according to the RPA primer design manual (Appendix TwistDx). However, electrophoresis of the RPA products revealed the presence of primer-dimers for *K. armiger*, and consequently the primers were slightly modified. Following primer optimisation, the estimated molecular weight of the products as visualised using gel electrophoresis was as expected, and no other bands were observed (**Fig. S1**).

3.2. Optimisation of RPA conditions

RPA conditions were optimised using synthetic ssDNA and subsequent ELONA detection. RPA was performed maintaining some conditions (at 37 ˚C for 30 min with purification step) and concentrations (14 mM magnesium acetate and 5 µL of DNA), whilst varying primer (240-480 nM), rehydration buffer (0.5-1x) and enzyme pellet (0.5-1x) concentrations. Results demonstrated that the LOD was remarkably decreased 110-fold using optimised RPA component concentrations. Furthermore, in an effort to shorten assay time, different RPA reaction times (5, 10, 20, 30 and 40 min) were evaluated. As expected, higher absorbance values were obtained at longer reaction times. Nevertheless, since the LODs obtained at 30 and 40 min were very similar (the LOD at 40 min was only ~2-fold lower than the LOD at 30 min), 30 min was chosen as the optimum amplification time. Finally, the need for an RPA product purification step prior to detection was evaluated. Results showed that without a purification step the LOD increased significantly (52-fold higher). This lower performance might be explained by the presence of proteins and primers in the RPA reaction that could hinder hybridisation events and/or increase the nonspecific adsorption. Optimised RPA conditions (see section 2.6) were used in subsequent experiments.

3.3. Specificity of RPA-ELONA

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

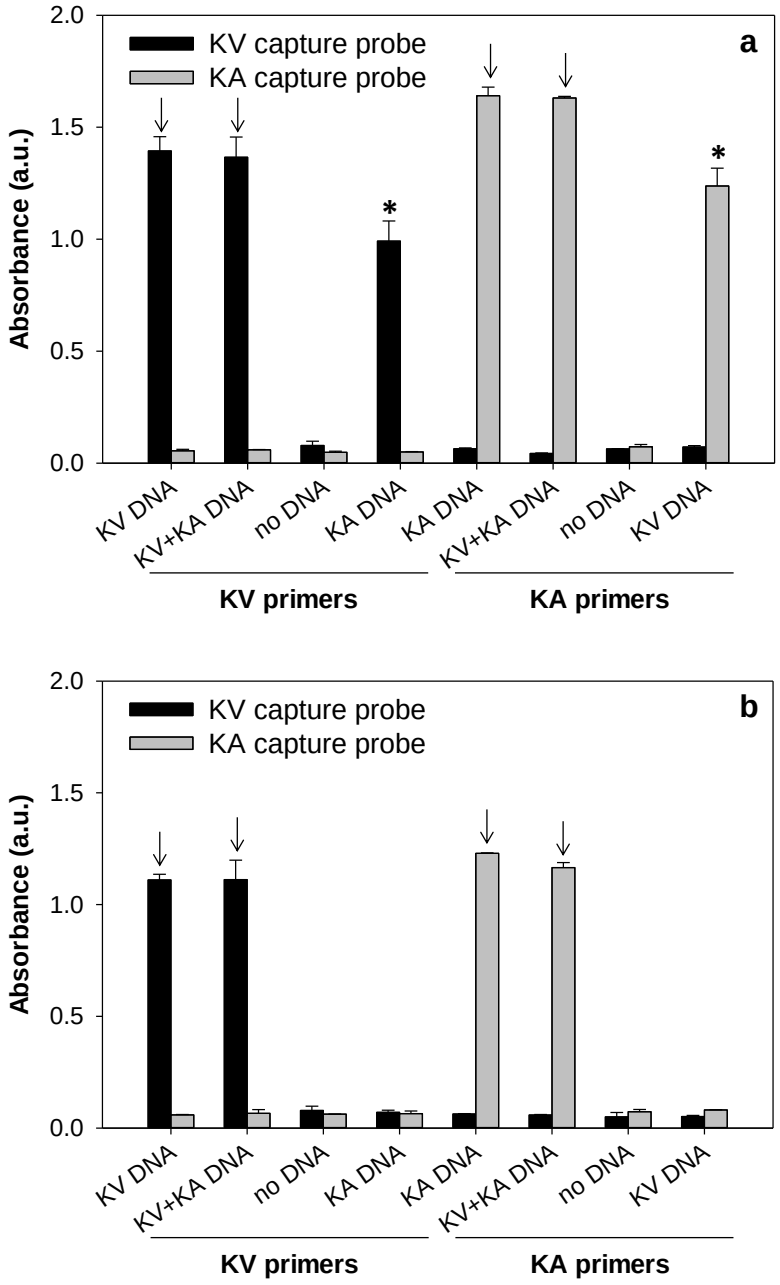

Fig. 2. RPA-ELONA results using different capture probes, primers and DNA: (**a**) synthetic ssDNA at 1nM and (**b**) genomic DNA at 2.3 ng/µL. Error bars represent the standard deviation for 3 replicates. Arrows and asterisks represent specific and non-specific detection, respectively. KV = *K. veneficum*,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

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

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

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

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

3.4. Calibration curves and LOD determination

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

2,417 cells for *K. armiger* (~25 cells/well for both species). Taking into account that the protocol involves the analysis of 50-mL samples, the LODs can be translated to 49,660 cells/L for *K. veneficum* and 48,340 cells/L for *K. armiger*.

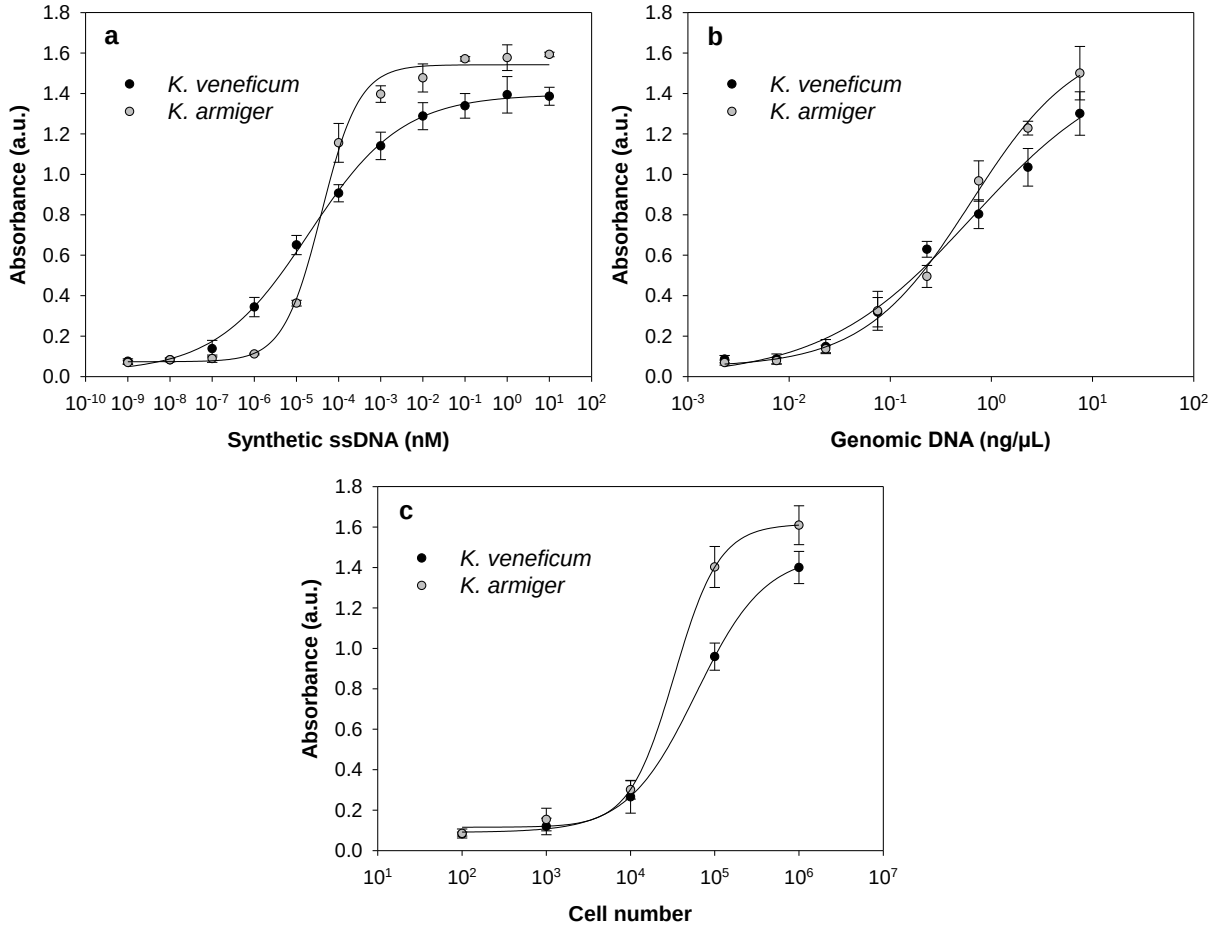

Fig. 3. Calibration curves for *K. veneficum* and *K. armiger* obtained with different concentrations of (**a**) synthetic ssDNA, (**b**) and genomic DNA, and (**c**) different number of cells from the pellets. Errors bars represent the standard deviation for 3 replicates.

There are only a few reports detailing the use of molecular methods for the detection of *Karlodinium* species. In the qPCR assays described, cell dilutions have been most commonly used to construct calibration curves, achieving LODs of 2,000 cells/L for *K. veneficum* and *K. armiger* (Toldrà *et al.* 2018) and 10 cells/reaction for *K. veneficum* (Eckford-Soper and Daugbjerg 2015). On the other hand, LAMP-LF used genomic DNA to determine the LOD of the assay, which was 7 pg/µL of *K. veneficum* genomic DNA (Huang *et al.* 2017). However, it was only qualitatively applied to the analysis of field samples, without being correlated with the number of cells. A limited number of molecular methods to detect microalgae without a prior amplification step have been reported, but they have not been applied to *Karlodinium* and they are limited by poor sensitivity. For example, the electrochemical DNA-biosensor for the detection of *Alexandrium ostenfeldii* achieved an LOD of 16 ng/µL (Metfies *et al.* 2005). Our RPA-

ELONA method, although it has a lower sensitivity compared to some of the described molecular methods, facilitates the quantification of *Karlodinium* species below the proposed alert threshold of 200,000 cells/L, which will enable early warnings of *Karlodinium* spp. blooms before they proliferate to critical levels. It is important to mention that this quantification limit could be reduced by centrifuging a larger sample volume or reducing the volume of elution buffer used for the DNA extraction.

3.5. Analysis of environmental spiked samples

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

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

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

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

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

4. Conclusions

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

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

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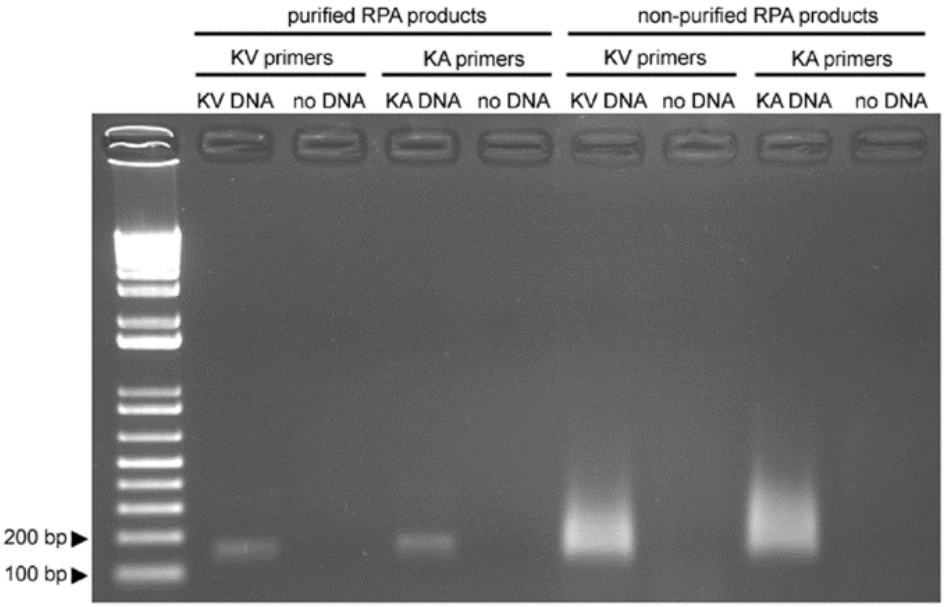
Supplementary information

Fig. S1. Gel electrophoresis analysis of target synthetic ssDNA (100nM) amplified by RPA. KV = *K. veneficum*, KA = *K. armiger*, L = 1 Kb Plus DNA Ladder.



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CHAPTER 4

Ostreopsis: colorimetric assays and electrochemical biosensors

Chapter 4A

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.

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 ~10,000-30,000 cells/L of seawater and 100,000 cells/g fresh weight of macroalgae (fwm). Similarly, a warning threshold of 30,000 cells/L and an alarm threshold of 100,000 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.

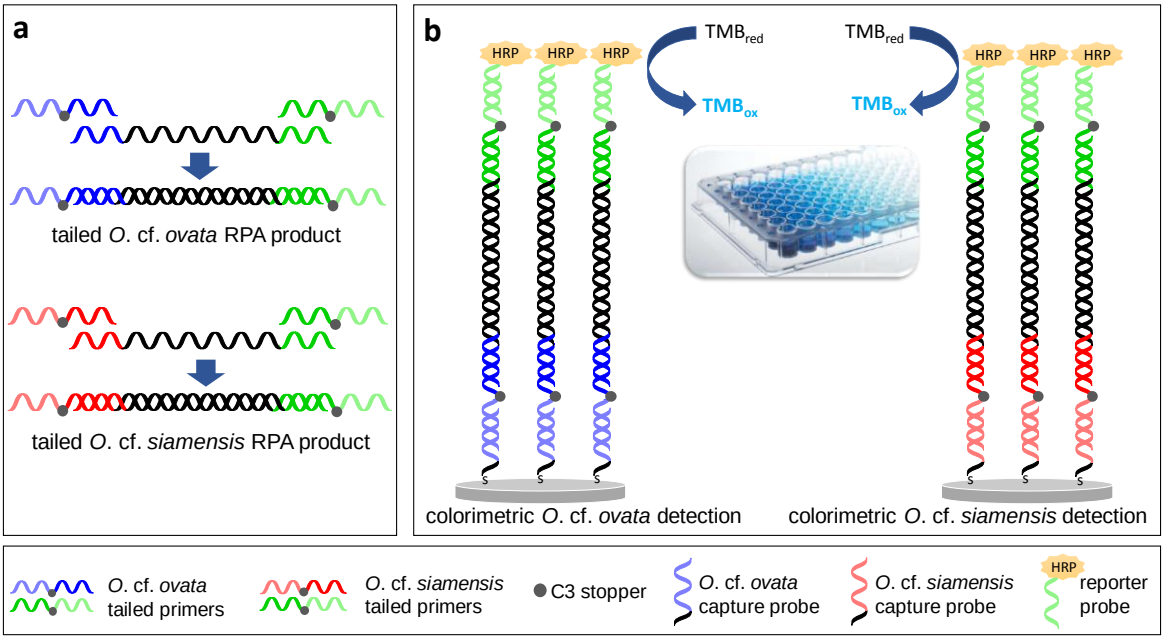

Fig. 1 Principle of the colorimetric DNA-based assay. The assay involves two steps: (**a**) recombinase polymerase amplification and (**b**) colorimetric detection.

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 synthesised 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 (4,500 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 S1**). 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 (4,500 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.

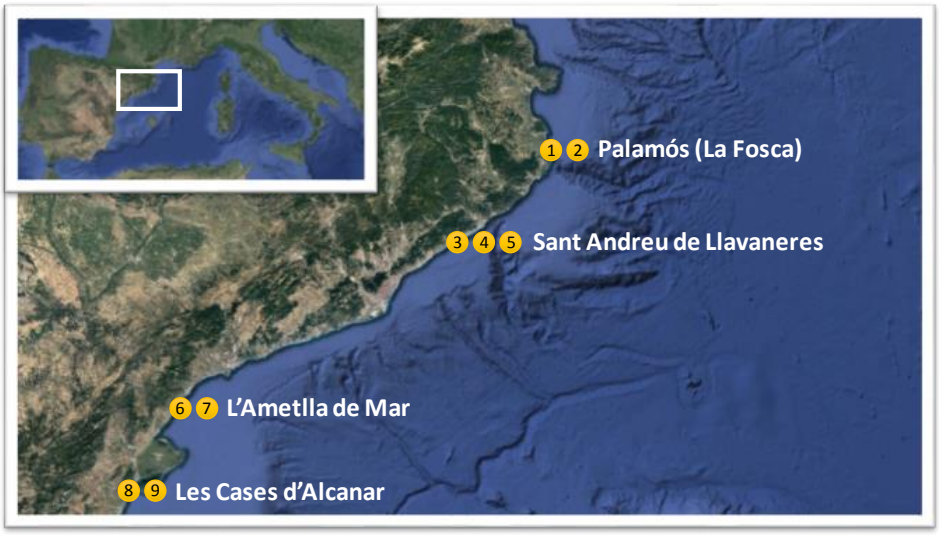

Fig. 2. Location of the sampling stations in the Catalan coast. At each station, planktonic and benthic samples were collected (see **Table S1** for geographic coordinates).

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.

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

Name	Sequence (5'-3')
Fw *O.* cf. *ovata* primer with tail	gtt ttc cca gtc acg ac-C3-aca atg ctc atg cca atg atg ctt gg
Fw *O.* cf. *siamensis* primer with tail	att acg acg aac tca atg aa-C3-tga gtt tgt gtg tat ctt gca cat gc
Rv *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

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, ½ 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 counts for both benthic (cells/g fwm) and planktonic (cells/L) samples. Due to the different type of samples and sampling methodology, the regression analysis was performed separately for planktonic and benthic samples. Predicted cell abundances were obtained from the regression model. The correlation between predicted and observed values was then analysed using Pearson's correlation coefficient (*r*). Data analyses were performed with IBM SPSS Statistics 23.0 (IBM 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 TwistAmp): 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*.

