This document is a postprint version of an article published in Science of The Total Environment© Elsevier after peer review. To access the final edited and published work see https://doi.org/10.1016/j.scitotenv.2020.141605

Document downloaded from:

Detecting harmful algal blooms with nucleic acid amplification-based biotechnological tools

Anna Toldrà[a,b*], Ciara K. O'Sullivan[c,d], Jorge Diogène[a], Mònica Campàs[a]

[a]IRTA, Ctra. Poble Nou km 5.5, 43540 Sant Carles de la Ràpita, Tarragona, Spain

[b]Department of Fibre and Polymer Technology, KTH Royal Institute of Technology, 100 44 Stockholm, Sweden

[c]Departament d'Enginyeria Química, Universitat Rovira i Virgili, Av. Països Catalans 26, 43007 Tarragona, Spain

[d]Institució Catalana de Recerca i Estudis Avançats (ICREA), Pg. Lluís Companys 23, 08010 Barcelona, Spain

*corresponding author: anna.toldra@irta.cat / anntf@kth.se

Abstract:

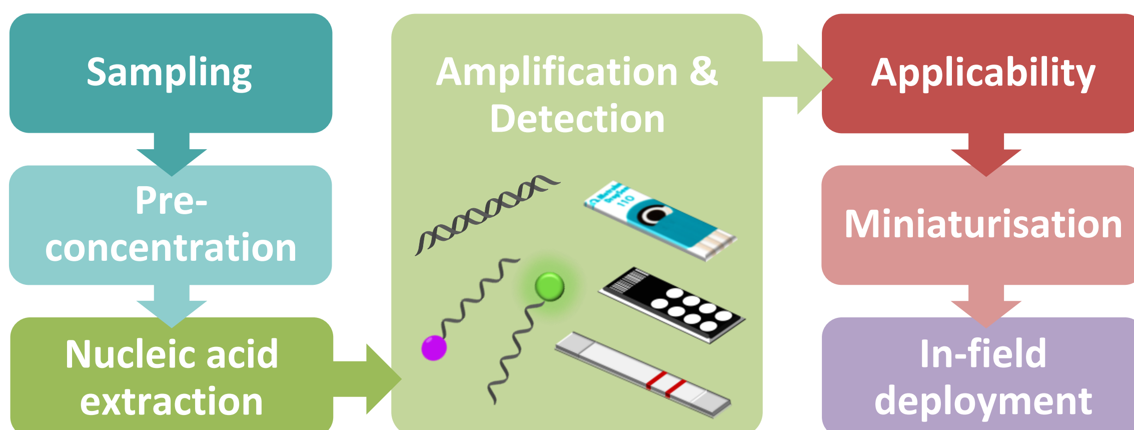
Harmful algal blooms (HABs) represent a growing threat to aquatic ecosystems and humans. Effective HAB management and mitigation efforts strongly rely on the availability of timely and *in-situ* tools for the detection of microalgae. In this sense, nucleic acid-based (molecular) methods are being considered for the unequivocal identification of microalgae as an attractive alternative to the currently used time-consuming and laboratory-based light microscopy techniques. This review provides an overview of the progress made on new molecular biotechnological tools for microalgal detection, particularly focusing on those that combine a nucleic acid (DNA or RNA) amplification step with detection. Different types of amplification processes (thermal and isothermal) and detection formats (e.g. microarrays, biosensors, lateral flows) are presented, and a comprehensive overview of their advantages and limitations is provided Although isothermal techniques are an attractive alternative to thermal amplification to reach *in-situ* analysis, further development is still required. Finally, current challenges, critical steps and future directions of the whole analysis process (from sample procurement to *in-situ* implementation) are described.

Keywords: harmful algal blooms (HABs); microalgae; molecular method; isothermal DNA amplification; *in-situ* HAB monitoring.

Highlights:

- A description of PCR and isothermal techniques to amplify microalgal DNA/RNA is provided
- Isothermal techniques are being used but major development is still required
- The critical steps to achieve true implementation of the molecular tools are considered
- Application of these new sensing tools is possible for an unlimited number of microalgal species

Graphical abstract:



1. Why molecular methods for HABs detection?

Microalgae are essential members of aquatic communities, since they constitute the base of the food web by fixing carbon and producing oxygen. However, under certain circumstances, some microalgae species can proliferate and cause negative impacts on the ecosystems, human health and/or socioeconomic related activities (e.g. aquaculture and recreational activities) (Glibert et al., 2018). These events are referred to as harmful algal blooms (HABs). Although HABs can also originate from other organisms such as macroalgae and cyanobacteria affecting marine and freshwater habitats, this review specifically focuses on marine microalgae due to their major occurrence and relevance.

Although difficult to classify, harmful microalgae can be largely divided into those that do and do not produce toxins (Table 1) (Anderson et al., 2012; Glibert et al., 2018) . The first group includes microalgae that, after reaching high abundances, cause mortality of fish and invertebrates through oxygen depletion as well as microalgae affecting fish by mechanically damaging their gills. The second group comprises ichthyotoxic microalgae that produce haemolytic toxins or oxidising substances that can kill fish. It also includes microalgae that produce toxins that bioaccumulate in fish or shellfish causing foodborne diseases in humans (and eventually affecting marine birds and marine mammals too), as well as microalgal species associated with respiratory and skin irritations.

Monitoring the presence of HAB species is therefore of utmost importance to understand, prevent, manage and mitigate their negative impacts. There are many well established monitoring programs, which periodically take seawater samples to screen for HAB microalgae in shellfish harvesting areas and, to a lesser extent, in specific fishing areas. Given the increase in frequency, magnitud and geographical distribution of HABs over the two past decades (Anderson et al., 2012; Glibert et al., 2018), such monitoring programs are becoming more and more necessary, not only to prevent cases of seafood poisoning, but also to protect ecosystems and human health. Light microscopy observation using the Utermöhl cell counting method (Utermöhl, 1958) is the standard and most frequently used method to identify microalgae in water samples. A clear advantage of this method is the possibility to perform quantitative analysis and to pre-concentrate the samples based on the sedimentation of an aliquot of a known volume. Although currently used, 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 microalgae at the species or even genus level. Eventually, this may hamper the implementation of appropriate preventive measures and to react in a timely and effective manner against the possible presence of risk situations. Molecular (nucleic acid-based) methods appear well suited as alternative or complementary tools to traditional microscopy techniques because: 1) they are more specific, allowing the correct identification of similar species or genera, and being in some cases fundamental, since species identification is not possible with optical microscopy observation; 2) they may be faster, especially when

dealing with numerous samples, which facilitates their use as early warning tools; 3) fewer hours of training are required to attain a level of expertise sufficient for routine laboratory screening; and 4) they are more compatible for integration into small, low-cost and portable devices to perform analysis in the field which, although potentially feasible, is more difficult to envisage for microscopy (Antonella and Luca, 2013).