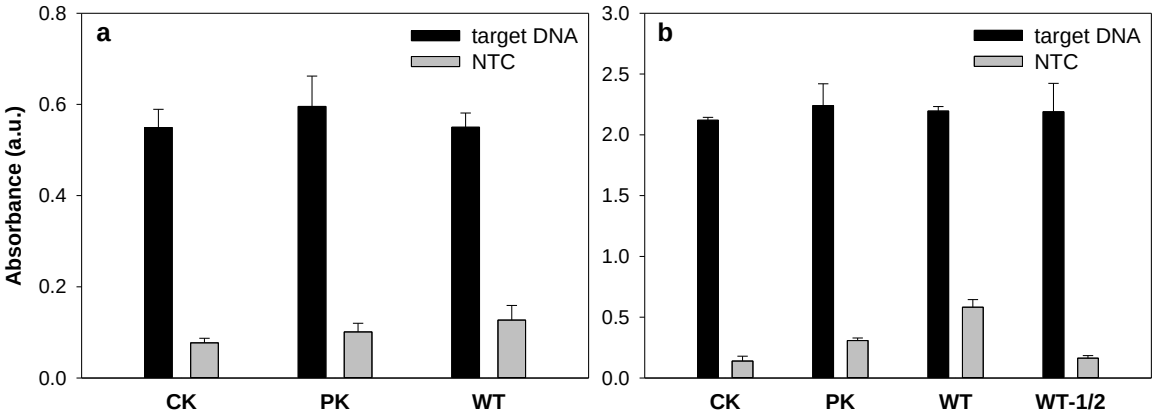

Fig. 3 Comparison of different cleaning treatments (commercial kit, CK; proteinase K, PK; without treatment, WT) on RPA-ELONA results: (**a**) *O*. cf. *ovata* and (**b**) *O*. cf. *siamensis*. Target genomic DNA (1 ng/µL) and NTC were tested. *O*. cf. *siamensis* results without treatment and using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

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.

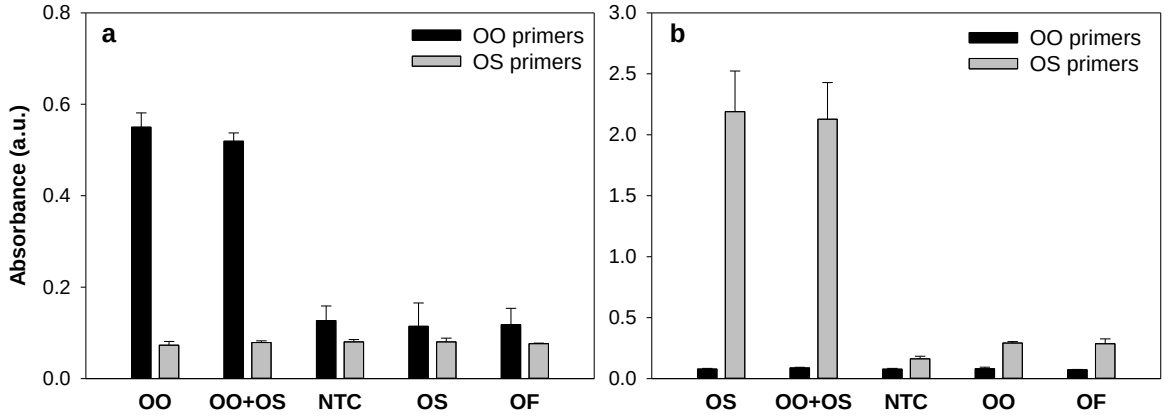

Fig. 4 RPA-ELONA cross-reactivity experiments. A combination of different capture probes (*O.* cf. *ovata* in **a** and *O.* cf. *siamensis* in **b**), primers and genomic DNA (1 ng/µL) was tested. Error bars represent the standard deviation for 3 replicates. OO = *O.* cf. *ovata*, OS = *O.* cf. *siamensis*,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

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 3,800 cells/L, respectively, both far below the Spanish alarm thresholds.

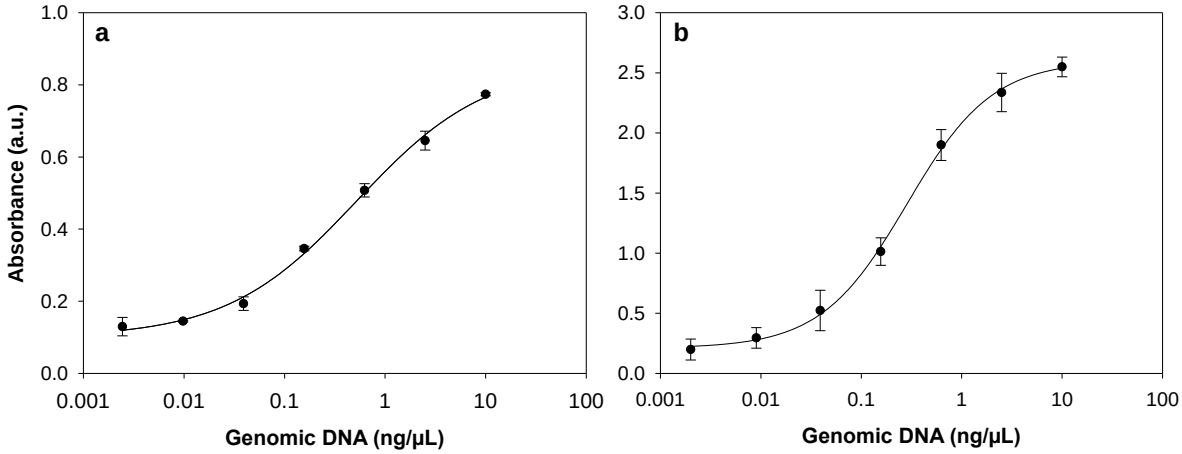

Fig. 5 Calibration curves obtained using different concentrations of genomic DNA: (**a**) *O.* cf. *ovata* and (**b**) *O.* cf. *siamensis*. Errors bars are the standard deviation (3 replicates).

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.

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.	2,840
1, benthic	63.721 ± 11.896	78.781 ± 6.367	n.d.	0.003 ± 2E-04	60,710
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.	7,600
3, benthic	1.369 ± 0.185	2.748 ± 0.248	n.d.	n.d.	32,831
4, planktonic	0.149 ± 0.069	0.098 ± 0.016	n.d.	n.d.	6,620
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.	1,680
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.	2,071
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.	6,015
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

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 S2**). *Ostreopsis* spp. abundances in planktonic samples were always below the Spanish alarm threshold established for *Ostreopsis* spp. in seawater (10,000 – 30,000 cells/L) and ranged from 300 to 7,600 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 100,000 cells/g fwm, and ranged from 210 to 60,710 cells/g fwm, with the highest density observed in La Fosca (station 2).

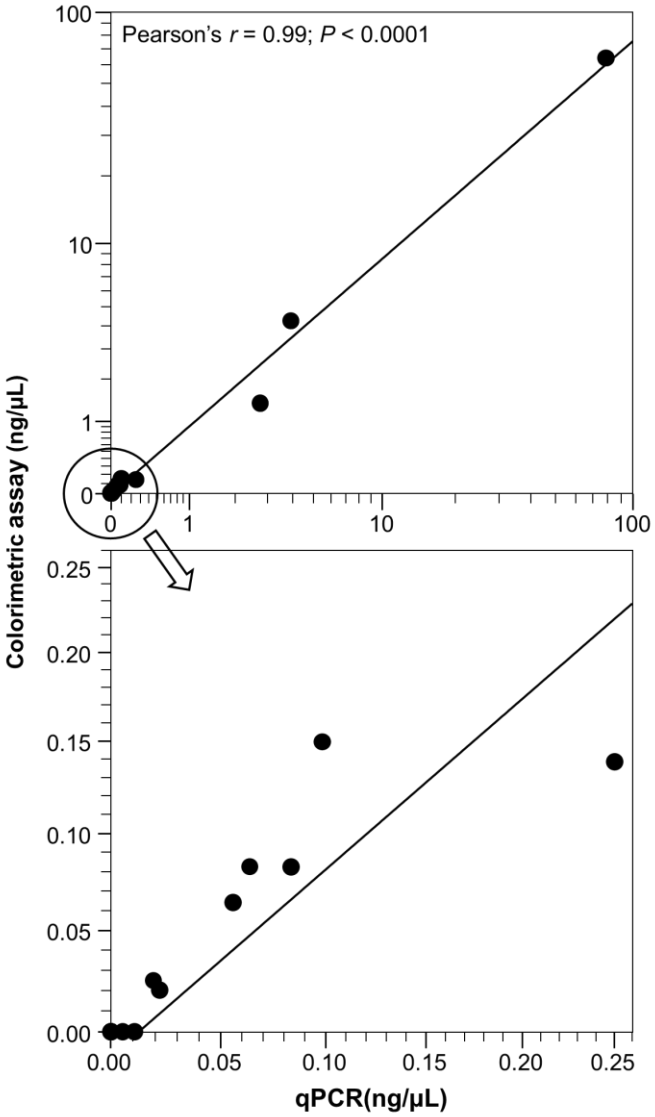

Fig. 6 Correlation between *O*. cf. *ovata* DNA quantifications obtained by RPA-ELONA and qPCR in all examined planktonic and benthic samples. Pearson's correlation coefficient (*r*) is shown.

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 (**Fig. 7**).

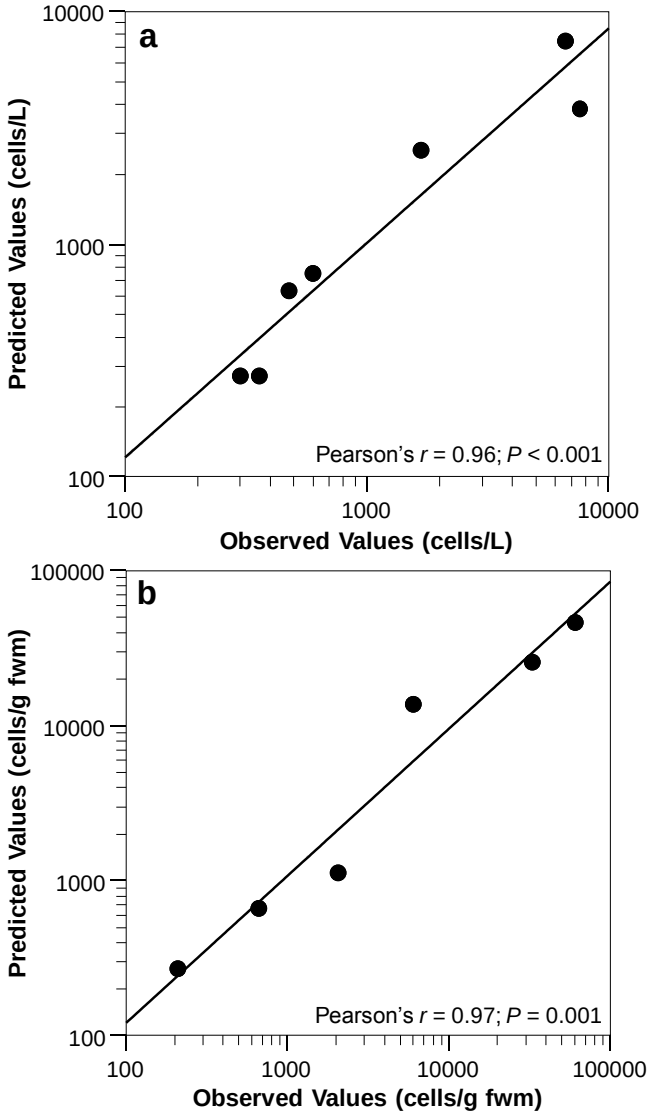

Fig. 7 Relationship between the observed and predicted cell abundance values by the regression analysis: (**a**) Relationship between the observed and predicted cells/L in in planktonic samples and (**b**) Relationship between the observed and predicted cells/g fwm in benthic samples. Pearson's correlation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

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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Supplementary information

Table S1. 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 S2. 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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Chapter 4B

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

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Abstract

Ostreopsis cf. *ovata* is a benthic microalga distributed in tropical and temperate regions worldwide which produces palytoxins (PlTXs). Herein, an electrochemical biosensor for the detection of this toxic microalga 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 (qPCR and colorimetric assays) and allowed the construction of a predictive regression model to estimate *O*. cf. *ovata* cell abundances. This new technology offer great potential to improve research, monitoring and management of *O*. cf. *ovata* and harmful algal blooms.

1. Introduction

Harmful algal blooms (HABs) include *Ostreopsis* species, a benthic microalga 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. In this regard, PCR and quantitative PCR (qPCR) have been developed for *Ostreopsis* species (Battocchi et al., 2010; Casabianca et al., 2014; Casabianca et al., 2013; Perini et al., 2011; 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). 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 biosensors for the detection has never been reported for microalgae.

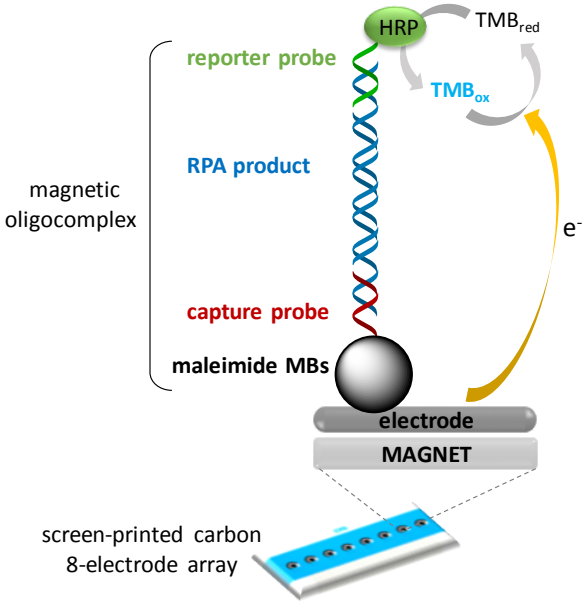

Fig. 1. Schematic illustration of the electrochemical biosensor.

In this work, we report an electrochemical biosensor for the detection of *O*. cf. *ovata* that exploits RPA, tailed primers and maleimide-activated magnetic beads (MBs). Tailed primers consist of a single-stranded DNA (ssDNA) sequence (tail) that is added to the species-specific primer using a C3 stopper, which prevents polymerase from 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. Oligocomplexes consisting of the RPA amplicon hybridised to the 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 reagents (rehydration buffer, pellet and magnesium acetate) 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 (≥96.5%), skimmed milk powder, bovine serum albumin (BSA), chloroform (≥99.5%), phenol:chloroform:isoamylalcohol (25:24:1, v:v:v), potassium phosphate dibasic (≥98.0%), potassium phosphate monobasic (≥99.0%) and sodium chloride (≥99.0%) 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). Briefly, 2 L of seawater (planktonic) samples and 100-200 g fresh weigh of macroalgae (fwm) (benthic) samples were collected. Microalgae substrates were mixed with 2 L of seawater, vigorously shaken and finally filtered through a 200-µm mesh. Cultured cells and environmental samples were fixed in 3% lugol's iodine solution. Pellets from both *Ostreopsis* cultures and 50-mL environmental samples were prepared by centrifugation (4,500 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 (Toldrà et al., 2019). 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, as detailed in Toldrà et al., 2019. 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 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 (100 mM potassium phosphate, 150 mM NaCl, 0.05% v/v Tween-20, pH 7.4). 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 washing buffer, 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 (10 pg since 1 µL of genomic DNA was used for each measurement) was attained, which corresponds to ~800 cells/L

considering 50-mL samples and taking into account the DNA extraction, RPA, detection protocols and the amount of DNA per *O*. cf. *ovata* cell (Toldrà et al., 2019).

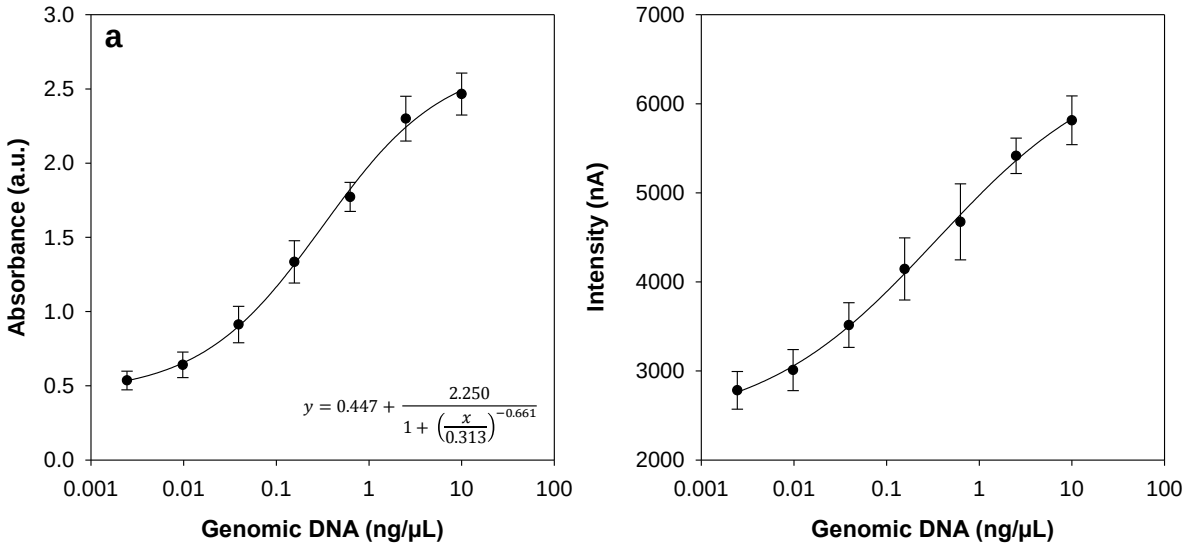

Fig. 2. Calibration curves obtained using different concentrations of *O*. cf. *ovata* genomic DNA: (**a**) colorimetric assay and (**b**) electrochemical biosensor. Error bars are the standard deviation of the mean, *n* = 3.

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 (~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.

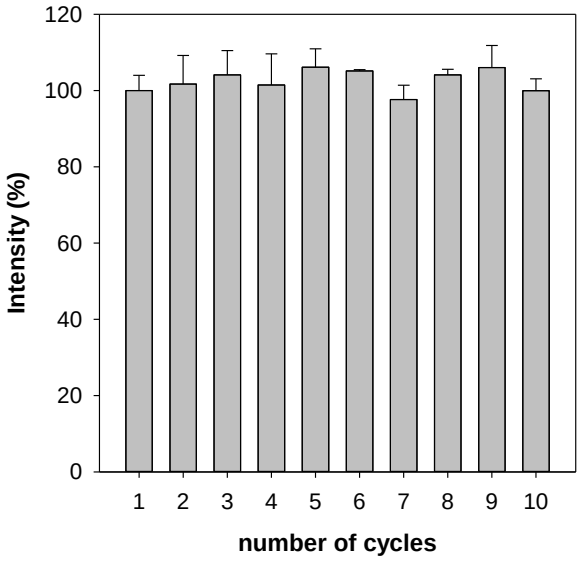

Fig. 3. Electrochemical responses of the electrochemical biosensor after 10 cycles of magnetic immobilisation/electrochemical measurement/cleaning. Error bars are the standard deviation of the mean, *n* = 3.

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 immobilised 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 functionalised 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 (Pearson's r = 0.999; P < 0.001). Similarly, good agreement was achieved when comparing quantifications provided by the electrochemical biosensor and qPCR (Pearson's r = 0.993; P < 0.001).