Whilst most molecular methods are developed for medical diagnostics, in recent decades, they have been tested, modified and refined for their application in environmental monitoring. Among them, traditional molecular methods including standard PCR (Litaker et al., 2003; Penna and Magnani, 1999), PCR with restriction fragment length polymorphism (RFLP) analysis (Adachi et al., 1994; Lyu et al., 2017), fluorescence *in-situ* hybridisation (FISH) (Miller and Scholin, 2000; Simon et al., 2000) and real-time quantitative (qPCR) (Galluzzi et al., 2004; Nishimura et al., 2016; Toldrà et al., 2018b; Zhang et al., 2016) have been widely used for the detection of microalgae. However, all these methods still suffer from limited rapidity and portability. This review provides a comprehensive overview of the advances achieved in the development of new molecular biotechnological tools for microalgal detection, especially focusing on those that combine a nucleic acid amplification process together with different detection strategies (e.g. microarrays, biosensors, lateral flows), which currently constitute an important emerging field of research.

2. Amplification-based biotechnological tools for HABs detection

Molecular tools are based on the hybridisation between a nucleic acid target (DNA or RNA) and its complementary primer/probe, which is present in solution and/or on a solid support. Molecular tools for HABs detection can be divided into two groups: amplification-based strategies (which target DNA genes, usually present at a low copy number) and amplification-free strategies (which target ribosomal RNA genes, naturally present at a high copy number). Although some RNA-based biotechnological tools have been developed for the detection of HABs (Anderson et al., 2006; Diercks-Horn et al., 2011; Greenfield et al., 2006; Orozco et al., 2016), RNA is inherently unstable (i.e. it is susceptible to ribonucleases), which may compromise the reliability of these assays. Therefore, the vast majority of biotechnological tools for HABs detection developed in recent years exploit DNA genes and include a DNA amplification step prior detection. Nevertheless, some examples of molecular methods that amplify RNA have been reported, which are also described in the present work.

PCR, the gold standard amplification technique, has been extensively used for microalgal detection. However, PCR-based tools require a power supply with precise temperature control, which may hinder its incorporation into miniaturised devices for *in-situ* testing. An alternative approach to avoid the need for thermal cycling is the use of isothermal amplification methods, which do not require specialised and expensive equipment. 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). Although the application of isothermal amplification techniques to detect microalgae is still in its infancy, some of them have already been reported in the literature. This section describes the existing amplification-based biotechnological tools developed for microalgal detection according to the amplification technique used, with a summary of these tools provided in Figure 1 and Table 2.

2.1. PCR-based tools

New PCR-based approaches have been described in recent years, with innovations in the detection strategy and/or the PCR technology itself. In these assays, amplification is usually performed in solution, followed by optical/electrochemical detection of the amplified product. In PCR, exponential amplification of the target occurs when the double-stranded DNA (dsDNA) target is thermally denatured into single-stranded DNA (ssDNA), allowing primers to bind to their complementary strands and facilitating DNA polymerase-mediated extension (Figure 1a).

The first PCR-based tool described for microalgae exploited a microarray format (Ki and Han, 2006). Although DNA microarrays were originally designed for gene expression analysis (Schena et al., 1995), they have been

increasingly used for pathogen detection due to their high-throughput and multiplexing capacity. Amplification-based multiplexed systems are generally divided into: "one-spot" (which amplify multiple targets in a single reaction, either using several species-specific primers or a set of universal primers) and "parallelised" amplification systems (which amplify multiple targets in single parallelised reactions) (Mayboroda et al., 2018). DNA microarrays consist in many species-specific oligonucleotide capture probes immobilised on defined spots of a solid support. After "one-spot" PCR using a set of universal primers, the fluorescent dye-labelled PCR product is denatured by heating to produce ssDNA, then hybridised with the capture probe and finally detected using a fluorescence scanner (Figure 1b). Therefore, in contrast to most molecular systems, the specificity of microarrays is not determined by the primers but by the capture probes. The feasibility of using microarray technology to detect microalgae has been reported in some publications (Galluzzi et al., 2011; Gescher et al., 2008; Ki and Han, 2006; Noyer et al., 2015), which differ according to the fluorescent label used, the type of glass surface modification (e.g. epoxy, amino, streptavidin) and the target detected. Variations of the classical planar surface microarrays have also been described such as the use of bead arrays, which use specific capture probes attached to multiple color-coded fluorescent microspheres and a Luminex flow cytometer detection system (Diaz et al., 2010; Scorzetti et al., 2009). Microarray and bead array technologies have been used to detect multiple microalgal species (from 2 to 23) simultaneously, including species of the genera *Alexandrium*, *Chatonella, Heterosigma, Karenia* and *Prorocentrum*, amongst others.

An alternative to fluorescence is colorimetry, such as the case of dot blots reported for the detection of 10 species of the genus *Pseudo-nitzschia*, in which the PCR products are spotted onto positively charged nylon membranes, incubated with digoxigenin (DIG)-labelled specific reporter probes and finally with anti-DIG antibody conjugated with alkaline phosphatase (ALP) (Barra et al., 2014). Alternatively, in a reverse dot blot, the probe is spotted onto the membrane, followed by incubation with DIG-labelled PCR products (Chen et al., 2015b; Zhang et al., 2014a). More recently, a lateral flow strip test has also been reported for the detection of 5 *Alexandrium* species (Nagai et al., 2016). With this method, different capture probes are immobilised on a nitrocellulose membrane. After "one-spot" PCR using species-specific primers, the amplified product migrates through the membrane *via* capillary action, and detection is achieved using a colloidal gold-labelled oligonucleotide. Unlike fluorescence DNA microarrays, results obtained with colorimetric DNA arrays can be visualised by naked eye without the need for any special instrument.