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	n.d.	n.d.	n.d.	0.010 ± 0.002
Sample 2	85.573 ± 8.968	50.516 ± 1.870	63.721 ± 11.896	78.781 ± 6.367
Sample 3	n.d.	n.d.	n.d.	n.d.
Sample 4	n.d.	n.d.	n.d.	n.d.
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	n.d.	n.d.	n.d.	0.005 ± 2E-04
Sample 14	4.403 ± 1.042	2.686 ± 0.321	4.220 ± 0.855	3.918 ± 0.257
Sample 15	n.d.	n.d.	n.d.	n.d.
Sample 16	n.d.	n.d.	n.d.	n.d.

n.d.: not detected

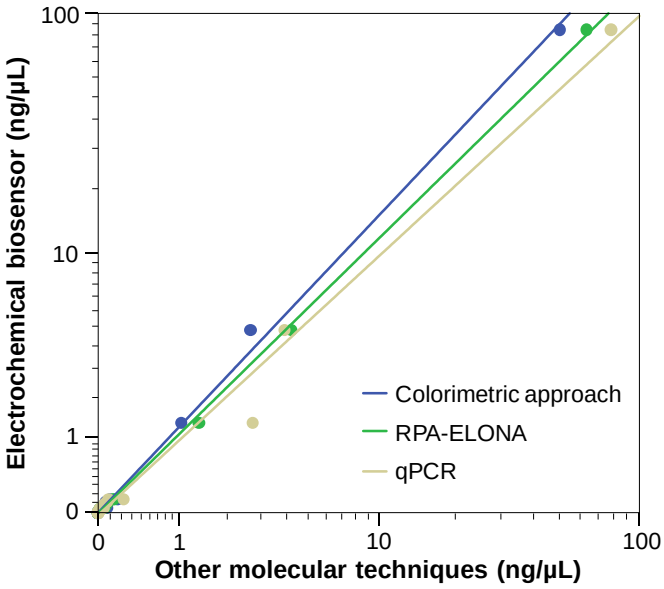

Fig. 4. Correlation between *O*. cf. *ovata* DNA quantifications provided by the electrochemical biosensor and those obtained by the colorimetric assay, RPA-ELONA and qPCR in all examined environmental samples.

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 (10,000-30,000 cells/L and 100,000 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.

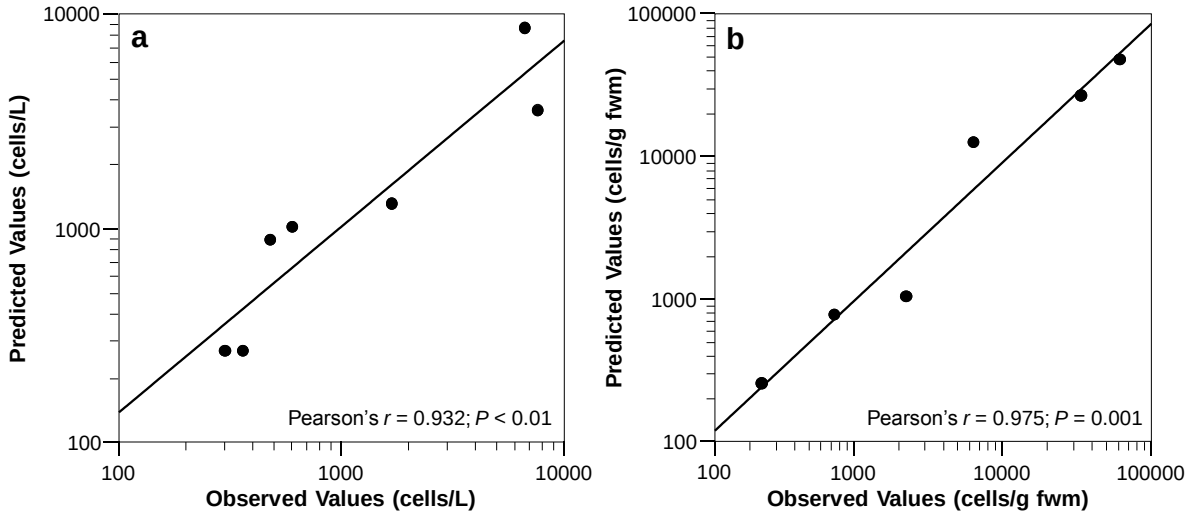

Fig. 5. Relationship between predicted cell abundances provided by the regression model and those counted by light microscopy in: (**a**) planktonic samples (cells/L) and (**b**) benthic samples (cells/g fwm). Pearson's correlation coefficient is shown.

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 immobilisation 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, this biosensor offers the potential to improve routine microalgae monitoring programs as either a quantitative or screening tool. However, further validation and inter-laboratory studies should be performed and some issues such as DNA extraction and rDNA copy number variability should be addressed to implement this alternative technology. Nonetheless, 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.

Acknowledgements

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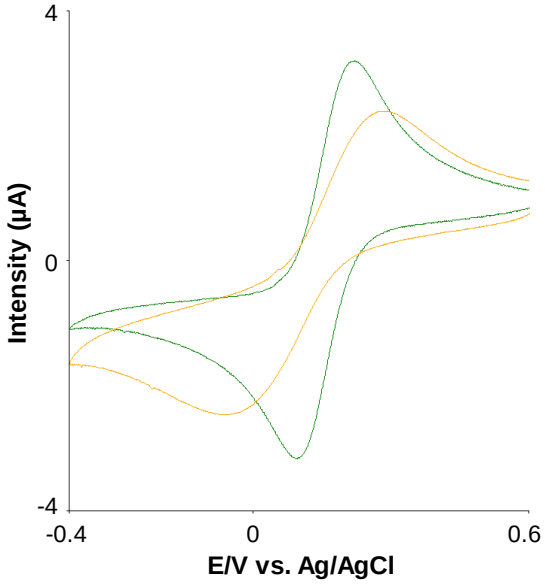
Table S1. Environmental samples collected in August 2017 along the Catalan cost. Location and geographical coordinates of the sampling stations as well as *Ostreopsis* spp. abundances obtained by light microscopy for planktonic (cells/L) and benthic (cells/g fwm) samples are shown. Data obtained from Toldrà *et al.* 2019.

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''	2,840
Sample 2	benthic	Palamós, La Fosca	1	N 41°51'20.71'' E 3°8'32.01''	60,710
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''	7,600
Sample 6	benthic	Sant Andreu de Llavaneres	3	N 41°33'7.69'' E 2°29'31.66''	32,831
Sample 7	planktonic	Sant Andreu de Llavaneres	4	N 41°33'12.25'' E 2°29'45.20''	6,620
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''	1,680
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''	2,071
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''	6,015
Sample 15	planktonic	Les Cases d'Alcanar	9	N 40°33'15.71'' E 0°31'58.71''	n.d.
Sample 16	benthic	Les Cases d'Alcanar	9	N 40°33'15.71'' E 0°31'58.71''	n.d.

n.d.: not detected

Table S2. List of primers and probes and their respective modifications. Tails ate underlined.

Name	Sequence (5'-3')
Fw *O.* cf. *ovata* primer	gtt ttc cca gtc acg ac-C3-aca atg ctc atg cca atg atg ctt gg
Rv *Ostreopsis* spp. primer	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



	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. 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 maleimide-activated MBs were used, which corresponds to 10 µL of oligocomplex.

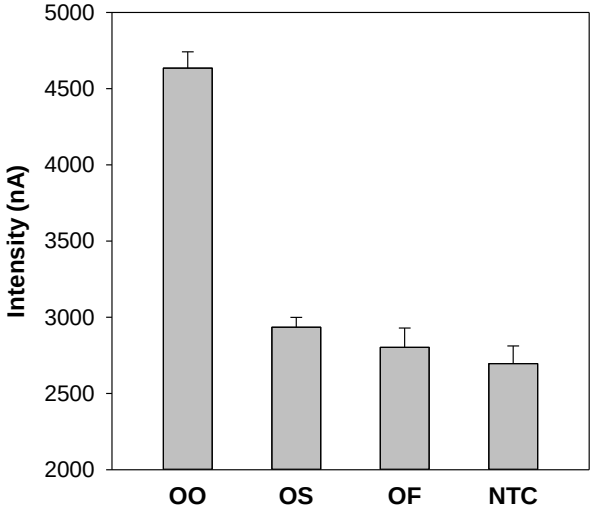


Fig. S2. Electrochemical responses of the electrochemical biosensor using DNA from: *O.* cf. *ovata* (OO), *O.* cf. *siamensis* (OS), *O. fattorussoi* (OF) and no template control (NTC). Error bars are the mean standard deviation, *n* = 3.

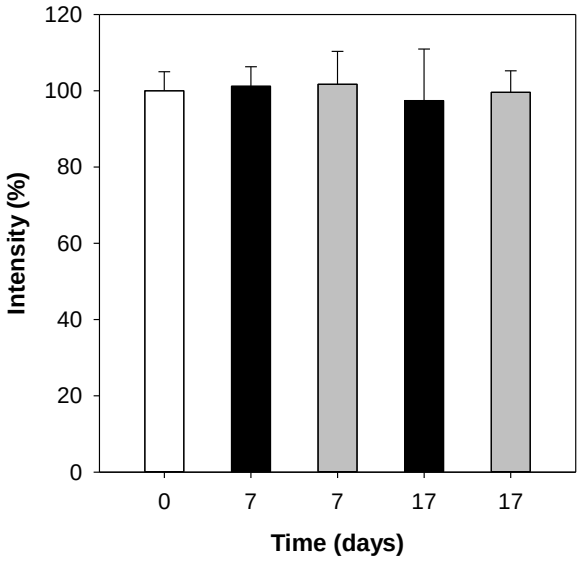


Fig. S3. Electrochemical responses corresponding to the stability study of functionalised MBs stored at 4 °C (black bars) and at -20 °C (grey bars), respect to day 0 (white bar, reference value). Error bars are the standard deviation of the mean, *n* = 3.

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CHAPTER 5

Ostreid herpesvirus-1: electrochemical biosensors and MB-based capture strategies

Chapter 5A

Detection of isothermally amplified ostreid herpesvirus-1 DNA in Pacific oyster (*Crassostrea gigas*) using a miniaturised electrochemical biosensor

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Abstract

Given the threat that ostreid herpesvirus-1 (OsHV-1) poses to shellfish aquaculture, the need for rapid, user-friendly and cost-effective methods to detect this marine pathogen and minimise its impact is evident. In this work, an electrochemical biosensor for the detection of OsHV-1 based on isothermal recombinase polymerase amplification (RPA) was developed. The system was first tested and optimised on maleimide microtitre plates as a proof-of-concept, before being implemented on miniaturised gold electrodes. Amperometric detection of the isothermally amplified product was achieved through a sandwich hybridisation assay with an immobilised thiolated capture probe and a horseradish peroxidase (HRP)-labelled reporter probe. Calibration curves were constructed using PCR-amplified OsHV-1 DNA, achieving a limit of detection of 207 OsHV-1 target copies. The biosensor was applied to the analysis of 16 oyster samples from an infectivity experiment, and results were compared with those obtained by qPCR analysis, showing a strong degree of correlation (r = 0.988). The simplicity, rapidity, cost-effectiveness and potential for *in situ* testing with the developed biosensor provide a valuable tool for the detection of OsHV-1 in aquaculture facilities, improving their management.

1. Introduction

Shellfish aquaculture has become an important component of the aquaculture industry, representing 40% of the aquaculture production in terms of weight and 28% in value in Europe. However, the shellfish industry has suffered from slow growth as a consequence of various infectious diseases (STECF 2016). Among pathogens affecting molluscs, ostreid herpesvirus-1 (OsHV-1) (Davison *et al*. 2005) is currently one of the major threats to shellfish aquaculture, particularly to the production of Pacific oysters (*Crassostrea gigas*) (EFSA 2015; Pernet *et al*. 2016; Rodgers *et al*. 2018). Recurrent mass mortality outbreaks of *C. gigas* have been reported worldwide, especially in European counties such as France (Segarra *et al*. 2010) and Spain (Roque *et al*. 2012), but also in Australia (Jenkins *et al*. 2013) and New Zealand (Keeling *et al*. 2014), where industry is suffering from this disease. Since oysters are farmed in open waters and there are neither vaccination nor antiviral therapies available, the efforts of the sector to manage the impact of this virus are essentially focused on the development of prevention strategies, mitigation approaches and early diagnostic tools.

Traditionally, surveillance of OsHV-1 has relied on the detection of the virus in oysters through conventional molecular techniques such as PCR (Webb *et al*. 2007) and *in situ* hybridisation (Lipart and Renault 2002). However, these techniques are time-consuming and laborious, and also suffer from low sensitivity and specificity. Recently, quantitative PCR (qPCR) protocols have been developed for OsHV-1 detection (Pepin *et al*. 2008), and are routinely used for OsHV-1 screening. Despite being sensitive and specific, qPCR requires a highly precise heat source to perform the thermal cycling as well as an expensive fluorescent detector, limiting its practical use for deployment for use in the field. Therefore, the development of rapid, user-friendly and cost-effective analysis tools is of great importance to better anticipate the presence of OsHV-1 and ultimately mitigate the effects of the disease.

Isothermal DNA amplification techniques have emerged in recent years as an effective replacement of PCR technology for molecular diagnostics (Deng and Gao 2015). These techniques achieve DNA amplification at a constant temperature, facilitating their combination with miniaturised analytical devices to perform *in situ* tests. To date, several isothermal methods exist including nucleic acid sequence-based amplification (NASBA), loop-mediated isothermal amplification (LAMP), rolling circle amplification (RCA), helicase dependent amplification (HDA) and recombinase polymerase amplification (RPA), which have been successfully applied in several areas including medicine, agriculture, aquaculture and food safety. Specifically for OsHV-1 detection, only LAMP followed by gel electrophoresis/fluorescent read-out (Ren *et al*. 2010) and RPA with real-time fluorescent detection (Gao *et al*. 2018) have been reported. Although both techniques allow sensitive and specific detection within a few minutes

(20-40 min), LAMP is performed at a relatively high temperature (60-65 °C) and requires the design of 4 primers, whereas RPA can be conducted at a much lower temperature (37-40 °C) and only requires 2 primers, rendering it particularly attractive. However, the recently described RPA methodology for OsHV-1 detection, while effective, still requires the use of expensive and bulky optical detection equipment, thereby efforts should be devoted to the development of cost-effective and miniaturised systems for OsHV-1 detection that can be easily deployed in the field. Electrochemical biosensors can certainly address this demand since they do not require complex instrumentation, handle small volumes of sample and can be integrated into microfluidic platforms for subsequent automation (Ronkainen *et al*. 2010). Although there are few reports describing the combination of RPA with electrochemical detection (de la Escosura-Muniz *et al*. 2016; del Rio *et al*. 2016; Lau *et al*. 2017; Ng *et al*. 2016), this promising strategy is still in its infancy, and efforts need to be focused on developing new proof-of-concept strategies and demonstrating their applicability in real-life settings.

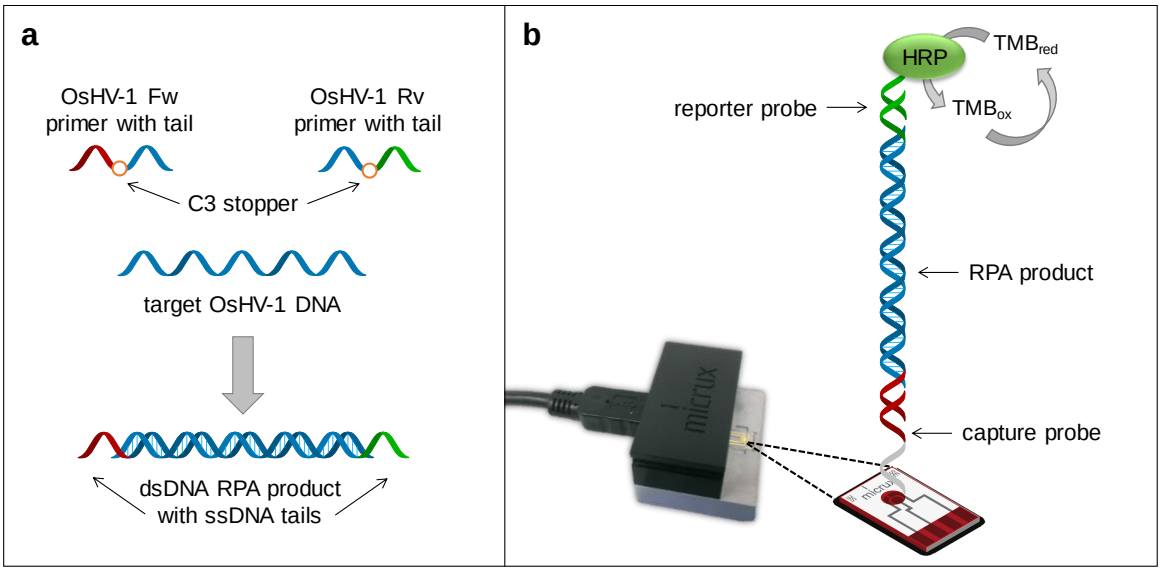

Fig. 1. Schematic representation of the strategy: (**a**) recombinase polymerase amplification and (**b**) electrochemical biosensor.

Within this context, we propose a simple, low-cost and miniaturised biosensor that combines RPA with an electrochemical read-out for the detection of OsHV-1 (**Fig. 1**). Our strategy starts with isothermal DNA amplification using primers modified with tails. A carbon stopper is located between the tail and the primer in order to prevent further elongation of the tail during amplification. Hence, amplification results in a double-stranded DNA (dsDNA) product with single-stranded DNA (ssDNA) tails at each end (**Fig. 1a**). This configuration allows subsequent detection of the RPA product through a capture probe immobilised on an electrode *via* a thiol group (which

hybridises to one tail) and a reporter probe labelled with horseradish peroxidase (HRP) (which hybridises to the other tail) (**Fig. 1b**). A colorimetric approach was first used to optimise the RPA conditions. The system was then transferred to a thin-film gold electrode for development of the corresponding DNA-based amperometric biosensor. Finally, the biosensor was applied to the analysis of OsHV-1 DNA in Pacific oyster (*C. gigas*) samples, and quantifications were compared with qPCR analysis.

2. Materials and methods

2.1. Reagents and solutions

Custom oligonucleotide primers and probes were synthesised by Biomers (Ulm, Germany). TwistAmp Basic kit was obtained from TwistDx Ltd. (Cambridge, UK). Qiagen DNeasy Blood and Tissue kit, QIAquick gel extraction kit, GeneJET PCR purification kit, Taq DNA polymerase, SYBR green and pierce maleimide-activated microtitre plates were supplied by Thermo Fisher Scientific (Madrid, Spain). Tween-20, 3,3',5,5'-tetramethylbenzidine (TMB) liquid substrate, 6-mercapto-1-hexanol, skimmed milk powder, potassium phosphate dibasic, potassium phosphate monobasic, sodium chloride, sulphuric acid, hydrogen peroxide and all other reagents were acquired from Sigma-Aldrich (Tres Cantos, Spain).

2.2. Equipment

Colorimetric measurements were performed with a Microplate Reader KC4 from BIO-TEK Instruments, Inc. (Vermont, USA). Gene 5 was used to collect and evaluate data.

Thin-film gold single electrodes (ED-SE1-Au) and a Drop-cell connector were provided by MicruX Technologies (Gijón, Spain). Electrodes (10 x 6 x 0.75 mm) are fabricated on a glass substrate and consist of a gold working electrode of 1 mm in diameter with its own gold counter electrode and gold reference electrode and a resin protective layer is used to delimit the electrochemical cell. Chronoamperometric measurements were performed with an AUTOLAB PGSTAT128N potentiostat from Metrohm Autolab (Utrecht, The Netherlands). Data were collected and evaluated with Nova 2.1.4 software.

PCR and RPA reactions were performed using a Nexus Gradient Thermal Cycler from Eppendorf Ibérica (Madrid, Spain). qPCR reactions were performed in 7300 real-time PCR system (Thermo Fisher Scientific, Madrid, Spain) and 7300 system 1.4.0 software was used to collect and evaluate data. A NanoDrop 2000 spectrophotometer (Thermo Fisher Scientific, Madrid, Spain) was used to quantitatively and qualitatively check amplified and extracted DNA.

2.3. Target OsHV-1 DNA

Target OsHV-1 DNA was prepared from OsHV-1 virus particles isolated using anionic magnetic beads (Toldrà *et al*. 2018a). Genomic OsHV-1 DNA from the captured particles was extracted with the Qiagen DNeasy Blood and Tissue kit as reported in our previous work (Toldrà *et al*. 2018a). Afterwards, target DNA was amplified from genomic OsHV-1 DNA using the ORF95F/ORF95R primer set described by Gao *et al.* (2018) (**Table S1**). Each 25-µL reaction mixture contained 2.5 µL PCR Buffer 1X, 600 µM dNTP, 2 mM $MgCl_2$, 0.2 µM of each primer, 1 U of Taq polymerase and 2 µL of genomic OsHV-1 DNA. PCR conditions included 40 cycles of 95 °C for 30 s followed by primer annealing at 65 °C for 30 s and extension at 72 °C for 1 min. PCR products were checked by agarose gel electrophoresis, purified using the QIAquick gel extraction kit and quantified using a NanoDrop to calculate the target OsHV-1 DNA concentration. DNA was stored at -20 °C until use.

2.4. Pacific oyster samples

Spat *C. gigas* oysters (*n* = 16) were obtained from an infectivity experiment described in our previous work (Toldrà *et al*. 2018a). Oyster samples were sourced from two aquaria: a control aquarium (oysters injected with sterile water) and a treatment aquarium (oysters injected with naturally OsHV-1-contaminated oyster homogenate). Mortality was checked daily, and any dead/moribund oysters being removed from the treatment aquarium (*n* = 6). At day 11, alive oysters were also collected from both control (*n* = 5) and treatment (*n* = 5) aquaria. Total genomic DNA from 16 oyster samples was extracted using the Qiagen DNeasy Blood and Tissue kit according to the manufacturer's protocols with 100 µL of AE buffer used for the final elution step (Toldrà *et al*. 2018a). DNA quality and quantity were measured using a NanoDrop. DNA was diluted to 50 ng of total OsHV-1 DNA/µL (to be analysed by qPCR) or to 100 ng of total OsHV-1 DNA/µL (to be analysed by the biosensor) and stored at -20 °C until use.

2.5. Primers and probes

The primers used in this work were based on the ORF95F/ORF95R OsHV-1 real-time RPA primer set reported by Gao *et al*. (2018). Primers have a length of 30 bp and amplify a product of 164 bp (**Table S1**). The specificity of the primers towards a variety of viruses and bacteria that commonly infect bivalve molluscs had been previously confirmed (Gao *et al*. 2018). In this study, these primers were modified with oligonucleotide tails, which enable the direct detection of the RPA product through complementary capture and reporter probes (**Fig. 1**). Primer and probe sequences are detailed in **Table 1**. Tails and probes were tested to confirm the absence of non-specific binding with the primer sequences and the OsHV-1 genome (AY509253.2) (Davison *et al*. 2005) using Multiple Primer analyser software.

Table 1. List of primers and probes and their respective modifications. Tails are underlined.

Name	Sequence (5'-3')
OsHV-1 forward primer	gtt ttc cca gtc acg ac-C3-cat gtt tac gtg gaa atg ttg gat tgg cta
OsHV-1 reverse primer	tgt aaa acg acg gcc agt-C3-atg tca aat agg ttg ttg gca gtg atg gtc
Capture probe	gtc gtg act ggg aaa act ttt ttt ttt ttt tt-C3-SH
Reporter probe	HRP-act ggc cgt cgt ttt aca

2.6. Recombinase polymerase amplification (RPA)

The RPA of OsHV-1 DNA was performed using the TwistAmp Basic kit. Following systematic optimisation, the RPA reaction (50 µL) contained 14.75 µL of rehydration buffer, ½ enzyme pellet, 120 mM of each primer, 14 mM of magnesium acetate and 5 µL of DNA, which corresponded to: a) target OsHV-1 DNA for the RPA optimisation and the storage stability of the capture probe-modified electrodes (10^5 OsHV-1 copies) and for the construction of the calibration curves (10-fold dilutions, from 10^8 to 10^2 OsHV-1 copies); and b) DNA extracted from oyster samples (100 ng of total OsHV-1 DNA/µL). Magnesium acetate was added to initiate the RPA reaction. Reactions were performed at 37 ˚C for 30 min. Blanks (NTC = no template control) were included in all experiments.

Optimisation of the RPA conditions was carried out using colorimetric detection (section 2.7). Primer concentration (2-fold dilutions, from 480 to 120 mM) and the need to clean-up the RPA product before detection were evaluated, while maintaining all other RPA parameters constant. Purification of the RPA product was carried out using the GeneJET PCR purification kit according to the manufacturer's instructions, with 50 µL of TE buffer for the final elution step. Once the optimal conditions were selected, the effect of RPA reaction time (30, 40 and 60 min) on the LOD was investigated.

2.7. Colorimetric assay

Colorimetric assays were carried out on 96-well maleimide-activated microtitre plates. Microtitre wells were incubated with 50 µL of 500 nM thiolated capture probe in PBS (100 mM phosphate, 150 mM NaCl, pH 7.4). Blocking of any non-functionalised maleimide groups was achieved using 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. In the following step, 45 µL of RPA product were added and, subsequently, 50 µL of 10 nM HRP-conjugated reporter probe in PBS-Tween (100 mM potassium phosphate, 150 mM NaCl, 0.05% v/v Tween-20, pH 7.4) were incubated.

Finally, 100 µL of TMB liquid substrate were added, and 10 min later the absorbance was read at 620 nm. After each step, wells were rinsed three times with 200 µL of PBS-Tween. During incubations, microtitre plates were placed on a plate shaker. All steps were carried out at room temperature for 30 min except for the immobilisation of the capture probe, which was performed at 4 °C overnight.

2.8. Electrochemical biosensor

The electrochemical biosensor protocol was essentially the same as the colorimetric assay with the exception of some minor volume adjustments for use with the MicruX electrodes in the detection step. Regarding volumes, 10 µL of non-purified RPA product were placed on each working electrode, whereas 50 µL were used in all other steps. For the electrochemical measurement, 2 µL of TMB were added to each electrode and incubated for 5 min and, finally, the TMB reduction current was measured using chronoamperometry applying -0.2 V for 5 s. After each step, electrodes were rinsed, first with PBS-Tween and then with Milli-Q water, and finally dried. All steps were carried out at room temperature for 30 min except for the immobilisation of the capture probe, which was performed at 4 °C overnight in a water-saturated atmosphere.

Following electrochemical measurements, electrodes were cleaned by immersion in Piranha solution (3:1 H_2SO_4:H_2O_2) rinsed with Milli-Q water, dried and stored at room temperature for subsequent use. To evaluate the stability of the functionalised electrodes, gold electrodes modified with the thiolated capture probe were prepared as described before, washed and stored at 4 °C and −20 °C. The initial electrochemical response (reference value) and the response after 7 and 17 days was measured.

2.9. Quantitative PCR (qPCR) analysis

Quantification of OsHV-1 DNA in oyster samples by qPCR was performed as described in our previous work (Toldrà *et al*. 2018a). Briefly, each 20-µL reaction mixture contained 10 µL 2X SYBR Green dye, 0.5 µM OsHVDPFor/OsHVDPRev primers (Webb *et al*. 2007) (**Table S1**) and 1 µL of extracted DNA from oyster samples (50 ng of total OsHV-1 DNA/µL). The qPCR conditions included 45 cycles of amplification following a three-step protocol (95 °C for 30 s, 60 °C for 1 min and 72 °C for 45 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). Quantification of OsHV-1 DNA copies was carried out using an interlaboratory-validated standard curve based on 10-fold dilutions of OsHV-1 plasmid DNA.

2.10. Data analysis

Measurements were performed in triplicate for the colorimetric assay and qPCR and in quadruplicate for the electrochemical biosensor. Calibration curves obtained using the colorimetric assay and the electrochemical biosensor were fitted to a sigmoidal logistic four-parameter equation using SigmaPlot 12.0 software. The LOD was defined as the blank (NTC) signal plus 3-fold its standard deviation. OsHV-1 DNA quantifications in oyster samples obtained by the biosensor and qPCR are expressed as OsHV-1 DNA copies per 50 ng of total DNA. Linear regression was used to evaluate the correlation between OsHV-1 DNA quantifications determined with the electrochemical biosensor and those obtained from qPCR analysis. Correlation between both techniques was assessed by means of Pearson's correlation coefficient (*r*). Data analyses were performed with SigmaStat 3.1 software.

3. Results and discussion

3.1. Colorimetric assay and RPA optimisation

Due to the similar affinity of maleimide and gold for thiol groups, colorimetric assays on maleimide-coated microtitre plates were first developed to evaluate the feasibility of the approach and to optimise the experimental parameters of the RPA.

According to the TwistDx manual (Appendix TwistDx), purification of RPA products before their detection is required to avoid non-specific signals. However, DNA purification involves an extra step, adding complexity, reagents and an equipment requirement to the overall process. With the aim of simplifying the protocol, both the need to purify RPA products and the effect of the RPA primer concentration were evaluated using the colorimetric assay, with a positive control consisting of a non-saturated concentration of OsHV-1 copies (i.e. 10^5 OsHV-1 copies) and a negative control (NTC).

The effect of using non-purified RPA product was first investigated (**Fig. 2**). When the primer concentration recommended by the manufacturer (480 mM) was used, the absorbance value corresponding to the non-specific adsorption (NTC) was as high as that achieved with the positive control. By reducing the primer concentration by half (240 mM), the non-specific adsorption value was also reduced by half, while the positive signal was maintained. Finally, the use of 120 mM primer concentration resulted in a greatly reduced non-specific adsorption (non-detectable signal), whereas the positive signal was maintained again. These results suggest that the presence of residual primers may cause the formation of primer-dimers, leading to an increase of the non-specific adsorption values in our configuration. When RPA products were purified, a decrease in the specific signals was observed for all primer concentrations

tested when compared to the responses obtained with non-purified ones, suggesting that the purification step may cause some loss of RPA product. On the other hand, although non-specific adsorption values were always lower than their corresponding positive ones, they were still significant at the highest primer concentration. This could indicate that not all residual primers are efficiently removed when purifying. In summary, the use of non-purified RPA product in combination with the lowest primer concentration tested provided the highest NTC-subtracted signals, thereby these conditions were chosen for the construction of the calibration curve.

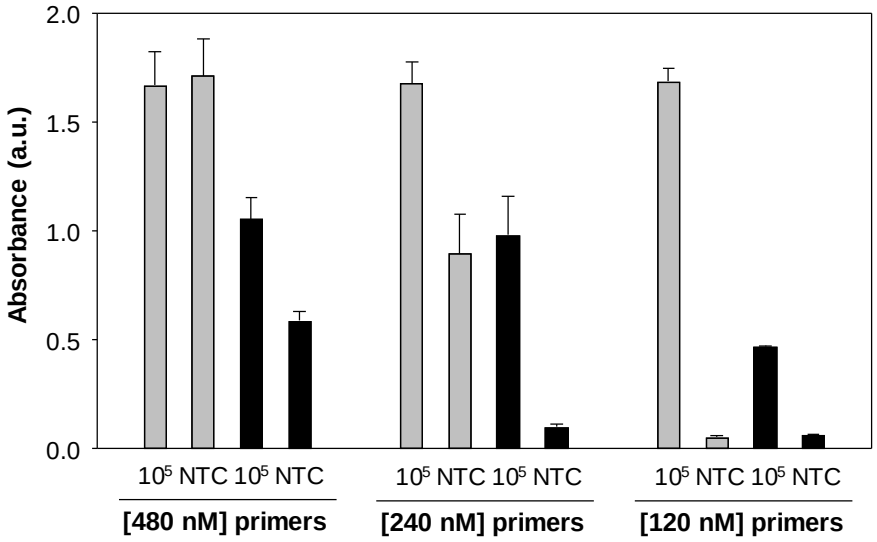

Fig. 2. Optimisation of the RPA conditions by the colorimetric assay using 10^5 OsHV-1 target copies and NTC. Black bars = purified RPA products; grey bars = non-purified RPA products. Error bars are the standard deviation of the mean, *n* = 3.

The calibration curve constructed using target OsHV-1 DNA is shown in **Fig. 3**. An LOD of 426 OsHV-1 target copies was achieved, which was higher than other reported molecular methods for OsHV-1 detection (see section 3.2). With the aim of decreasing the LOD the system, the effect of extending the RPA reaction time to 40 and 60 min was evaluated. However, the same LODs were reached, thus the reaction time was maintained to 30 min, as recommended by the manufacturer and also used in our previous works (Toldrà *et al*. 2019; Toldrà *et al*. 2018b).

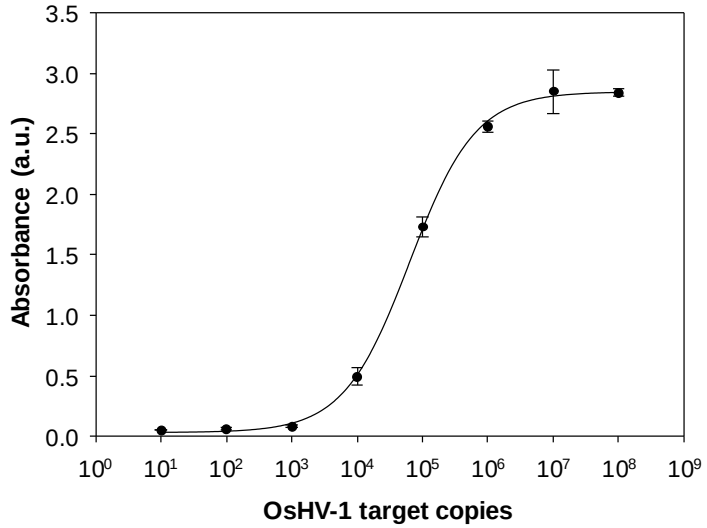

Fig. 3. Calibration curve obtained with the colorimetric assay. Errors bars are the standard deviation of the mean, *n* = 3.

3.2. Electrochemical biosensor

After protocol optimisation using the colorimetric assay, the strategy was transferred to gold electrodes to develop the corresponding electrochemical DNA-based biosensor. Maleimide plates were replaced by thin-film gold electrodes and the electrochemical signal was recorded instead of the colorimetric one. The use of miniaturised electrodes enabled a 1/5 reduction of the volume of RPA product required.

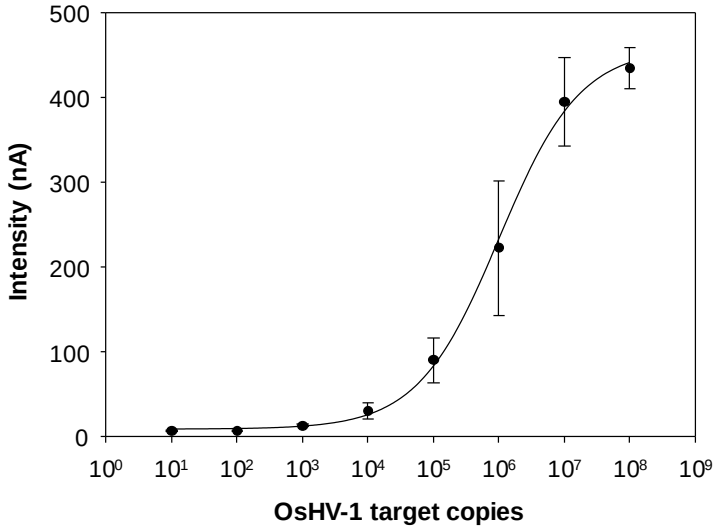

Fig. 4. Calibration curve obtained with the electrochemical biosensor. Error bars are the standard deviation of the mean, *n* = 4.

Fitting the calibration curve to the sigmoidal logistic four-parameter equation (**Fig. 4**) resulted in an LOD of 207 OsHV-1 target copies, which is ~2-fold lower than that obtained with the colorimetric approach. The biosensor has an LOD lower than that

attained with PCR followed by gel electrophoresis (1,000 copies) (Webb *et al*. 2007), but higher than those achieved by other molecular methods described to date, including qPCR (4 copies) (Pepin *et al*. 2008), LAMP (20 copies) (Ren *et al*. 2010) or real-time RPA (5 copies) (Gao *et al*. 2018). Nevertheless, the biosensor offers potential advantages in terms of portability, miniaturisation and automation, which make it more applicable to field detection, and strategies for signal enhancement and decrease in LOD will be explored.

3.3. Stability of the functionalised electrodes

To investigate the possibility to shorten the biosensor protocol time, the storage stability of the capture probe-modified electrodes was tested at 4 and -20 °C over 17 days. The intensity values achieved were constant with time (**Fig. S1**), clearly demonstrating that the functionalised electrodes were stable for at least 17 days, stored both at 4 °C and −20 °C. Moreover, such real-time stability can be used to predict shelf-life of functionalised electrodes using the Q Rule method, which states that a product degradation rate changes exponentially with the temperature, and is proportional to $(Q10)^n$, where n is the temperature change divided by 10 (Anderson and Scott 1991). 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 functionalised electrodes are stable at least for 17 days, an n value of 2.4 and a conservative Q10 value of 2, the predicted stability [17 days/$(2)^{2.4}$] of the product at -20 °C is at least 3 months. This great storage stability significantly reduces the assay time, as multiple electrodes can be prepared on the same day and stored until use, providing ready-to-use modified electrodes and allowing to perform the assay within the same day.

3.4. OsHV-1 detection in Pacific oyster samples

A total of 16 oyster samples from an experimental infection were analysed using the electrochemical biosensor to demonstrate its applicability. OsHV-1 target copies in the oysters were quantified using the sigmoidal logistic equation. As shown in **Table 2**, samples from the control aquarium were all negative, and 2 out of 11 samples from the treatment aquarium also provided negative results. Such negative oysters were alive when collected at the end of the experiment. The rest of the oysters from the treatment aquarium, which were dead/moribund or alive, resulted positive for OsHV-1 detection, with contamination levels from 1.50 x 10^2 to 3.34 x 10^5 OsHV-1 DNA copies/50 ng of total DNA. As expected, dead/moribund oysters presented higher viral DNA amounts than the live ones.