Electrochemical detection has attracted particular attention due to its high sensitivity, short measurement times, simple and inexpensive instrumentation, possibility of miniaturisation and compatibility with microfluidic systems (Ronkainen et al., 2010). However, there are very few reports describing electrochemical amplification-based biosensors for microalgal detection. LaGier and coworkers (LaGier et al., 2007) reported

an electrochemical biosensor for the detection of *Karenia brevis*, where the PCR product was labelled with biotin and then immobilised on neutravidin-coated carbon electrodes. Following product denaturation and addition of a fluorescein (FITC)-labelled reporter probe, amplicons are detected by amperometry after incubation with an anti-FITC antibody conjugated with horseradish peroxidase (HRP). This approach was successfully applied to the semi-quantitative analysis of *K. brevis* cells in environmental samples. To avoid the need to generate ssDNA and also the use of labels that require to be added in a subsequent step, Magriñá and collaborators (Magriñá et al., 2019) used primers with ferrocene-labelled dATP to develop an electrochemical genosensor for the detection of *Karlodinium armiger*. The redox-labelled PCR amplicons are hybridised with a thiolated capture probe immobilised on a gold electrode array and detected using square wave voltammetry. This biosensor has been successfully used to quantitatively detect *K. armiger* target copies in spiked seawater samples.

Advances in PCR technology have also been applied to the detection of microalgae. For instance, a device based on solid phase PCR (SP-PCR) has been reported for the detection of *Cochlodinium polykrikoides* and 2 other aquatic pathogens (i.e. *Salmonella* spp. and *Staphylococcus aureus*), allowing amplification followed by immediate detection of DNA (Kon et al., 2016). During SP-PCR, the immobilised forward primers and solution-phase fluorescent dye-labelled reverse primers cooperate to generate PCR products, which remain covalently attached to the device for fluorescence detection. Although this technology is very innovative and promising, it has not been yet applied to the *in-situ* analysis of real samples. An alternative approach exploits insulated isothermal PCR (iiPCR) with fluorescence readout, recently developed for *Alexandrium tamiyavanichii* (Pham et al., 2018). The portable iiPCR device heats the reaction tube from the bottom, creating a temperature gradient inside the column of the reaction tube that drives the fluid convection and the reaction components, PCR cycles being therefore driven by thermal convection.

2.2. LAMP-based tools

LAMP is the most exploited isothermal technique, representing approximately half of the publications describing isothermal methods for microalgal detection. LAMP was first proposed in 2000 by Notomi and co-workers (Notomi et al., 2000) and it takes advantage of the strand-displacing *Bacillus stearothermophilus* (*Bst*) DNA polymerase and 4 primers (2 inner and 2 outer) that recognise 6 distinct regions in the target DNA. The amplification is performed at a constant temperature between 60 and 65 °C in a reasonably short time (60 min). To increase the amplification efficiency, some improvements in the amplification process have been reported, such as: the use of an additional set of 2 loop primers (Huang et al., 2020; Huang et al., 2017a; Huang et al., 2017b; Nagai and Itakura, 2012; Trinh and Lee, 2018), the incorporation of an incubation step at 95 °C before amplification (e.g. Qin et al., 2019; Wang et al., 2019) and/or at 80 °C after amplification (e.g. Zhang et al., 2009; Zhang et al., 2014b) during few minutes, as well as the use of rRNA instead of rDNA, which

unfortunately implies an additional reverse-transcriptase step, even longer that the amplification itself (Chen et al., 2013). The resulting LAMP products are a mixture of oligonucleotide sequences of different lengths, several target inverted repeats and multiple loops (Figure 1c). These products can be detected by observing a white amplification by-product (magnesium pyrophosphate) or by colour change upon addition of a DNA intercalating fluorescent dye, as reported for the detection of *Alexandrium* (Wang et al., 2008), *Karenia* (Zhang et al., 2009) and *Prorocentrum* (Chen et al., 2013; Zhang et al., 2014b) species. However, these strategies may generate false positives because they cannot distinguish between specific and non-specific amplification products such as primer dimers. An alternative strategy to detect LAMP products relies on lateral flow assays, which have been described for species of the genera *Alexandrium* (Wang et al., 2020), *Amphidinium* (Wang et al., 2019), *Chattonella* (Qin et al., 2019), *Karenia* (Huang et al., 2020), *Karlodinium* (Huang et al., 2017a), *Prymnesium* (Zhu et al., 2019) and *Skeletonema* (Huang et al., 2017b). In these systems, the use of a biotin-labelled primer results in biotinylated LAMP products that hybridise with a FITC-labelled reporter probe, and the addition of gold-labelled anti-FITC antibodies generates the characteristic red band (Figure 1d). In general, LAMP strategies achieve around 10 to 100-fold improvement in limits of detection (LODs, usually calculated as the blank plus three times its standard deviation) as compared to standard PCR. Some of the described LAMP strategies were able to detect between 2 and 5 cells per reaction and, although not all strategies were applied to the analysis of environmental and/or spiked samples, the lowest effective LOD achieved was 100 cells/L of the target microalgae.

Due to the requirement of several primers, "one-spot" amplification in LAMP is extremely difficult. For this reason, the only reports describing the detection of two microalgal species (*Alexandrium tamarense* and *Alexandrium catenella* (Nagai and Itakura, 2012) or *A. catenella* and *Alexandrium minutum* (Zhang et al., 2012)) are actually parallelised LAMP assays, in which amplifications are performed separately in single reactions and then detected by turbidity or fluorescence, respectively. Similarly, Trinh and Lee (Trinh and Lee , 2018) reported a microdevice for the parallel amplification of *C. polykrikoides* and 3 other foodborne pathogens (i.e. *Escherichia coli, S. aureus* and *Salmonella* spp.), followed by on-chip fluorescence detection using a DNA-intercalating fluorophore (i.e. fisetin). In this case, amplification and detection are integrated in a plastic microsystem. Papers infused with LAMP reagents and specific primers are embedded inside the multiple reaction chambers of the microdevice. After injecting the sample to the centre of the microdevice, itis divided into several smaller volumes *via* centrifugal force, making possible the detection of multiple pathogens in a parallelised manner. Although the approach looks promising, the prototype still needs to be fully characterised and applied to field samples. **2.3. RCA-based tools**

RCA is another isothermal enzymatic process, which uses a circular DNA template and unique DNA and RNA polymerases (Phi29, *Bst* and Vent *exo*-polymerase for DNA, and T7 RNA polymerase for RNA) with strand-

displacement activity. In the basic RCA reaction, once the primer has annealed to the circular ssDNA, the DNA polymerase synthesises the new strand, eventually completing a loop and reaching the point of initiation. The polymerase then displaces the new strand while initiating an iterative synthesis (Ali et al., 2014; Deng and Gao, 2015; Fire and Xu, 1995). Usually, to increase the amplification power, the RCA product is used as the template for further amplification with a second primer, resulting in hyperbranched RCA (H-RCA) (Zhang et al., 2001). In the case of microalgal detection, due to the lack of a circular template, ligation-HRCA (L-HRCA) is used instead of HRCA. In L-HRCA, a linear ssDNA sequence (padlock probe, PLP) consisting of two terminal fragments complementary to the linear target dsDNA is first used to form a circular DNA template. The two standard primers (derived from the central region of the PLP) are then extended (Figure 1e). Therefore, although L-HRCA is performed at a constant temperature (60-65 °C for a minimum of 15 min) it also requires two preliminary steps: 1) PLP ligation, at 60-95 °C for ~60 min, and 2) exonuclease digestion, at 37-80 °C for ~60 min. Current studies are focused on simplifying such steps, such as performing a double ligation HRCA (dL-HRCA) using two PLP probes, which minimises non-specific self-circularisation and also avoids the exonuclease step. However, this strategy has been observed to compromise sensitivity (Zhang et al., 2019b).