Quantifications obtained using the biosensor were compared with those obtained by qPCR analysis. qPCR also provided negative results for the control oysters, whilst for

the oysters from the treatment aquarium, OsHV-1 DNA was detected in all samples. The two samples that resulted negative using the biosensor were positive by qPCR, certainly due to the higher LOD of the biosensor. Linear regression was used to evaluate the correlation between techniques. Samples that resulted negative for both techniques were not included in the regression (n = 5), neither samples that were negative for one of the two techniques (n = 2). Samples with ~10^4 OsHV-1 DNA copies/50 ng of total DNA showed an excellent 1:1 correlation, while a slight overestimation and underestimation by the biosensor was observed in samples with <10^3 and >10^5 DNA copies/50 ng of total DNA, respectively. Nevertheless, as shown in **Fig. 5**, the overall correlation between techniques was highly significant (Pearson's r = 0.988; P < 0.001).

Table 2. OsHV-1 DNA concentrations (OsHV-1 DNA copies/50 ng of total DNA) in oysters obtained by the electrochemical biosensor and qPCR analysis.

Sample	Oyster physical state	Aquarium	Electrochemical biosensor	qPCR
1	dead/moribund	treatment	$3.34 \times 10^5 \pm 5.91 \times 10^4$	$7.13 \times 10^5 \pm 4.05 \times 10^4$
2	dead/moribund	treatment	$4.78 \times 10^5 \pm 1.02 \times 10^5$	$4.81 \times 10^5 \pm 7.65 \times 10^4$
3	dead/moribund	treatment	$7.26 \times 10^4 \pm 1.52 \times 10^4$	$1.79 \times 10^5 \pm 4.10 \times 10^4$
4	dead/moribund	treatment	$6.10 \times 10^3 \pm 4.76 \times 10^2$	$6.52 \times 10^3 \pm 2.34 \times 10^3$
5	dead/moribund	treatment	$5.21 \times 10^3 \pm 6.80 \times 10^1$	$4.38 \times 10^3 \pm 1.37 \times 10^3$
6	dead/moribund	treatment	$2.75 \times 10^3 \pm 6.18 \times 10^2$	$1.98 \times 10^3 \pm 4.46 \times 10^2$
7	alive	treatment	$1.97 \times 10^2 \pm 6.89 \times 10^1$	$1.21 \times 10^2 \pm 2.46 \times 10^1$
8	alive	treatment	$5.27 \times 10^2 \pm 6.98 \times 10^1$	$7.83 \times 10^1 \pm 1.97 \times 10^1$
9	alive	treatment	$1.50 \times 10^2 \pm 4.47 \times 10^1$	$3.24 \times 10^1 \pm 5.67$
10	alive	treatment	n.d.	$1.04 \times 10^1 \pm 5.83$
11	alive	treatment	n.d.	$7.93 \times 10^0 \pm 3.38$
12	alive	control	n.d.	n.d.
13	alive	control	n.d.	n.d.
14	alive	control	n.d.	n.d.
15	alive	control	n.d.	n.d.
16	alive	control	n.d.	n.d.

n.d.: not detected

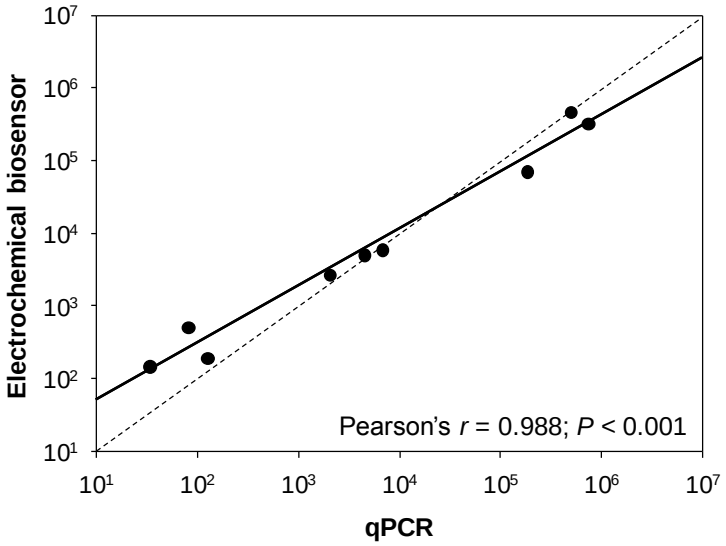

Fig. 5. Correlation between OsHV-1 DNA concentrations (OsHV-1 DNA copies/50 ng of total DNA) in oysters obtained with the electrochemical biosensor and qPCR analysis (n = 9) (black line). Oyster samples that were negative for both techniques were not included in the regression (n = 5), neither samples that were negative for one of the two techniques (n = 2). The dashed line represents the bisector, to which points should align in case of perfect identity of the methods.

4. Conclusions

Ostreid herpesvirus-1 (OsHV-1) infection is the biggest threat to the Pacific oyster (*C. gigas*) production around the world. Early detection of this pathogen is therefore of key importance to facilitate the rapid implementation of appropriate measures, thus avoiding the spread of the disease and, ultimately, minimising its impact. For this purpose, the development of biosensors able to perform rapid and *in situ* tests is of utmost importance for the sector.

In this work, an electrochemical biosensor for the detection of OsHV-1 has been described. Prior to biosensor development, RPA conditions were optimised using a colorimetric assay. The biosensor exhibits a good analytical performance, with the specificity, storage stability, sensitivity and reliability necessary to be applied to the detection of the pathogen in oyster samples. This was evidenced by the strong correlation found between the OsHV-1 DNA concentrations determined by the biosensor and qPCR in the analysis of oyster samples.

Our innovative biosensor configuration offers several advantages. Firstly, the use of an isothermal DNA amplification such as RPA circumvents the need for equipment-dependent and time-consuming PCR technology. Secondly, the electrochemical read-out instrumentation is less complex than the currently used optical detection systems. Thirdly, the use of miniaturised thin-film electrodes enables working with smaller sample volumes. Although not yet automated, the system offers great potential to be

easily integrated into microfluidic systems to develop compact devices that could be used in the field by end-users. Despite the multiple benefits, the LOD of the biosensor remains higher than other described molecular techniques. Future work will be focused on decreasing LODs by means of improving both the amplification and the detection steps or, alternatively, using pre-concentrating agents such as magnetic beads.

The economic loses associated to the OsHV-1 infection in aquaculture facilities make this bioanalytical tool highly interesting for screening and quantification purposes. Examples of applicability could be the fast screening of stocks before they are transferred to a depuration plant or another harvesting area, or the "on the spot" control by competent customs agencies. The present work not only reports the first electrochemical biosensor for OsHV-1 detection, but also constitutes a clear demonstration that progress in molecular biology in conjunction with simple detection systems can result in powerful analytical tools with wide applications.

Acknowledgments

This work was supported by the European Commission H2020 Framework Programme through grant agreement 678589 (VIVALDI project). The authors are grateful to Dr. K. B. Andree for his initial suggestions. The authors also acknowledge support from the CERCA Programme/Generalitat de Catalunya. Anna Toldrà acknowledges IRTA-Universitat Rovira i Virgili-Banco Santander for her PhD grant (2015PMF-PIPF-67).

Supplementary information

Table S1. List of primers used for PCR (section 2.3) and qPCR (section 2.9) reactions.

	Primer name	Sequence (5'-3')	Primer size (bp)	Amplicon size (bp)	Target gene
PCR OsHV-1 Fw primer	ORF95F	cat gtt tac gtg gaa atg ttg gat tgg cta	30	164	ORF95
PCR OsHV-1 Rv primer	ORF95R	atg tca aat agg ttg ttg gca gtg atg gtc	30		
qPCR OsHV-1 Fw primer	OsHVDPFor	att gat gat gtg gat aat ctg tg	23	197	ORF100
qPCR OsHV-1 Rv primer	OsHVDPRev	ggt aaa tac cat tgg tct tgt tcc	24		

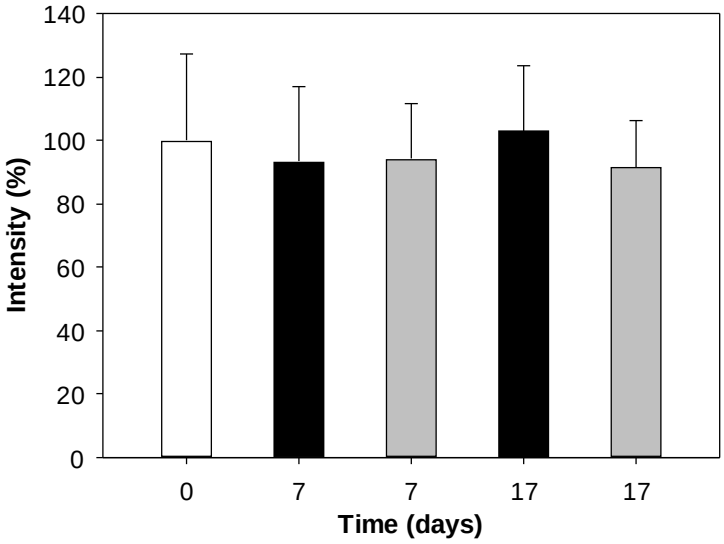

Fig. S1. Storage stability of the capture probe-modified gold electrodes. Bars represent the electrochemical responses obtained with electrodes stored at 4 °C (black bars) and at -20 °C (grey bars) respect to day 0 (white bar, reference value). Error bars are the standard deviation of the mean, *n* = 4.

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Chapter 5B

Rapid capture and detection of ostreid herpesvirus-1 from Pacific oyster *Crassostrea gigas* and seawater using magnetic beads

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Abstract

Ostreid herpesvirus-1 (OsHV-1) has been involved in mass mortality episodes of Pacific oysters *Crassostrea gigas* throughout the world, causing important economic losses to the aquaculture industry. In the present study, magnetic beads (MBs) coated with an anionic polymer were used to capture viable OsHV-1 from two types of naturally infected matrix: oyster homogenate and seawater. Adsorption of the virus on the MBs and characterisation of the MB-virus conjugates was demonstrated by real-time quantitative PCR (qPCR). To study the infective capacity of the captured virus, MB-virus conjugates were injected in the adductor muscle of naïve spat oysters, using oyster homogenate and seawater without MBs as positive controls, and bare MBs and sterile water as negative controls. Mortalities were induced after injection with MB-virus conjugates and in positive controls, whereas no mortalities were recorded in negative controls. Subsequent OsHV-1 DNA and RNA analysis of the oysters by qPCR and reverse transcription qPCR (RT-qPCR), respectively, confirmed that the virus was the responsible for the mortality event and the ability of the MBs to capture viable viral particles. The capture of viable OsHV-1 using MBs is a rapid and easy isolation method and a promising tool, combined with qPCR, to be applied to OsHV-1 detection in aquaculture facilities.

1. Introduction

Pacific oyster *Crassostrea gigas* represents the most important species for global aquaculture production of oysters. However, infectious diseases often result in massive losses and their management remains a major concern (EFSA 2015; FAO). During recent years, massive mortality outbreaks of *C. gigas* have been reported in many European countries (e.g. France, Spain, Italy and Ireland), New Zealand, Japan, USA and Australia (Burge *et al.* 2006; Jenkins *et al.* 2013; Keeling *et al.* 2014; Renault *et al.* 1994; Roque *et al.* 2012; Segarra *et al.* 2010). These mortalities mostly take place during the spring/summer period, when the seawater temperature reaches 16 °C (Carrasco *et al.* 2017; Pernet *et al.* 2014; Petton *et al.* 2013), and have been associated to the detection of ostreid herpesvirus type 1 (OsHV-1). OsHV-1 is an enveloped virus of double-stranded DNA with an icosahedral structure and a diameter size of about 120 nm. It belongs to the *Malacoherpesviridae* family, and is the only member of the genus *Ostreavirus* (Davison *et al.* 2005).

The economic importance of *C. gigas* oyster aquaculture has favoured the development of diagnostic tools for the detection of OsHV-1 including conventional PCR (Arzul *et al.* 2001; Webb *et al.* 2007), *in situ* hybridisation (Lipart and Renault 2002) and immunohistochemistry (Martenot *et al.* 2016). However, these methods are time consuming, laborious and qualitative, limiting its practical use in disease prevention and control. Recently, more rapid, specific and sensitive methods such as real-time quantitative PCR (qPCR) have been described and applied to OsHV-1 detection (Pepin *et al.* 2008). Despite being routinely applied for OsHV-1 quantification, qPCR does not specify whether DNA correspond to viable viruses, which are necessary to cause viral infection (Rodriguez *et al.* 2009). Addressing the infectivity of viruses is essential to understand their persistence in the environment and the estimation of the risk of transmission. Presently, the only way to assess the infectivity of OsHV-1 involves experimental infections in bivalves, further evaluation of oyster mortality and/or DNA and mRNA analysis by qPCR and reverse transcription qPCR (RT-qPCR), respectively. However, infectivity assays are difficult and greatly depend on the assay conditions (e.g. age, physiology and genetics of oysters). The combination of qPCR with capture systems able to separate between viable and non-viable virus particles from the environmental matrix may provide a feasible approach to directly detect viable virus particles, as an alternative to the use of infectivity assays.

On the other hand, obtaining OsHV-1 viral particles to develop and validate diagnostic tools and for other research purposes (e.g. antibody development, functional studies and sequencing) is nowadays of utmost interest. Because no stable bivalve cell lines are available, the virus cannot be cultured and can only be obtained from infected bivalves (Burge and Friedman 2012; Schikorski *et al.* 2011b). A promising approach has

been recently described, which uses haemocytes collected from spat oysters to develop *in vitro* models and to study host/virus interactions (Morga *et al.* 2017). Additionally, subsequent virus isolation and purification involves high speed ultracentrifugation, which is time consuming and requires very expensive equipment. Although polyethylene glycol (PEG) precipitation has also been used for this purpose, it may be incompatible with virus detection techniques. Furthermore, both ultracentrifugation and PEG precipitation often decrease the infectivity of the isolated virus particles (Sakudo and Onodera 2012). As a result, there is a strong interest in developing rapid and efficient isolation/purification methods as well as new diagnostic tools for OsHV-1.

An alternative approach to isolate viruses is the use of magnetic beads (MBs) coated with antibodies or organic chemicals that efficiently bind viral particles. Poly (methyl vinyl ether-maleic anhydrate) [poly (MVE-MA)] and its derivatives are copolymers with molecular and physicochemical characteristics that allow them to be used as bioadhesives (Sakudo and Onodera 2012). In this sense, MBs coated with anionic poly (MVE-MA) have been used to capture different types of viruses. These include some enveloped viruses such as human immunodefiency virus (HIV) (Sakudo and Ikuta 2012), respiratory syncytial virus (RSV) (Sakudo *et al.* 2009a) or influenza virus (Sakudo *et al.* 2009b), as well as non-enveloped viruses like adenovirus (Sakudo *et al.* 2016). Although this technology has a broad potential for the isolation of diverse viruses, the binding capacity of the MBs has been reported to vary depending on the virus type, and even fails to effectively bind some virus types (Sakudo *et al.* 2016; Sakudo *et al.* 2011). Thus, its universal applicability in all virus types has not been proven. The exact mode of interaction between the virus particle and the anionic MB remains unclear, but it seems that electrostatic, hydrophilic and hydrophobic interactions are involved (Sakudo *et al.* 2016; Sakudo *et al.* 2009a). Several parameters such as physical characteristics of the virus (isoelectric point, particle size), membrane properties (electric charge, hydrophobicity) and sample characteristics (pH, ionic strength) may influence virus adsorption (Sakudo *et al.* 2009a). The ability of the MBs to capture virus has been demonstrated in only a few matrices, mainly cell culture media and phosphate-buffered saline, and its application in biological samples has not been tested yet (Patramool *et al.* 2013; Sakudo *et al.* 2016). Compared to traditional isolation techniques, the MB-mediated capture method has several advantages: i) it is fully compatible with subsequent conventional detection techniques (e.g. PCR, ELISA), ii) it is rapid, inexpensive and easy to perform, and iii) it is able to preserve the infective capacity of the isolated virus.

The aim of the present work was to investigate the use of MBs coated with an anionic polymer to capture viable OsHV-1 particles from two types of samples: oyster

homogenate and seawater (**Fig. 1**). Adsorption of OsHV-1 on the MBs was assessed by qPCR analysis, and characterisation of the MB-virus conjugates was performed. Afterwards, MB-virus conjugates were injected into naïve spat oysters. The ability of the captured virus to produce an active infection in oysters was evaluated through mortality monitoring as well as DNA and RNA analysis of oysters by qPCR and RT-qPCR, respectively.

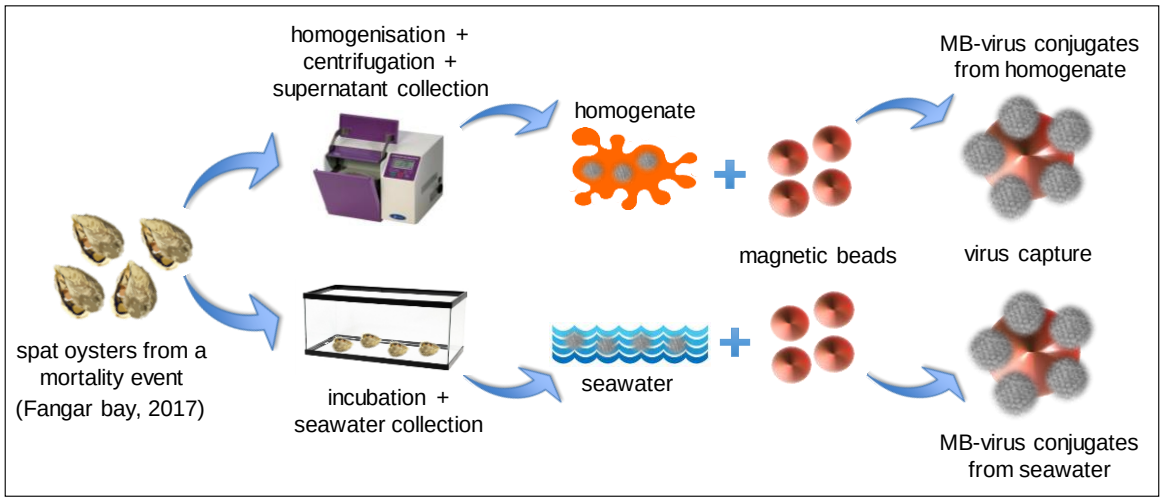

Fig. 1. Schematic representation of the protocol for the capture of viable OsHV-1 from the homogenate and the seawater using anionic MBs and subsequent virus detection.

2. Materials and Methods

2.1. Reagents

Anionic polymer-coated magnetic beads (Viro-adembeads) with a diameter of 300 nm were purchased from Ademtech (Pessac, France). Qiagen DNeasy Blood and Tissue kit, Qiagen RNeasy Mini kit, SYBR Green dye, SuperScript First-Strand cDNA Synthesis kit, RNA*later* and custom primers were supplied by Thermo Fisher Scientific (Madrid, Spain).

2.2. Infected material

C. gigas spat oysters naturally infected with OsHV-1 were collected from Fangar Bay (NW Mediterranean Sea, N 40˚46'27.62'' E 0˚44'12.27'') during a mortality event on April 2017, and OsHV-1 prevalence was evaluated by qPCR following the protocol described in section 2.5. These animals were used to prepare two types of samples: (1) Homogenate: 30 oysters (~23 mm in length) were mixed with 20 mL of UV-treated seawater and homogenised using a stomacher for 1 min at maximum speed; after centrifugation (1,000 g; 10 min), the supernatant was collected. (2) Seawater: 30 oysters were placed in a 500-mL UV-treated seawater tank and maintained at 22 °C;

after 22h, the seawater was collected. Both sets of samples were stored at 4 °C until use.

2.3. Capture of OsHV-1 by MBs

Virus capture by MBs was performed following the company's instructions with some modifications. Briefly, MB-virus conjugates were prepared as follows: (1) 10 µL of MB suspension were transferred to a tube and washed three times with binding buffer (supplied with the MBs); for the washing steps, the tube was placed on the magnetic separation stand and the supernatant was removed; (2) 100 µL of infected material (homogenate or seawater) was added and incubated for 20 min at room temperature with slow tilt-rotation; (3) MB-virus conjugates were washed once with washing buffer (100 mM potassium phosphate, 0.05 % v/v Tween-20, pH 7.4) (three times to evaluate the necessity to include several washing steps), resuspended in 100 µL of sterile water and subjected to DNA extraction (and subsequent qPCR analysis) or oyster infection. When other amounts of MB were used, volumes were adjusted proportionally. Characterisation of the conjugates was performed using serial dilutions of the homogenate (pure and 4-fold dilutions) and serial dilutions of MBs (4-fold dilutions; from 160 µL to 0.625 µL) while maintaining a constant amount of MB (10 µL) and using non-diluted homogenate, respectively. Conjugates were prepared and analysed in triplicate.

2.4. Infectivity experiment

Experimental infections were performed using naïve spat *C. gigas* oysters (~25 mm in length) from IRTA hatchery, which had been previously found to be negative for the presence of OsHV-1 DNA by qPCR following the protocol described in section 2.5. The experimental design included 6 treatments: MB-virus conjugates from the homogenate (7.7×10^1 DNA copies/µL), MB-virus conjugates from the seawater (2.9 DNA copies/µL), bare MBs, homogenate (1.1×10^4 DNA copies/µL), seawater (4.2×10^2 DNA copies/µL) and sterile water. Oysters were placed on ice and, once relaxed, 50 µL of the corresponding sample were injected into the adductor muscle of each animal using a syringe. Oysters were then placed in 6 aquaria (26-30 oysters per aquarium/treatment) containing 10 L of UV-treated seawater at 22 °C and an independent aeration source. Oysters were fed every 2 days for 11 days with a mix of *Isochrysis sp., Tetraselmis sp.* and *Chaetoceros gracilis* and water parameters (pH, salinity, oxygen concentration) were monitored. Mortality was checked daily, dead and moribund oysters being removed from aquaria. At day 11, living oysters were also collected. Oyster tissues (combinations of mantle, gill, digestive gland and adductor muscle) were frozen at -80 °C until DNA extraction. Tissues of some moribund and

living oysters (3 per treatment with infected material or MB-virus conjugates) were also stored at -80 °C with 700 µL of RNA*later* for further RNA extraction.

2.5. OsHV-1 DNA extraction and qPCR analysis

Total DNA was extracted using the Qiagen DNeasy Blood and Tissue kit, according to the manufacturer's protocols with some adjustments. For the infected material, 100 µL of homogenate or seawater were mixed with 100 µL of lysis buffer (1 M NaCl, 70 mM Tris, 30 mM EDTA, pH 8.6), 20 µL of proteinase K and 200 µL of AL buffer. After 1 h at 56° C, the content was mixed with 200 µL of ethanol and DNA extraction was carried out using spin columns. Elution was performed with 50 µL of AE buffer. The same protocol was used for MB-virus conjugates, the only exception being that MBs were removed after heating using a magnetic separation stand. For oysters, tissue samples were mixed with 180 µL of ATL buffer and 20 µL of proteinase K, and transferred to 2-mL cryotubes containing zirconium glass beads. The mixture was disrupted using a BeadBeater-8 (BioSpec) pulsed for 45 s at full speed and digested overnight at 56° C. Afterwards, 200 µl of buffer AL and 200 µL of ethanol were added, and the contents transferred into the spin columns. Elution was performed with 100 µL of AE buffer. DNA quality and quantity were measured in a NanoDrop 2000 spectrophotometer (Thermo Fisher Scientific). Total DNA from oysters was diluted to 50 ng/µL. Extracted DNA was stored at -20 °C until qPCR analysis.

Detection and quantification of OsHV-1 DNA was carried out by qPCR using the primer pair OsHVDPFor/OsHVDPRev (Webb *et al.* 2007). Reactions were performed using an ABI 7300 thermocycler (Thermo Fisher Scientific) according to the following conditions: 1 cycle of pre-incubation at 95 °C for 10 min; 45 cycles of amplification at 95 °C for 30 s, 60 °C for 1 min s and 72 °C for 45 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). Each 20-µL reaction mixture contained 10 µL 2X SYBR Green dye, primers (final concentration 0.5 µM) and 1 µL of extracted DNA. A negative control (without DNA) and a positive control (pure OsHV-1 genomic DNA) were included, and each qPCR reaction was performed in triplicate. Quantification of OsHV-1 DNA copies was carried out using a standard curve based on 10-fold dilutions of OsHV-1 genomic DNA. Results were expressed as total OsHV-1 DNA copies (per 100 µL of homogenate, seawater and MB-virus conjugates, section 3.1 and section 3.2) or as OsHV-1 DNA copies per ng of total DNA (oysters, section 3.3.2).

2.6. OsHV-1 RNA extraction, cDNA synthesis and RT-qPCR analysis

Total RNA extraction from oyster tissue samples was performed with the Qiagen RNeasy Mini kit, using 50 µL of diethylpyrocarbonate (DEPC) treated water for the elution. RNA quality and quantity were determined by spectrophotometry and

TAE/formamide agarose electrophoresis. First-stranded cDNA synthesis was carried out using SuperScript First-Strand cDNA Synthesis kit using 2 µg of RNA. To evaluate virus gene expression, primer pairs for 3 OsHV-1 genes (ORF4, ORF16 and ORF42) and one oyster housekeeping gene (Elongation factor 1-alfa, EF1) described by Segarra et *al*. (2014) were used. RT-qPCR reactions were performed in a total volume of 20 µL containing 2 µL of 5-fold diluted cDNA, 10 µL 2X SYBR Green dye and primers (final concentration 0.5 µM). Reactions were performed in triplicate and samples of RNA without reverse transcription were included in order to control absence of genomic DNA contamination. PCR cycling conditions were as follows: 10 min at 95 °C, followed by 45 cycles of amplification at 95 °C for 15 s, 60 °C for 20s. Melting curves were also plotted. Viral gene expression of the 3 viral genes was normalised to EF1 using the formula: Delta Ct = Ct viral ORF – Ct EF1.

2.7. Statistical analysis

Statistical analyses were carried out using SigmaStat software 3.1 (Systat Software Inc., California, USA). The normality of the data was tested using a Shapiro-Wilk test. To evaluate differences between means of viral DNA and RNA amounts quantified in dead/moribund and living oysters, a non-parametrical Mann-Whitney test was conducted. A p-value level of 0.05 was used in all tests to identify differences.

3. Results and discussion

3.1. Capture of OsHV-1 by MBs

Analysis by qPCR of the infected samples revealed the presence of OsHV-1 DNA: $1.1 \times 10^6 \pm 3.2 \times 10^4$ DNA copies in the homogenate and $4.2 \times 10^4 \pm 1.8 \times 10^3$ DNA copies in the seawater. To examine the capacity of the MBs to capture the virus from both sample types, the DNA samples extracted from MB-virus conjugates were also analysed by qPCR. OsHV-1 DNA was detected in both conjugates, confirming the ability of the MBs to capture virus from both types of samples: $7.7 \times 10^3 \pm 6.3 \times 10^2$ DNA copies in the MB-virus conjugates from homogenate and $2.9 \times 10^2 \pm 2.5 \times 10^1$ DNA copies in the MB-virus conjugates from seawater. It is important to mention that MB-virus conjugates from the homogenate were washed three times and the supernatants from these washing steps were analysed. No differences were obtained between these supernatants, the amount of viral DNA being always below 10% that of the conjugate, hence indicating that the viral DNA measured in the conjugate samples was the result of the MB-virus interaction (**Fig. 2**). In further experiments, only one washing step was included in the protocol.

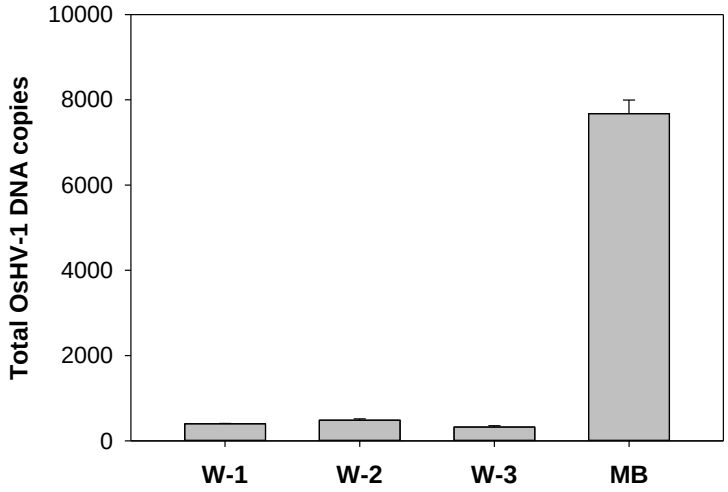

Fig. 2. OsHV-1 DNA detected in the supernatant after the first (W-1), second (W-2) and third (W-3) washing steps and in the MB-virus conjugates from homogenate (MB).

Under the current experimental conditions, capture efficiency from both homogenate and seawater samples was ~1% (percentage calculated using the amount of viral DNA from qPCR data, which only detects viral DNA, and thus may not be reflecting the capacity of the MBs to capture viable virus particles). This low efficiency could be explained by the non-specific adsorption of proteins on the MBs in the case on the homogenate sample, and by the high ionic strength of the seawater, which could screen charge attraction. When compared to other works, high capture efficiencies were achieved using simple matrices (i.e. cell culture media and buffer), whereas the capture of the virus was inhibited when using human blood serum, likely due to the non-specific binding of albumin (Patramool *et al.* 2013; Sakudo *et al.* 2016). In this work, despite the use of complex matrices, OsHV-1 capture was successfully achieved. It should be noted that experimental conditions can be modified (e.g. volume ratios) to improve virus capture efficiency by the beads (see section 3.2) or to get virus pre-concentration (see section 3.3).

3.2. MB-virus conjugates characterisation

To study the OsHV-1 virus capture by the MBs, two experiments were performed: 1) using serial dilutions of the homogenate and a constant amount of MBs, and 2) using serial dilutions of MBs and non-diluted homogenate. In the first experiment, the amount of OsHV-1 DNA on the MBs decreased with the homogenate dilution (**Fig. 3a**) (no OsHV-1 DNA from MBs was detected when using 1/1,024 homogenate dilution or less). Results show that at each homogenate dilution, there is an equilibrium between the number of virus particles captured by the MBs and the number of free virus particles in solution. Additionally, no saturation of the MBs by virus particles was

observed, suggesting that MBs could physically accommodate more virus particles, if present in the homogenate sample. **Fig. 3b** shows the total amount of OsHV-1 DNA on the MBs in the experiment where serial dilutions of MBs were used. Except when 160 µL of MBs were tested (at these conditions steric effects may influence the virus capture efficiency; thus, this point has was not included in the regression), the amount of OsHV-1 DNA on the MBs decreased with the MB dilution. Nevertheless, when the amount of OsHV-1 DNA is normalised to 10 µL of MBs, results indicate that the lower the number of MBs used, the higher the amount of virus particles captured per MB (as before, 160 µL amount was not included in the regression) (**Fig. 3c**). These results clearly indicate that the MB/virus ratio is a key factor in the capture event. Although the highest total amount of viral DNA was recovered using 40 µL of MBs, further experiments were performed with 10 µL of MBs for economic reasons.

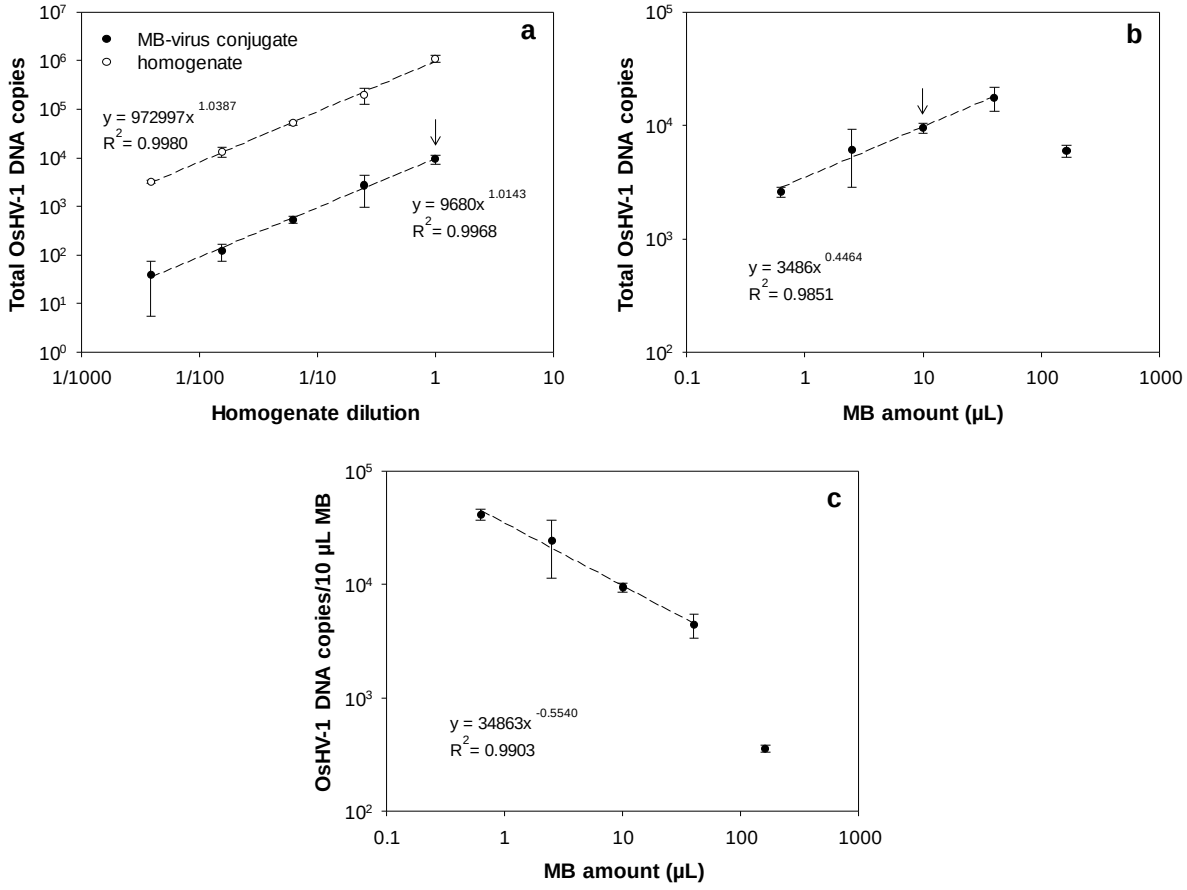

Fig. 3. Calibration curves using 4-fold serial dilutions of homogenate (**a**) and MBs (**b, c**) while maintaining MB amount (10 µL) and using non-diluted homogenate, respectively. Arrows indicate the same MB/homogenate ratio in (**a**) and (**b**). Fig (**c**) is normalised to 10 µL of MBs.

3.3. Infectivity experiment

3.3.1. Oyster mortality

No mortality was observed in the aquaria with oysters to which bare MBs or sterile water had been injected in the adductor muscle of naïve spat oysters. The mortality results for the other 4 treatments are shown in **Fig. 4**.

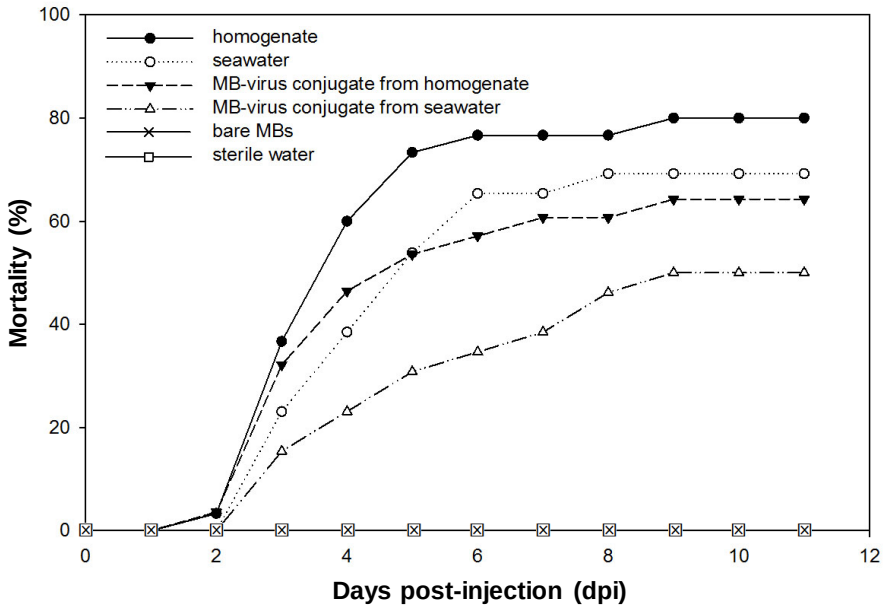

Fig. 4. Cumulative mortality of Pacific *Crassostrea gigas* oysters experimentally infected by intramuscular injection with homogenate, seawater, MB-virus conjugates from homogenate and MB-virus conjugates from seawater, bare MBs and sterile water.