As in LAMP, L-HRCA products have been detected with fluorescent dyes (for *Amphidinium* (Chen et al., 2015b), *Heterosigma* (Zhang et al., 2018b) and *Karenia* (Zhang et al., 2019b) species) and lateral flow formats (for *Karenia* (Zhang et al., 2019a) and *Karlodinium* (Liu et al., 2019) species). Additionally, dot blot (Nie et al., 2017) (Figure 1f) and reverse dot blot (Zhang et al., 2018a) arrays have been developed for the detection of *Chatonella* marina and for the "one-spot" detection of multiple microalgal species, respectively. L-HRCA products need to be denatured prior to their hybridisation with the capture probe (i.e. in the reverse dot blot) or the labelled reporter probe (i.e. in the LD dipstick and dot blot assays). As with LAMP, the described RCA strategies achieve 10 to 100-fold improvement in LODs as compared to standard PCR. All RCA-based tool have been applied to the analysis of environmental and spiked samples, achieving LODs between 1 and 10 cells per reaction and, in some cases, an effective LOD as low as 10 cells/L (Liu et al., 2019) was attained.

2.4. RPA-based tools

Developed by Piepenburg and collaborators in 2006 (Piepenburg et al., 2006), the RPA technology employs a mixture of three proteins to achieve amplification: a T4 uvsX recombinase, a ssDNA-binding (SSB) protein and a strand-displacing *Bsu* DNA polymerase. The process starts when the recombinase binds to the primers forming a recombinase-primer complex. This complex scans the dsDNA target for homologous sequences. When the homologous sequence is found, the recombinase facilitates a strand-displacement process and the formation of a D-loop, thus allowing primers 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 polymerase elongates the primers, achieving an

exponential amplification (Deng and Gao, 2015) (Figure 1g). RPA offers advantages over LAMP or RCA: it operates at a low and constant isothermal temperature (37–42 °C) without an initial DNA melting step, it uses only two primers, and amplification is achieved in 20-40 min. However, optimisation of primers and RPA conditions is required for each target, especially in multiplexed formats.

To avoid the need to denature the RPA-amplified products before their detection, and to decrease assay time and cost, an innovative approach based on the "tailed primer" concept has been proposed. A tailed primer consists of a ssDNA sequence ("tail"), a carbon chain spacer (which prevents the polymerase from further elongation) and the primer (Joda et al., 2015; O'Sullivan et al., 2013). Following amplification, dsDNA products flanked by ssDNA tails are generated, which can be easily detected in a sandwich hybridisation format using oligonucleotide probes complementary to the tails: an immobilised capture probe and an enzyme-labelled reporter probe. RPA with tailed primers has been coupled with an enzyme-linked oligonucleotide assay (ELONA) to detect two different microalgal species (*Karlodinium veneficum* and *K. armiger* (Toldrà et al., 2018c) or *Ostreopsis* cf. *ovata* and *Ostreopsis* cf. *siamensis* (Toldrà et al., 2019a)) in a parallelised format. Following the same strategy, an amperometric biosensor was recently reported for *O*. cf. *ovata*, where the capture probe was immobilised on magnetic beads (MBs) and the MB-complexes were integrated on screen-printed carbon electrode arrays (Toldrà et al., 2019b) (Figure 1h). The biosensor was applied to quantify *O*. cf. *ovata* cells in planktonic and benthic samples, and demonstrated to have an effective LOD of 640 cells/L. Additionally, as with PCR, LAMP and RCA, RPA products have been detected with lateral flow formats (Fu et al., 2019). This strategy enabled the detection of the presence/absence of *K. veneficum* at low concentrations (100 cells/L) in spiked samples, which represents a 100-fold lower LOD than conventional PCR.

2.5. NASBA-based tools

NASBA, first published by Compton in 1991 (Compton, 1991), specifically amplifies ssRNA targets owing to the combination of reverse transcription with the amplification process. Two primers and three enzymes (i.e. reverse transcriptase AMV RT, RNaseH and T7 RNA polymerase) are involved in NASBA, which usually amplifies at 41 °C for 90 min. The process starts when the first primer binds to the ssRNA target and synthesises a complementary DNA strand, producing a DNA-RNA heteroduplex. RNase then hydrolyses the target RNA, leaving ssDNA sequences. Following annealing of the second primer and generation of dsDNA, the RNA polymerase continuously transcribes the complementary RNA strands of this dsDNA template. The newly synthesised ssRNA strands serve as secondary RNA targets, and new and iterative cycles start with this second primer (Deng and Gao, 2015) (Figure 1i). Unlike the afore-mentioned techniques, NASBA amplifies messenger RNA instead of ribosomal DNA. NASBA has been used to detect *K. brevis* by fluorometry using molecular beacons (Casper et al., 2004). The molecular beacon is a single-stranded oligonucleotide labelled with a fluorophore and a quencher at each end. When the beacon is in the closed configuration (hairpin loop), the

fluorophore is quenched. Upon binding to the amplicon, the quencher is separated from the fluorophore and the probe fluoresces (Figure 1j). This strategy has been coupled with a hand-held battery-powered incubator and fluorescence reader (Casper et al., 2007), and successfully integrated in a microfluidic system (Loukas et al., 2017; Tsaloglou et al., 2013). NASBA-based tools have been used to quantify *K. brevis* cells in real time in environmental or spiked seawater samples, in some cases achieving an LOD as low as 1 cell per reaction and an effective LOD lower than 1,000 cells/L (Casper et al., 2007).