Mortality started at the 2nd day post-injection (dpi) for the treatments with homogenate and MB-virus conjugates from homogenate and at the 3rd dpi for the treatments with seawater and MB-virus conjugates from seawater, which may reflect the time needed for viral replication in oyster tissues (Sauvage *et al.* 2009). At the 9th dpi the cumulative mortality was 80%, 69%, 64% and 50% in the treatments injected with homogenate, seawater, MB-virus conjugates from homogenate and MB-virus conjugates from seawater, respectively, and no more mortalities were recorded afterwards. These results confirm that MBs are able to capture viable OsHV-1 virus particles from both the homogenate and the seawater, and indicate that naïve oysters were successfully infected after injection of MB-virus conjugates. Additionally, a clear dose-response relationship between the number of copies injected (see section 2.4) and the final cumulative mortality was observed. These mortality rates are in accordance with previous experimental works performed by intramuscular injection using OsHV-1-infected oyster homogenates (Paul-Pont *et al.* 2015; Schikorski *et al.* 2011b). Mortalities were detected earlier for the treatment with the MB-virus conjugates from homogenate than for the treatment with seawater (**Fig. 4**). Since

lower number of copies were injected with the MB-virus conjugates (see section 2.4), this earlier mortality may indicate that the presence of MBs could enhance infectivity in oysters. Although the mechanisms by which virus infect cells in the form of anionic poly (MVE-MA) MB-virus conjugates remain unknown, some studies have shown that poly (MVE-MA) derivatives can be used as a vehicle for intracellular transfer, and viruses may be incorporated into cells in the form of conjugates and infect cells (Sakudo *et al.* 2009b; Yonchevai *et al.* 2008). In fact, several phenomena (presence of viable and non-viable virus particles, capture efficiency and/or infectivity potency) may be simultaneously playing a role in this infectivity experiment.

3.3.2. OsHV-1 DNA quantification in oysters

Analysis of OsHV-1 DNA was performed by qPCR for each individual. OsHV-1 DNA was not detected in oysters to which bare MBs or sterile water had been injected. Viral DNA amounts were above 10^2 DNA copies/ng total DNA in almost all dead/moribund oysters regardless of the treatment (**Fig. 5**), which suggests that the quantity of viral DNA must reach a threshold in oyster tissue before causing death, as reported in other works (Paul-Pont *et al.* 2015). Only a few dead/moribund oysters contained lower viral DNA amounts: 1 oyster injected with homogenate (collected at the 6^{th} pdi), 2 oysters injected with MB-virus conjugates from homogenate (collected at the 7^{th} and 9^{th} pdi) and 4 oysters injected with MB-virus conjugates from seawater (collected at the 4^{th}, 8^{th} and 9^{th} pdi). This lower DNA viral content may be explained by DNA degradation processes occurring after death, or individual genetic differences making some individual animals more susceptible (Degremont 2013; Degremont *et al.* 2013). The OsHV-1 DNA amounts found in dead/moribund oysters are in accordance with other studies performed by intramuscular injection (Schikorski *et al.* 2011b).

OsHV-1 DNA was also detected in oysters that survived until the end of the experiment. Only 6 oysters injected with MB-virus conjugates from seawater displayed negative results for OsHV-1 DNA detection. For the rest, viral DNA concentrations were below 10^1 DNA copies/ng total DNA in all treatments, except for 1 oyster injected with seawater (5.7×10^1 DNA copies/ng total DNA). The presence of OsHV-1 DNA has been previously reported in asymptomatic OsHV-1 infected oysters (Sauvage *et al.* 2009; Schikorski *et al.* 2011a; Schikorski *et al.* 2011b). The presence of living oysters could be attributed to a higher antiviral immune response capacity, which reduces their susceptibility to develop the disease (Degremont 2013; Degremont *et al.* 2013; Schikorski *et al.* 2011a). Overall, the amount of viral DNA in dead/moribund oysters was significantly different ($p < 0.05$) from that found in living oysters in all treatments, which confirms that the oysters were infected by the injection of MB-virus conjugates.

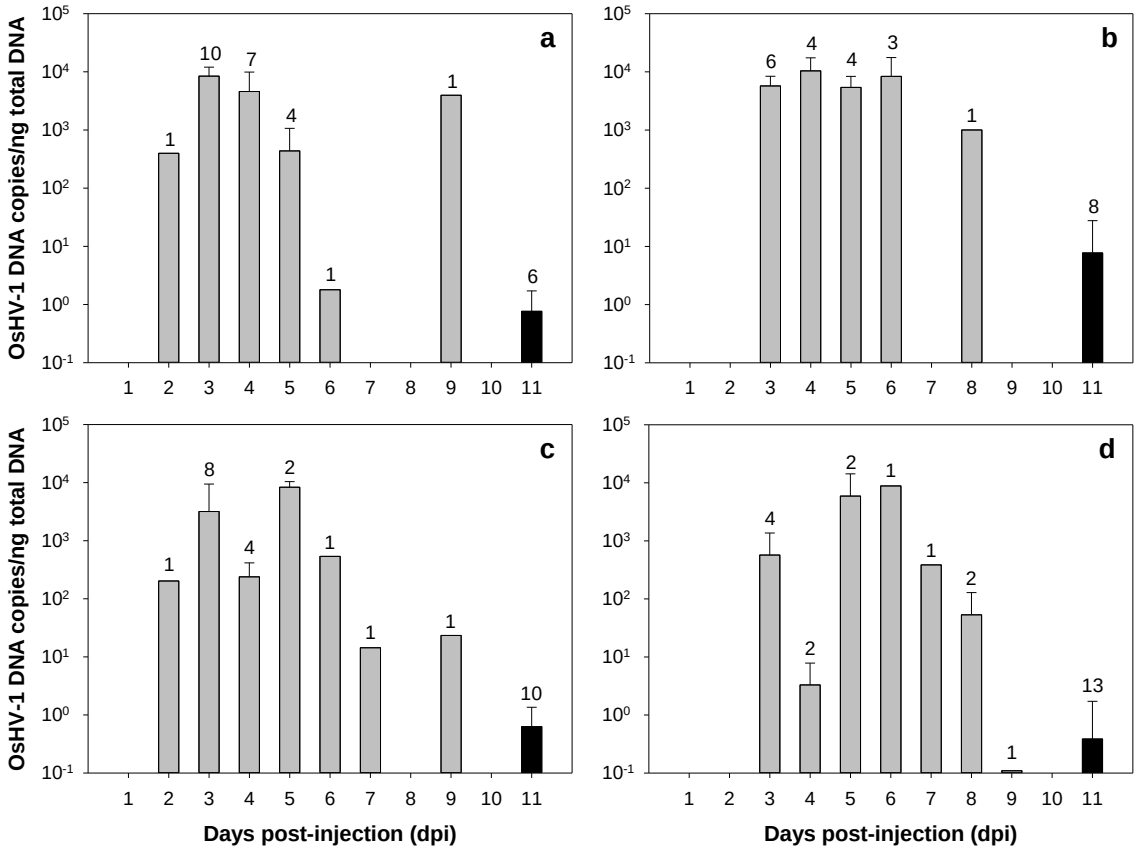

Fig. 5. Viral DNA quantifications by qPCR in dead/moribund (grey bars) and living (black bars) oysters after intramuscular injection of homogenate (**a**), seawater (**b**), MB-virus conjugates from homogenate (**c**) and MB-virus conjugates from seawater (**d**). Results (mean ± SD) are expressed as OsHV-1 DNA copies/ng of total DNA. The number above each bar represents the number of oysters analysed.

3.3.3. OsHV-1 gene expression

The 3 viral ORFs (ORF4, ORF16 and ORF42) selected to study virus gene expression are classified as early genes (expressed before 2h post-injection) and encode proteins with different functions/structures: ORF4 (unknown protein), ORF16 (putative membrane protein) and ORF 42 (putative apoptosis protein). These belongs to different groups/families of genes and were previously studied by Segarra *et al*. (2014). The oyster host EF1 gene was selected as an endogenous control because it has been frequently used in several marine species (Araya *et al.* 2008; Martenot *et al.* 2017; Morga *et al.* 2010; Morga *et al.* 2017) and it has been reported to be the most stable housekeeping gene in *C. gigas* oysters when challenged with OsHV-1 (Segarra *et al.* 2014). Moribund animals were collected before mortality occurred to avoid degradation of viral transcripts in dead individuals. Viral RNA expression in living oysters was significantly lower (higher Ct values) ($p < 0.05$) than in moribund oysters for the 3 viral ORFs (**Fig. 6**). These results indicate an active replication of the virus in moribund oysters and confirm that the mortalities occurring during the time course of

the experiment are due to the OsHV-1 infection after injection with MB-virus conjugates.

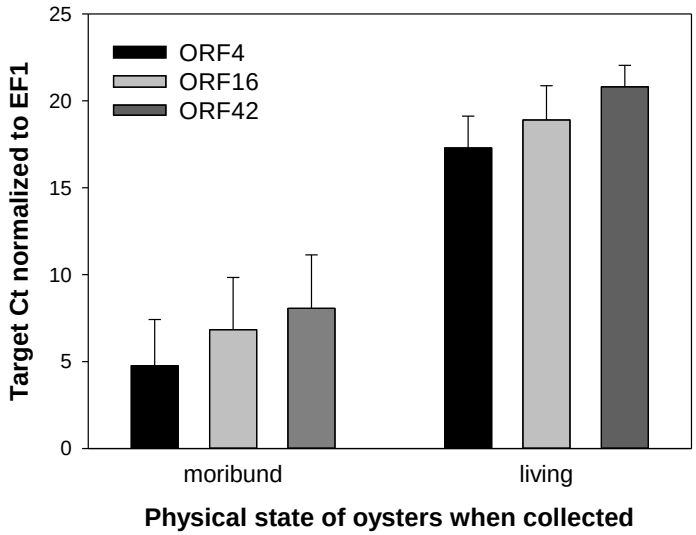

Fig. 6. Relative expression of the 3 viral gene transcripts (ORF4, ORAF16, ORF42) in moribund and living oysters. Ct results (mean ± SD) are normalised to EF1. Lower Ct values correspond to earlier detection (higher expression levels) of each transcript. A total of 12 moribund oysters and 12 living oysters were analysed.

Summarising, the mortality observed in the oysters to which MB-virus conjugates were injected, together with the high viral DNA loads in dead/moribund oysters and the active viral gene transcription in moribund oysters, clearly demonstrate that OsHV-1 was the causative agent of oyster spat mortality and the ability of the MBs to capture viable viral particles.

3.3. MBs as pre-concentrating agents

The utility of MBs as pre-concentrating agents was assessed using the homogenate samples. The volume of MBs was increased (50 µL), the ratio MBs volume/homogenate sample volume was decreased (50 µL/50 mL) and the ratio elution volume/homogenate sample volume was reduced (100 µL/50 mL). MBs conjugates were prepared as described in section 2.3 (the only exception being the above-mentioned modified volumes) using 10-fold dilutions of the homogenate (from un-diluted to 1:100 diluted). Total DNA was extracted from these conjugates and from 100 µL of 10-fold diluted homogenate samples, and subsequently analysed by qPCR for the presence of OsHV-1 DNA as described in section 2.5. As shown in **Fig. 7**, when using the non-diluted homogenate, viral DNA was detected either using MBs prior to qPCR or only qPCR. When the homogenate was diluted 10 and 100 times, detection was only achieved using qPCR in combination with MBs, indicating that MBs were able to pre-concentrate OsHV-1 particles around 100 times under these experimental conditions.

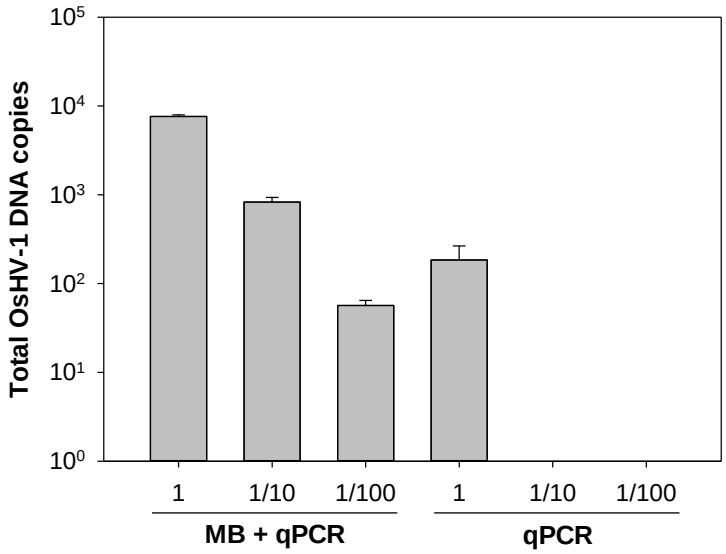

Fig. 7. OsHV-1 DNA detected in 10-fold homogenate dilutions (1 = non-diluted; 1/10 = 10 times diluted; 1/100 = 100 times diluted) using MBs in combination with qPCR or qPCR alone.

4. Conclusions

This study reports the use of anionic polymer-coated MBs to capture viable OsHV-1 from both oyster homogenate and seawater samples for the first time. The absorption of the virus on MBs in conjugates from homogenate and seawater was confirmed by qPCR. Furthermore, the viability of the isolated virus particles was demonstrated through experimental infections of naïve oysters and subsequent mortality monitoring as well as DNA and RNA analysis. Further research is needed to elucidate the role of the MBs in the oyster infection, which would be of great interest for pathogenicity studies.

The isolation of viable OsHV-1 is especially important because no stable bivalve cell lines are available to culture the virus and it can only be obtained from naturally infected material. In this sense, another merit of this study is that MBs can be used successfully for virus collection from samples of a complex nature, such as an oyster tissue homogenate, a semi-viscous matrix with a high protein content. Conventional virus isolation techniques are not straightforward, while the method presented herein is simple and provides minimal sample preparation to obtain useful material. Additionally, the isolation of viruses from seawater is key to understanding mechanisms of horizontal transmission of OsHV-1 (direct host-host transmission, or indirect by way of some other vector).

The use of anionic polymer-coated MBs is a rapid, easy and cost-effective strategy to isolate viable OsHV-1 particles from complex matrices, and could be of great utility in research activities such as studies on genetics, physiology, immunology and pathology.

Additionally, it could provide a valuable tool when integrated with qPCR or other detection techniques, either to enable viable virus detection (provided that only viable particles are captured by the MBs), to enhance sensitivity by previously concentrating virus particles. The assay may contribute to increase the knowledge of the OsHV-1 and limit the impact of OsHV-1 infection in the oyster industry.

Acknowledgements

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CHAPTER 6

General discussion

General discussion

The presence of biological hazards in the marine environment has become a serious concern due to their direct impact on marine life, human health and the economy via fishery closures, water contamination issues and impact on tourism and recreational water use. Nevertheless, the application of monitoring programs can help to prevent and mitigate these impacts. Driven by the need to have alternative methods to current light microscopy and conventional molecular methods, the development of new, rapid, reliable, user-friendly and *in situ* analysis tools is of immense interest in environmental research.

In this thesis, DNA-based assays and biosensors including qPCR assays, colorimetric assays, electrochemical biosensors and MB-based capture strategies were developed for the detection of toxic marine microalgae and/or viruses. Moreover, the applicability of such tools to the analysis of contaminated samples was demonstrated. The application/implementation of bioanalytical tools requires other steps besides amplification and detection, such as sampling, sample pre-treatment and DNA extraction, which can compromise the rapidity and simplicity of the overall analysis process. Accordingly, a part of this thesis also focused on developing novel approaches to improve sample pre-treatment and DNA extraction steps. Even though the improvement of the sampling step was not addressed, some challenges were identified, such as the lack of a standardised sampling methodology for benthic microalgae, which is currently approached by the use of artificial substrates (Jauzein *et al.* 2018).

The present section does not aim to provide a list of insights based on independent chapters, but a common discussion of the findings achieved in this thesis due to the close relation among them, together with future work and challenges that still need to be tackled. Thus, findings achieved in sample pre-treatment, DNA extraction, amplification, detection and application are discussed.

Sample pre-treatment is crucial when dealing with environmental samples such as seawater, since microalgae and viruses are normally present in the sample in a very low number. Centrifugation was successfully applied in Chapters 3 and 4 to concentrate microalgae from 50-mL environmental samples. The volume of water chosen (from millilitres to several litres) should be perceived as a compromise. On one hand, the more sample volume used, the higher the probability to detect the target organism at the given limit of detection (LOD) of the method. On the other hand, using a large volume of sample inherently adds time and complexity to the analysis. In contrast to microalgae, pre-concentrating viruses is more difficult and it usually

requires ultracentrifugation technology, which increases the time and cost of sample processing. In Chapter 5B, magnetic beads (MBs) coated with an anionic polymer were successfully used to capture ostreid herpesvirus-1 (OsHV-1) from both infected seawater and oyster tissue homogenates. Additionally, they were able to pre-concentrate viral particles around 100 times. Although in this thesis the use of MBs as pre-concentrating agents was specifically demonstrated using the homogenate, they could certainly be applied to seawater samples. The use of MBs in combination with qPCR to detect OsHV-1 in aquaculture facilities would be of great interest.

Extraction of nucleic acids from environmental samples is another bottleneck in rapid and *in situ* molecular diagnostics. In fact, both traditional standard procedures and commercial kits are highly dependent on laboratory infrastructure (e.g. centrifuges and fume hood) and take several hours. To address these challenges, a novel method was proposed to extract DNA from microalgae in Chapter 3A. This method uses a syringe that is passed through five different buffers, and offers several advantages: (i) simplicity (it requires few steps), (ii) versatility (it was successfully applied to three microalgae representing different cell morphology and structural composition), and (iii) rapidity (it takes less than 4 min). The incorporation of a mechanical disruption step using a lab-bench bead beater notably improved the DNA yield for the three microalgae tested. Since small hand-held versions of bead beaters do exist, their combination with Biomeme would allow the rapid and simple DNA extraction in the field.

Amplification of the target DNA is also critical, since current detection techniques are not sensitive enough to detect low copy numbers of the target sequence. Even though the amplification step can be avoided when targeting ribosomal RNA genes (Orozco and Medlin 2013), such a strategy is more complex and inaccurate, and has not been addressed in this thesis. In Chapter 3A, a qPCR assay was developed for the identification, discrimination and quantification of two *Karlodinium* species, *K. armiger* and *K. veneficum*, which cannot be differentiated using light microscopy. The developed assay is well suited to improve monitoring and/or research activities. As an example of the latter, the application of an established qPCR assay to distinguish between *O*. cf. *ovata* and *O*. cf. *siamensis* was extremely useful for the preliminary work on Chapter 4A in order to identify different *Ostreopsis* species from a wide range of monoclonal cultures, without the need to sequence each of them.

Despite these advantages, qPCR is quite costly and requires thermocycling, which renders it unsuitable for miniaturisation and use at point-of-need. Accordingly, a substantial part of this thesis describes assays and biosensors based on isothermal recombinase polymerase amplification (RPA), which can circumvent qPCR's drawbacks. In particular, colorimetric assays to detect two *Karlodinium* species (i.e. *K*.

veneficum and *K*. *armiger*, Chapter 3B) and two *Ostreopsis* species (i.e. *O*. cf. *ovata* and *O*. cf. *siamensis*, Chapter 4A) as well as electrochemical biosensors to detect *O*. cf *ovata* (Chapter 4B) and OsHV-1 (Chapter 5A) were developed exploiting RPA.