3. Challenges in developing and implementing DNA-based tools

Different parameters need to be carefully controlled and defined during the development of DNA-based biotechnological tools. Moreover, to make these tools suitable for environmental monitoring activities and also in-field applications, other steps besides amplification/detection itself need to be taken into consideration. These steps are: sample pre-concentration, nucleic acid extraction, applicability and miniaturisation/portability (Figure 2). Herein, a description of all these steps is provided, together with approaches currently used and challenges that still need to be tackled. Although these steps are presented separately, they are intrinsically interconnected with each other.

3.1. Sample pre-concentration

A pre-concentration step is necessary in microalgal detection, since they are normally present at low abundances in seawater samples. Two traditional approaches are commonly used to pre-concentrate microalgae: filtration (Huang et al., 2017a) and centrifugation (Toldrà et al., 2018c), which have been used to pre-concentrate samples of volume between 10 mL (Wang et al., 2020) and 150 L (Pham et al., 2018). Faster and simpler technologies such as the use of MBs also exist and have been used to pre-concentrate marine toxins (Bragg et al., 2018), marine viruses (Toldrà et al., 2018a) and, more recently, for the harvesting of the microalgal species *Nannochloropsis oculata* (Chu et al., 2020).

3.2. Nucleic acid extraction

Nucleic acid extraction is another crucial pre-treatment step. Due to the large amount of polysaccharides and polyphenols in microalgae, the isolation of high-purity DNA/RNA is not straightforward (Greco et al., 2014). At present, nucleic acid extraction methods are mostly based on phenol/chloroform (Barra et al., 2014) or standard spin kits (Qin et al., 2019), and a bead-beat protocol is normally implemented to disrupt the cells. Unfortunately, these methods are time consuming and require many reagents and laboratory infrastructure (e.g. centrifuges), limiting the speed of the whole assay and their use in the field, respectively. Innovative nucleic acid extraction methods based on the use of syringes (Casper et al., 2007; Toldrà et al., 2018c) or MBs (Loukas et al., 2017; Tsaloglou et al., 2013) have also been coupled with RPA and NASBA-based biotechnological tools for the detection of *Karlodinium* and *Karenia* species, respectively, moving towards the implementation of simple and rapid nucleic acid extraction methods for *in-situ* testing.

3.3. Amplification/detection

Different parameters are used to assess the performance of an analytical method and thus prove that the method is acceptable for its intended purpose. The classical performance characteristics are: specificity, LOD or LOQ (limit of quantification, usually calculated as the blank plus ten times its standard deviation), linearity

and application range, precision (reproducibility and repeatability), stability, recovery and regeneration. Among them, specificity and LOD or LOQ are the two most reported parameters in molecular-based methods for microalgal detection. The specificity of a molecular method is essentially determined by the primers/probes used. Such primers/probes are generally designed within ribosomal DNA (rDNA) genes (i.e. small subunit (SSU), large subunit (LSU) and internal transcribed spacer (ITS1-5.8S-ITS2)), due to: 1) a high number of sequences available in molecular databases; 2) their extensive use in taxonomy, 3) the high copy number present and 4) the different degrees of sequence variability, which can be exploited to target microalgae at a genus or species level (Penna et al., 2014). However, primer specificity does not only refer to the capability of amplifying the target species, but also not amplifying non-target DNA. Therefore, primer design involves *in silico* comparison with other sequences of species that could co-exist with the target in the sample. However, the design of reliable primers is challenging due to the limited sequences of marine microorganisms currently available. Consequently, assessing primers/probes specificity also implies performing cross-reactivity tests with non-target microalgae from pure cultures and finally applying the method to field samples containing a wide range of unknown microorganisms that show a rich molecular diversity. Next-generation sequencing technologies (NGS) could contribute an expansion of the molecular databases for marine species as well as achieving the entire genome sequencing of microalgae, which could ultimately facilitate the design of reliable primers. Additionally, the use of NGS could facilitate the identification of genes involved in toxin biosynthesis, which may be useful to discriminate between toxin-producing and non-toxin-producing genotypes, thus being closer to a real estimation of the microalgal associated risk.

LOD is another critical point that should be considered. Comparing LODs among the described molecular biotechnological tools is difficult because different types of analytes can be used to define such LODs, which include: synthetic DNA (or target copies), genomic DNA and cells (Table 2). Nevertheless, when using the same type of analyte, nucleic acid-based biotechnological tools achieve around 10 to 100- fold improvement in LOD as compared to standard PCR. The effective LOD is even more important than the LOD of the method. The effective LOD takes into account the whole analytical process (from sampling to detection), and therefore depends on the volume of sample, the nucleic acid extraction protocol and the LOD of the method. Establishing the effective LOD is fundamental from a practical perspective, and it is usually expressed in cells/L (Table 2). 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 highlight that the practical LOD can be improved not only by decreasing the LOD of the method, but also by increasing the initial volume of sample or decreasing the volume of DNA extract. Additionally, it is even more important to have in mind the final application of the method, and whether achieving very low LODs is of crucial importance. For instance, for monitoring purposes, being

able to detect microalgal species below the proposed alarm thresholds is the key point, rather than being able to detect a single microalgal cell. This issue has been taken into consideration in some reports where, despite not achieving extremely low LODs, the utility of the developed tools has been fully demonstrated to be able to detect the target microalgae below the alarm thresholds. This is the case of an RPA and an RCA-based tools that were able to detect 640 *O*. cf *ovata* cells/L (Toldrà et al., 2019b) and 5,000 *Heterosigma akashiwo* cells/L (Zhang et al., 2018b), respectively, where both LODs are well below the established alarm levels of 10,000-30,000 *O*. cf *ovata* cells/L and 100,000 *H. akashiwo* cells/L.

To demonstrate the reliability of the developed method, an in-depth assessment of several performance parameters is required. Therefore, besides specificity and LOD/LOQ, other parameters should be properly addressed, which include: linearity, reproducibility, repeatability, stability, recovery and regeneration. Given that the development of molecular methods for the detection of microalgae is still in its infancy, such parameters, while crucial, have not been always thoroughly evaluated.

3.4. Applicability

In the past few years, most of the advances have been focused on the development of molecular systems but not on their application. The application of nucleic acid-based biotechnological tools to the analysis of environmental samples is fundamental to verify their usefulness and facilitate their eventual implementation in monitoring activities. In the case that environmental samples are not available, these methods can be applied to environmental seawater spiked with microalgal cells from a laboratory culture for validation purposes.

As explained before, specificity and LODs can be only properly determined after the analysis of environmental samples. Moreover, a subsequent validation step is required, which involves comparing the results of the analysis of samples using the developed molecular tool with other established methodologies. In the case of microalgae, ideally, the developed tool should be compared with light microscopy identification and counts, since it is the current reference analysis method for microalgal detection. Such a comparison with cell counts can be qualitative, semi-quantitative or quantitative (Table 2), with the latter being the most desirable. However, when estimating microalgal cell abundances, a good correlation between molecular methods and light microscopy is not always obtained. As stated in section 3.3., most molecular methods that detect microalgae target the rDNA. However, since rDNA copy number per microalgal cell may vary depending on the species, strain, growth phase and/or environmental conditions, it is challenging to determine cell abundances in environmental samples using molecular methods (Galluzzi et al., 2010). Although some approaches have been used to minimise this problem, such as the use of site-specific environmental calibration curves (Perini et al., 2011) or predictive models (Toldrà et al., 2019a), they have only been applied to *Ostreopsis* species from a specific geographical area. A proper validation should include enough environmental samples, also from

different geographical sites. Since qualitative approaches may be insufficient and quantitative approaches still remain highly challenging, it should be considered whether semi-quantitative approaches may be adequate and fulfil the requirements of a monitoring program. Furthermore, it would be interesting to consider the suitability of expressing the thresholds for microalgae in terms of DNA/RNA contents.

3.5. Miniaturisation/portability

Given that current techniques to monitor microalgae require samples to be sent to highly equipped and centralised laboratories, resulting in a considerable time lag between sample collection and data generation/availability, lot of effort is being placed on the development of *in-situ* tools using portable or deployable instrumentation. To move molecular diagnostics from the laboratory to the field, the whole analysis process (including sample pre-concentration, nucleic acid extraction, amplification and detection) needs to be simple, energy-efficient and compatible with miniaturisation. In this context, the use of isothermal molecular techniques has gained popularity due to the reduction in power consumption. Nonetheless, it should be highlighted that portable PCR systems do already exist. Additionally, the use of solar energy instead of portable batteries in such systems could allow the use of PCR technology in the field. Regarding detection systems, electrochemical systems usually require less complex instrumentation than optical systems, besides being more compatible for integration into automated microfluidic platforms. Nevertheless, battery-operated portable systems able to perform amplification and florescence detection do exist, such as the NASBA-based tool for *K. brevis* detection (Loukas et al., 2017; Tsaloglou et al., 2013). Although this system does not feature sample pre-concentration and nucleic acid extraction, they could be combined with the aforementioned portable/simpler concentration/extraction methods (section 3.2 and 3.3), thus allowing the execution of analysis in the field by non-specialist end users (e.g. fish/shellfish producers or coastal managers). Future improvements in microfluidics and nanotechnology could lead to lab-on a-chip devices that integrate sample concentration, nucleic acid extraction, amplification and detection in a single platform, deployable for *in-situ* analysis and not requiring any operator intervention. Such autonomous platforms exist for molecular methods based on rRNA without an amplification step. 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, currently under development), and it is able to acquire water samples, conduct sample pre-treatment and apply the molecular analytical technology (Scholin et al., 2017).

4. Concluding remarks and perspectives

The use of modern molecular biotechnology to identify and detect microalgae responsible for HABs is promising. This review has specifically focused on nucleic acid-based biotechnological tools including an amplification step (thermal or isothermal). To date, much progress has been made on the amplification technology and the detection strategy (colorimetric, fluorescence or electrochemical). Future work should also consider other steps of the analysis process (e.g. sample pre-concentration, nucleic acid extraction, validation and miniaturisation/portability) to ultimately implement these biotechnological tools in the field, either using hand-portable or autonomous instrumentation. Furthermore, other issues such as the necessity to quantify cells or DNA/RNA or the necessity to decrease the LODs should be addressed. The use of portable/autonomous amplification-based biotechnological platforms is one solution to reducing the time interval from sample collection to HABs warning and acquiring species-specific data. Although coordinated science/engineering efforts are still necessary to implement such tools, these new sensing modalities will undoubtedly soon become available, offering new opportunities for enhancing monitoring, research and management of HABs.

Acknowledgements

This research has received funding from the Ministerio de Ciencia, Innovación y Universidades (MICINN), the Agencia Estatal de Investigación (AEI) and the Fondo Europeo de Desarrollo Regional (FEDER) through the CIGUASENSING (BIO2017-87946-C2-2-R) project. The authors also acknowledge support from CERCA Programme/Generalitat de Catalunya.

Figure 1. Schematic representation of the nucleic acid amplification techniques used in microalgal detection: a) PCR, c) LAMP, e) L-HRCA, g) RPA, h) NASBA; and examples of detection formats: b) microarray, d) lateral flow, f) reverse dot blot array, h) electrochemical biosensor, j) real-time fluorescence.