Primer design is a key factor in RPA reaction. There are several reports describing that PCR primers can also be used in RPA (Yamanaka *et al.* 2017), which was also demonstrated in this thesis for *Karlodinium*. However, species-specific PCR primers for *O*. cf. *ovata* and *O*. cf. *siamensis* were observed not to be suitable for RPA. By increasing both the length and GC content of the primers, a 100-fold improvement of the LOD was achieved. The need for longer primers in RPA may be explained by the different mechanisms for amplification: thermal *versus* isothermal for PCR and RPA, respectively. Although some recommendations to design useful primers for RPA exist (primers of 30-35 bp in length with a GC content between 40-60% that amplify targets between 100 and 200 bp) (Appendix), more research would be needed in this direction to make RPA a more robust technique. On the other hand, in this thesis, primers were specifically designed to minimise the number of required primers. For example, instead of using four primers to detect two *Karlodinium* or *Ostreopsis* species, three primers were designed: two species-specific primers and one genus-specific primer. Although amplifications were performed in parallel for each target, such a strategy could eventually facilitate multiplex amplification.

Experimental RPA conditions including temperature, time and reagent concentration (i.e. primers, enzyme pellet and dehydration buffer) were optimised for *Karlodinium* and further maintained for the other targets, with the only exception being the primer concentration. The requirement for a purification step following amplification was evaluated for *Ostreopsis* and OsHV-1. It was demonstrated that the presence of un-reacted primers interferes with the subsequent detection when the purification step is avoided. Nevertheless, by adjusting the primer concentration, direct analysis of the RPA product was achieved, thus simplifying the assay. Unfortunately, such an adjustment depends on the set of primers used and therefore, ideally, it should be optimised for each target.

Colorimetric assays and electrochemical biosensors to **detect** amplified RPA products were based on a sandwich hybridisation format. This was achieved by modifying primers with a stopper and a tail, thus resulting in double-stranded product flanked by single-stranded tails, which can be easily detected using an immobilised capture probe and a labelled reporter probe (O'Sullivan *et al.* 2013). This strategy allows the avoidance of any post-processing of the amplified product prior to its detection. Colorimetric assays are usually performed in standard microtitre plates allowing simple and high-throughput analysis. Generally, colorimetric assays are presented as a previous step for the subsequent development of electrochemical biosensors.

Nevertheless, as clearly demonstrated in this thesis for *Karlodinium* and *Ostreopsis*, they can constitute a powerful analytical tool by themselves. DNA-based electrochemical biosensors have garnered broad interest in the environmental field since they combine inherent DNA specificity with the advantages of electrochemical detection including high sensitivity, low cost, possibility of miniaturisation and performing *in situ* analysis. Herein, electrochemical biosensors for the detection of *O.* cf*. ovata* and OsHV-1 have been developed. Although the "tailed primer concept" has been used in colorimetric assays and electrochemical biosensors for *Karlodinium*, *Ostreopsis* and OsHV-1, it is a versatile approach that opens up the possibility to be easily adaptable to many other targets, to be exploited with other detection systems (e.g. lateral flow assays) and to be formatted in a multiplex configuration.

Detection by colorimetry or electrochemistry basically involves the same steps (i.e. immobilisation of the capture probe, blockings, hybridisation of the amplified product, hybridisation of the reporter probe and substrate addition) but, whilst in colorimetry the capture probe is usually immobilised on a microtitre plate and the absorbance is read, in electrochemistry the capture probe is immobilised on an electrode and the reduction current is measured. As an example, the capture probe was immobilised on a miniaturised thin-film gold electrode with a diameter size of 1 mm for the detection of OsHV-1. Alternatively, as demonstrated for *O*. cf. *ovata*, capture probes can be immobilised on MBs, which are subsequently placed on a magnetised screen-printed carbon electrode. In this case, the use of MBs as immobilisation supports provided advantages in terms of improved washing steps, easy handling and possibility to regenerate the electrode surface. Additional well-known advantages include the large surface area available for immobilisation, improved assay kinetics and reduction of matrix effects. Moreover, the transition from MB-based colorimetric assays to MB-electrochemical biosensors is straightforward and only requires the immobilisation of the functionalised MBs on magnetised electrodes and electrochemical measurement. Despite the multiple benefits, mass transfer limitations were observed when anchoring bare MBs on the electrode surface.

The time of each incubation step (i.e. overnight for the capture probe and 30 min for the other steps) was not optimised in this thesis. Nevertheless, it was calculated that capture-probe functionalised MBs and electrodes could be stable at least 3 months, thus providing ready-to-use platforms that enable to shorten the assay time. Therefore, the assay (amplification not included) could be completed in just ~2.5 h instead of 1 day when using already-prepared platforms. Future work may include the reduction of the incubation times to further reduce the assay time.

Specificity and LOD are two major parameters used to assess the performance of an analytical method. The specificity of the developed methods is essentially determined by the primers used in the amplification process, which are designed based on available genomic sequences of the target of interest. Whilst the genome of OsHV-1 is fully available, this is not the case for microalgae. Most abundant sequences for microalgae include ribosomal DNA (rDNA) genes, since they have been widely exploited in taxonomy. Among the different regions of the rDNA, the ITS region was used to design primers for *Karlodinium* and *Ostreopsis* species because of their high variability. However, primer specificity does not only mean the ability to amplify the target analyte, but also the ability to not amplify non-target species. Therefore, primer design also implies comparison with other sequences of species that could coexist with the target in the sample. Given the broad range of microorganisms and high molecular diversity in marine environmental samples, limited sequences of marine microorganisms are currently available, which may hamper the development of reliable primers (and also reliable tails). Next-generation sequencing technologies could contribute to broaden the molecular databases for marine species as well as achieving the entire genome sequencing of microalgae. The latter could eventually facilitate the identification of other genes rather than ribosomal ones that may be useful to target microalgae at species level. Herein, after confirming the specificity of the designed primers *in silico*, the specificity of the whole assay/biosensor (which includes primers specificity) was evaluated using cross-reactivity experiments with species closely related to the target species. Furthermore, the good agreement found with other methods (see below), regardless of the presence of non-target species at high concentrations, was also an indicator of the high specificity of the systems.

Nowadays, most efforts in analytical science are focused on decreasing the LODs of the methods by means of incorporating signal amplification approaches, developing fashionable strategies and/or exploiting nanotechnology. However, it is important to have in mind the final application of the method, and whether achieving very low LODs is of crucial importance. For instance, whilst the detection of a single OsHV-1 particle could represent a hazard to the aquaculture sector, the presence of a single microalgae cell does not pose a risk to humans neither to aquaculture, but its risk is defined by specific alarm thresholds.

Quantitative PCR assays for *Karlodinium*, *Ostreopsis* and OsHV-1 achieved better LODs than the developed colorimetric assays and electrochemical biosensors for the same targets. Nevertheless, these new analytical tools offer other advantages regarding simplicity and possibility of in-field analysis. In addition, as stated above, a higher LOD is not necessarily a limitation. In fact, both colorimetric and electrochemical biosensors developed for *Karlodinium* and *Ostreopsis* attained LODs below the

proposed alarm thresholds: 200,000 cells/L for *Karlodinium* (Fernández-Tejedor *et al.* 2004) and 10,000-30,000 cells/L for *Ostreopsis* (Vassalli *et al.* 2018). From a practical perspective, this is the key point. Consequently, it should be considered if quantitative approaches are required in the analysis of microalgae or if qualitative or semi-quantitative approaches could be also adequate. A different case is OsHV-1. Despite the multiple benefits of the electrochemical biosensor for OsHV-1, its LOD remains higher than qPCR assay. Future work will focus on decreasing this LOD by means of improving both the amplification and the detection steps or, alternatively, using pre-concentrating agents such as MBs.

To determine the LOD of a method, a calibration curve using the target analyte needs to be obtained. Different approaches have been reported in the literature to construct standard curves in molecular methods that, from less to more complex, are: (i) using dilutions of a synthetic target sequence (ssDNA or dsDNA); (ii) using dilutions of genomic DNA extracted from a pool of cells/particles; and (iii) using genomic DNA extracted from dilutions of cells/particles. In this thesis, the three approaches have been used. Although it is easier to work with synthetic DNA, genomic DNA better reflects what happens in nature, as demonstrated for microalgae in Chapter 3B, where the use of synthetic sequences generated an artefact that resulted in non-specific signals. Unfortunately, working with genomic DNA is not always feasible. Whilst genomic DNA from microalgae can be obtained by culturing them, OsHV-1 cannot be cultured due to the lack of adequate bivalve cell lines. Accordingly, electrochemical biosensors for OsHV-1 were developed using short dsDNA target sequences. Obtaining pure genomic OsHV-1 DNA usually involves collecting infected bivalves and subsequent virus purification with high-speed ultracentrifugation. The use of a simple and rapid isolation/purification methods such as magnetic beads (MB) as described in Chapter 5B, would be of great interest to the multidisciplinarian community of researchers studying OsHV-1 (e.g. analytical science, studies on genetics, immunology and pathogenesis), whose studies highly depend on obtaining useful material.

The **application** of the developed tools to the analysis of contaminated samples is essential to demonstrate their usefulness and reliability. Tools developed for microalgae were applied to environmental planktonic, benthic and/or field samples spiked with microalgae cultured in the lab. Regarding OsHV-1 detection, they were applied to seawater, oyster tissue homogenate and/or oyster samples. When developing new analytical methods, results are compared with those obtained by current reference analysis methods or, in their absence, with those currently used, namely: light microscopy for microalgae and qPCR for OsHV-1. A good agreement between techniques implies both a good correlation (evaluated with the Pearson's correlation coefficient: r) and a 1:1 relationship. OsHV-1 DNA concentrations

determined by the electrochemical biosensor and qPCR in the analysis of oyster samples showed an excellent agreement (r = 0.988). However, it is important to highlight that some samples that gave a negative result using the biosensor, were deemed positive using qPCR, which can be attributed to the higher LOD of the biosensor.

Obtaining a good agreement between molecular methods and light microscopy when targeting microalgae is much more challenging, since the copy number of rDNA genes per microalgae cell is not stable (Galluzzi *et al.* 2010). In fact, rDNA copy number per cell may vary depending on the species, strains, growth phase, environmental conditions and/or geographical sites. Although the variation of rDNA copy number has not been studied for *Karlodinium* species, some differences have been reported for *Ostreopsis* depending on the geographical area (e.g. differences between the Spanish and Italian Coasts) (Vassalli *et al.* 2018). Therefore, this problematic does not arise, obviously, from a limitation of the molecular methods themselves.

In Chapter 3A, calibration curves based on cultured cells were used to calculate cell abundances. Although *Karlodinium* cell abundances obtained with qPCR were 2.8-fold higher than microscopy counts, a good correlation (r = 0.876) was observed, which indicated the ability of the qPCR to quantify *Karlodinium* species in environmental samples. This 2.8-fold difference may be explained by the variation of target copy number between *Karlodinium* cells in the environmental samples analysed and those in cultures, which were used to develop the method and construct the calibration curves. In contrast, in Chapter 3B, a good agreement between the colorimetric assay and light microcopy was observed for *Karlodinium* abundances, which make sense since, instead of environmental samples, spiked samples containing cultured *Karlodinium* cells were analysed, potentially reducing the variation in target copy number.

To increase the reliability of the molecular methods when analysing environmental samples, the use of site-specific environmental calibration curves has been described (Casabianca *et al.* 2014; Perini *et al.* 2011). However, this strategy is time-consuming and not useful when a rapid response is required. In Chapter 4, genomic DNA from cultures was used to construct the calibration curves. A predictive model to estimate *Ostreopsis* cell abundances in environmental planktonic and benthic samples was constructed based on the relationship between *Ostreopsis* DNA quantifications and *Ostreopsis* counted by light microscopy, thus allowing reduction in the variation in target copy number between cultured and environmental cells. An excellent agreement was obtained between the colorimetric assay (r for planktonic samples = 0.96; r for benthic samples = 0.97) as well as the electrochemical biosensor (r for planktonic samples = 0.932; r for benthic samples = 0.975) with respect to light

microscopy. This strong agreement demonstrated the low differences in *Ostreopsis* rDNA copy among environmental samples obtained from the Catalan coast and that, besides dilutions of cells (which involves counting and performing several DNA extractions, thus being complicated and time-consuming), the use of genomic DNA from a pool of cells is also a valid approach to construct calibration curves. Despite the good agreement achieved, the analysis of an increased number of environmental samples, also including other geographical sites, should be carried out to validate the robustness of these tools and implement them in monitoring programs. Furthermore, whilst current thresholds for microalgae are expressed in cell abundances, it should be considered whether it is necessary to quantify cells rather than DNA using molecular methods. Perhaps DNA can provide additional and/or more convenient information than cell abundances.

Current techniques to monitor marine microalgae and viruses (i.e. light microscopy and qPCR) require samples to be sent to centralised laboratories, resulting in a considerable time lag between sample collection and data generation/availability, which may hamper the application of adequate mitigation actions. To address this limitation, considerable work is being directed at developing field-deployable technologies (Doucette and Kudela 2017; Scholin et al. 2017). Remote sensing *via* satellite and airborne technologies, although unable to discriminate microalgae at species level, is used to study and predict the distribution of high-biomass microalgae blooms by detecting chlorophyll. Fluorescent microscopes and flow cytometers have already been integrated into autonomous underwater platforms. Nevertheless, a certain level of taxonomic expertise is still required to accurate identify microalgae using these technologies. Similarly, molecular-based methods have been coupled with autonomous underwater platforms. This is the case of the Environmental Sample Processor (ESP), which combines a fluorescence-based molecular assay with a mooring system (2nd generation ESP) or an underwater vehicle (3rd generation ESP). Ideally, for a DNA-based method to be field-deployable, it needs to be simple and energy-efficient. The use of isothermal amplification and electrochemical biosensors presented in this thesis could reduce power consumption and simplify the required detection instrumentation, facilitating their integration into hand-held battery-operated portable devices. These smaller instruments are also more amenable to be incorporated in autonomous in-water platforms including fixed-location (moorings and buoys) and mobile platforms (buoyancy gliders, wave gliders and underwater autonomous vehicles). These platforms are able to acquire water samples, conduct sample pre-treatment and apply the molecular analytical technology, without any operator intervention.

I expect that the diverse DNA-based methods presented throughout this thesis can help to improve both research and monitoring activities in the environmental field. Although further validation and inter-laboratory studies should be performed to implement these assays and biosensors in monitoring programs, I believe that they could be highly useful as screening tools that complement current methods. Thus, a faster and more cost-effective system to protect shellfish harvesting and bathing areas could be achieved. Furthermore, this thesis also contributed to the identification and description of issues (e.g. variation in DNA copy number) that are currently hindering the implementation of molecular tools, whilst also describing the potential of the application of these tools directly in the field by exploiting isothermal amplification methods. With the advances in miniaturisation and microfluidics, these DNA-based tools have the potential to be integrated in fully automated systems capable of performing *in situ* analysis. Overall, this thesis demonstrates the opportunities that nucleic acids offer to environmental analytical chemistry, and will serve to guide future research in this field.

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CHAPTER 7

Conclusions

Conclusions

The findings achieved in this thesis lead to draw the following conclusions:

- Different tools based on DNA including qPCR, colorimetric assays, electrochemical biosensors and magnetic bead (MB)-based capture strategies have been developed to detect *Karlodinium*, *Ostreopsis* and ostreid herpesvirus-1 (OsHV-1), and successfully applied to the analysis of environmental samples (seawater and oyster samples).
- A rapid and simple method to extract DNA has been applied to different microalgae species, and successfully coupled with qPCR for the quantitative analysis of *K. veneficum* and *K. armiger.*
- The use of isothermal recombinase polymerase amplification (RPA) with tailed primers, followed by a sandwich hybridisation assay, has been demonstrated to be useful for the development of both colorimetric assays and electrochemical biosensors.
- Primer design has been demonstrated to be a key factor for RPA performance. Furthermore, the optimisation of primer concentration in RPA enabled the avoidance of a purification step of amplified products prior to their detection.
- The design of species-specific primer sets for two *Karlodinium* and two *Ostreopsis* species facilitated the development of dual qPCR and colorimetric systems to detect, quantify and discriminate between *K. veneficum* and *K. armiger* as well as *O*. cf. *ovata* and *O*. cf. *siamensis*.
- The use of MBs as immobilisation supports was shown to be useful for the development of an electrochemical biosensor for *O*. cf. *ovata*, offering advantages in terms of improved washing steps, ease of handling and regeneration of electrodes.
- The developed methods showed enough specificity to be applied in surveillance activities. Additionally, qPCR, colorimetric assays and electrochemical biosensors developed for *Karlodinium* and *Ostreopsis* achieved LODs below the proposed current thresholds.
- The use of genomic DNA demonstrated to perform better than synthetic DNA. In particular, both the use of genomic DNA coming from a pool of cells or from cell dilutions proved useful to construct calibration curves.

- DNA quantifications provided by the developed molecular tools and qPCR were in good agreement. A high degree of correlation between cell abundances provided by the developed molecular methods and microscopy was obtained. In addition, the construction of a predictive regression model allowed the estimation of cell abundances from DNA quantifications.
- MBs coated with an anionic polymer have been successfully used to capture viable OsHV-1 form both seawater and oyster tissue homogenate samples, and to concentrate OsHV-1 from the homogenate.

Future work:

- To apply the DNA-based methods to a higher number of samples, also coming from different geographical areas, to further validate these new approaches.
- To develop multiplex systems ("one-pot" reaction) for the dual qPCR and colorimetric assays by mixing the primers in the same amplification reaction and using different labels/physical separation for the sequent detection.
- To decrease the LODs of the electrochemical biosensor for OsHV-1 by improving the amplification and the detection steps and/or using pre-concentrating agents such as MBs.
- To use MBs in combination with qPCR to capture and detect OsHV-1 in seawater samples from aquaculture facilities.

Potential applications:

- The use of MBs to obtain purified and viable OsHV-1 for research activities.
- The implementation of the developed bioanalytical tools, following proper validation and inter-laboratory studies, in environmental monitoring activities.
- The integration of the developed methods into microfluidics platforms and easy-to-handle compact devices for their deployment into the market.

ANNEXES

Biological hazards such as virus and microalgae presently represent a clear threat to marine life and human health. Consequently, the development of new detection tools is of current interest in environmental research. This thesis aims to contribute to the development and applicability of DNA-based assays and biosensors for the detection of toxic marine microalgae and viruses, as well as to other steps in the environmental analysis process, such as sample pre-treatment and DNA extraction. These new DNA-based approaches include qPCR assays, colorimetric assays, electrochemical biosensors and magnetic bead-based capture strategies, which will pave the way towards the implementation of rapid, specific, easy-to-use, low-cost and *in situ* analysis tools to improve research, monitoring and management of marine biological hazards.

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