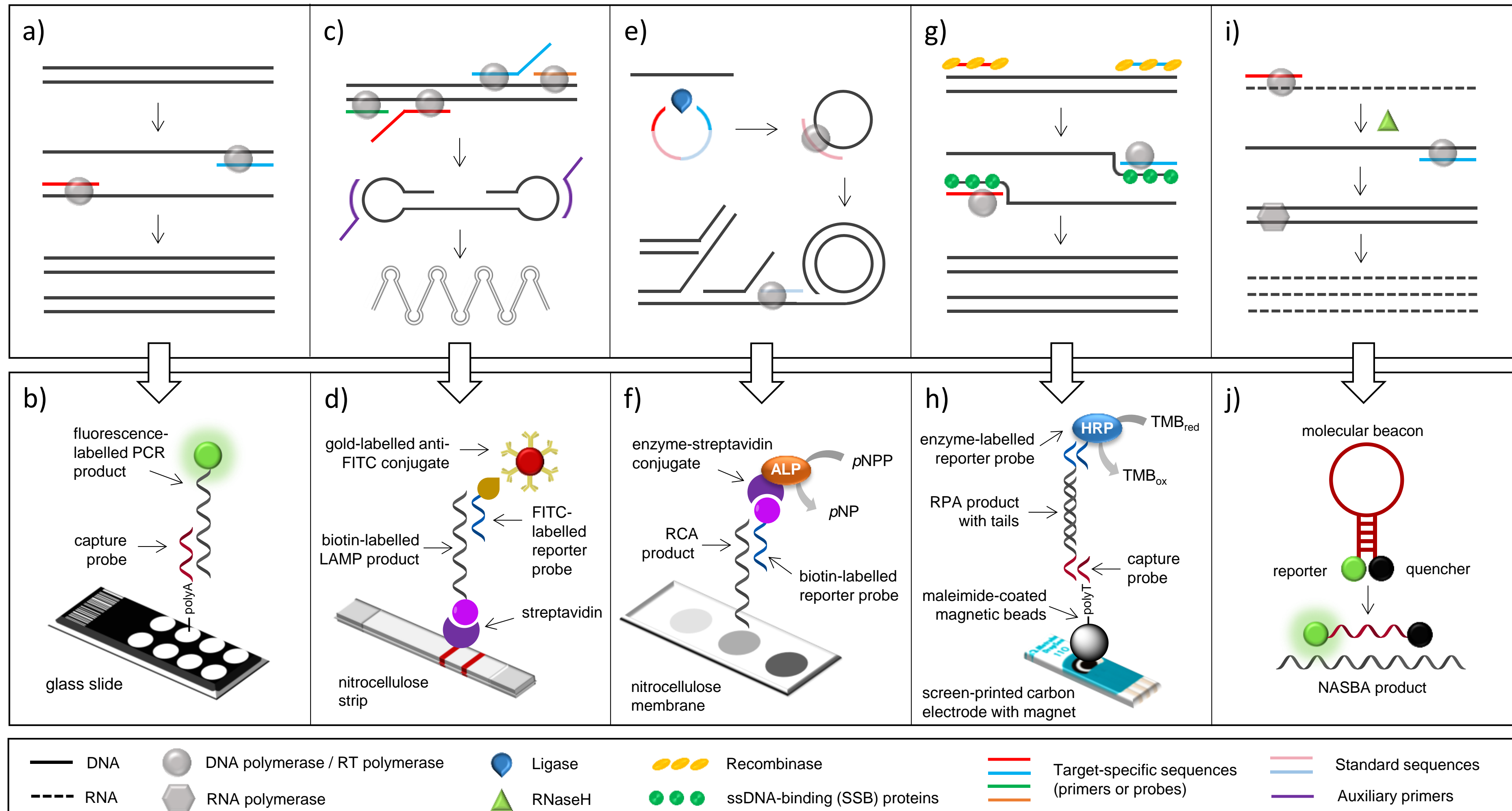


Figure 2. Overview of the analysis workflow in microalgal detection.

Table 1. Classification of harmful marine microalgae.

GROUP 1: non-toxin producing microalgal species	**Examples**
Species reaching high concentrations	*Scripsiella, Skeletonema, Amphidinium*
GROUP 2: toxin producing microalgal species	**Examples**
Ichthyotoxic species	*Karlodinium, Cochlodinium, Prymnesium, Karenia mikimotoi, Chatonella, Heterosigma, Chaetoceros*
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)	*Gambierdiscus*
Species associated with respiratory/skin irritations	*Ostreopsis*

Table 2. Overview of the molecular biotechnological tools developed for the detection of microalgae including: PCR, LAMP, RCA, NASBA and RPA-based methods. LM: light microscopy; Env.: environmental.

Amplification technique	Detection technique	Detection format or singularity	Primers region	Microalgae	Limit of detection (LOD)	Applicability	Applicability comparison techniques	Ref.
PCR	Fluorescence	Microarray	LSU (D2)	10 microalgal species	1 pM PCR product	Env. samples, qualitative	-	(Ki and Han, 2006)
PCR	Fluorescence	Microarray	LSU (D1/D2)	5 *Alexandrium* species/clades	-	Spiked samples, qualitative	LM	(Gescher et al., 2008)
PCR	Fluorescence	Microarray	5.8S-ITS	9 microalgal species/clades	2 ng PCR product; 2,000-8,000 cells/L	Spiked samples, qualitative	LM	(Galluzzi et al., 2011)
PCR	Fluorescence	Microarray	LSU, SSU, 5.8S-ITS	23 microalgal species	50-500 cells	Env. and spiked samples, qualitative	LM, sequencing	(Noyer et al., 2015)
PCR	Fluorescence	Bead array	LSU (D1/D2)	14 microalgal species	-	Env. samples, qualitative	LM	(Scorzetti et al., 2009)
PCR	Fluorescence	Bead array	LSU (D1/D2)	*Karenia brevis* and *Karenia mikimotoi*	0.05-43 target copies; 1-10 pg DNA	Env. samples, semi-quantitative	LM	(Diaz et al., 2010)
PCR	Colorimetry	Dot blot array	LSU	10 *Pseudo-nitzschia* species	-	-	-	(Barra et al., 2014)
PCR	Colorimetry	Reverse dot blot array	LSU (D1/D2)	6 microalgal species	0.6 cells	Env. and spiked samples, qualitative	LM	(Zhang et al., 2014a)
PCR	Colorimetry	Reverse dot blot array	ITS	5 microalgal species	10 cells	Spiked samples, qualitative	LM, PCR	(Chen et al., 2015b)
PCR	Colorimetry	Lateral flow	LSU (D1/D2), 5.8S-ITS	5 *Alexandrium* species	5-500 target copies; 0.1-10 pg DNA	Env. samples, qualitative	PCR, sequencing	(Nagai et al., 2016)
PCR	Electrochemistry	Hybridisation assay	LSU (D1/D2)	*Karenia brevis*	10 cells; 1,000 cells/L	Env. samples, semi-quantitative	LM, sequencing, colorimetry	(LaGier et al., 2007)
PCR	Electrochemistry	Redox labelled dNTPs	5.8-ITS	*Karlodinium armiger*	277 aM target copies	Spiked samples, qualitative	PCR, LM	(Magriñá et al., 2019)
PCR	Fluorescence	SP-PCR	LSU	*Cochlodinium polykrikoides* and other pathogens	32.5 target copies	-	-	(Pham et al., 2018)

PCR	Fluorescence	iiPCR	ITS	*Alexandrium tamiyavanichii*	5 cells	Env. and spiked samples, qualitative	qPCR	(Kon et al., 2016)
LAMP	Fluorescence	Intercalating dye	5.8S	*Alexandrium minutum*	5 cells	-	-	(Wang et al., 2008)
LAMP	Fluorescence	Intercalating dye	ITS	*Alexandrium catenella* and *Alexandrium minutum*	5 pg DNA; 2 cells	-	-	(Zhang et al., 2012)
LAMP	Fluorescence	Intercalating dye	5.8S-ITS	*Cochlodinium polykrikoides* and other pathogens	-	-	-	(Trinh and Lee, 2018)
LAMP	Fluorescence and turbidity	Intercalating dye and by-product	ITS	*Karenia mikimotoi*	6 pg DNA	-	-	(Zhang et al., 2009)
LAMP	Fluorescence and turbidity	Intercalating dye and by-product	LSU (D1/D2)	*Prorocentrum donghaiense*	0.6 cells	Spiked samples, qualitative	LM	(Chen et al., 2013)
LAMP	Fluorescence and turbidity	Intercalating dye and by-product	LSU (D2)	*Prorocentrum minimum*	36 pg DNA	Spiked samples, qualitative	PCR, LM	(Zhang et al., 2014b)
LAMP	Turbidity	by-product	LSU (D1/D2)	*Alexandrium tamarense* and *Alexandrium catenella*	1 cell	Spiked samples, qualitative	LM	(Nagai and Itakura, 2012)
LAMP	Colorimetry	Lateral flow	ITS	*Karlodinium veneficum*	15 pg DNA	Env. samples, qualitative	PCR, LM, sequencing	(Huang et al., 2017a)
LAMP	Colorimetry	Lateral flow	LSU	*Skeletonema costatum*	1.9 pg DNA	Spiked samples, qualitative	PCR, LM	(Huang et al., 2017b)
LAMP	Colorimetry	Lateral flow	5.8-ITS	*Prymnesium parvum*	60 pg DNA	Spiked samples, qualitative	PCR, LM	(Zhu et al., 2019)
LAMP	Colorimetry	Lateral flow	ITS	*Amphidinium carterae*	3.7 10^4 target copies; 1.7 pg DNA; 10,000 cells/L	Spiked samples, qualitative	LM, PCR, LAMP (dye)	(Wang et al., 2019)
LAMP	Colorimetry	Lateral flow	ITS	*Chattonella marina*	1.3 target copies; 0.34 pg DNA; 1,000 cells/L	Env. and spiked samples, qualitative	LM, PCR	(Qin et al., 2019)
LAMP	Colorimetry	Lateral flow	ITS	*Alexandrium catenella*	2.5 10^4 target copies; 1 pg DNA; 100 cells/L	Spiked samples, qualitative	LM, PCR, LAMP (dye)	(Wang et al., 2020)
LAMP	Colorimetry	Lateral flow	5.8S-ITS	*Karenia mikimotoi*	72 pg DNA	Env. and spiked samples, qualitative	PCR, LM	(Huang et al., 2020)

L-HRCA	Fluorescence	Intercalating dye	LSU (D1/D2)	*Amphidinium carterae*	283 target copies; 1 cell	Env. and spiked samples, qualitative	PCR, LM	(Chen et al., 2015a)
L-HRCA	Fluorescence	Intercalating dye	LSU (D1/D2)	*Heterosigma akashiwo*	80 fg target copies; 1 cell; 5,000 cells/L	Env. and spiked samples, qualitative	PCR, LM	(Zhang et al., 2018b)
dL-HRCA	Fluorescence	Intercalating dye	LSU (D1/D2)	*Karenia mikimotoi*	10 cells; 50,000 cells/L	Env. and spiked samples, qualitative	PCR, LM, L-HRCA (dye)	(Zhang et al., 2019b)
L-HRCA	Colorimetry	Lateral flow	ITS	*Karenia mikimotoi*	4 target copies; 4 fg DNA; 100 cells/L	Env. and spiked samples, qualitative	PCR, LM	(Zhang et al., 2019a)
L-HRCA	Colorimetry	Lateral flow	LSU (D1/D2)	*Karlodinium veneficum*	54 target copies; 0.3 fg DNA; 10 cells/L	Env. and spiked samples, qualitative	PCR, LM	(Liu et al., 2019)
L-HRCA	Colorimetry	Dot blot array	LSU (D1/D2)	*Chattonella marina*	4 target copies; 100 cells/mL	Env. and spiked samples, qualitative	PCR, LM, L-HRCA (dye)	(Nie et al., 2017)
L-HRCA	Colorimetry	Reverse dot blot array	LSU (D1/D2)	6 microalgal species	40 target copies; 100 cells/L	Env. and spiked samples, qualitative	PCR, LM	(Zhang et al., 2018a)
RPA	Colorimetry	Tailed primers	ITS	*Karlodinium veneficum* and *Karlodinium armiger*	0.043-0.7 fM target copies; 55-60 pg DNA; 50,000 cells/L	Spiked samples, quantitative	qPCR, LM	(Toldrà et al., 2018c)
RPA	Colorimetry	Tailed primers	5.8-ITS	*Ostreopsis* cf. *ovata* and *Ostreopsis* cf. *siamensis*	50-70 pg DNA; 800-3,800 cells/L	Env. samples, quantitative	qPCR, LM	(Toldrà et al., 2019a)
RPA	Colorimetry	Lateral flow	ITS	*Karlodinium veneficum*	71 target copies; 5 pg DNA; 100 cells/L	Spiked samples, qualitative	PCR, LM	(Fu et al., 2019)
RPA	Electrochemistry	Tailed primers, MBs	5.8-ITS	*Ostreopsis* cf. *ovata*	45 pg DNA; 640 cells/L	Env. samples, quantitative	qPCR, LM, RPA-ELONA	(Toldrà et al., 2019b)
NASBA	Fluorescence	Real-time	*rbcL* mRNA	*Karenia brevis*	80 fg target RNA; 1 cell; <1,000 cells/L	Env. samples, quantitative	LM	(Casper et al., 2004)
NASBA	Fluorescence	Real-time	*rbcL* mRNA	*Karenia brevis*	1 cell; <1,000 cells/L	Env. samples, quantitative	LM	(Casper et al., 2007)
NASBA	Fluorescence	Real-time	*rbcL* mRNA	*Karenia brevis*	500 cells; 1,750,000 cells/L	Env. samples, quantitative	LM	(Tsaloglou et al., 2013)
NASBA	Fluorescence	Real-time	*rbcL* mRNA	*Karenia brevis*	50 cells	Spiked samples, quantitative	LM	(Loukas et al., 2017)

References:

Adachi M, Sako Y, Ishida Y. Restriction-fragment-length-polymorphism of ribosomal DNA internal transcribed spacer and 5.8S-regions in Japanese *Alexandrium* species (Dinophyceae). Journal of Phycology 1994; 30: 857-863.

Ali MM, Li F, Zhang ZQ, Zhang KX, Kang DK, Ankrum JA, et al. Rolling circle amplification: a versatile tool for chemical biology, materials science and medicine. Chemical Society Reviews 2014; 43: 3324-3341.

Anderson DM, Cembella AD, Hallegraeff GM. Progress in understanding harmful algal blooms: paradigm shifts and new technologies for research, monitoring, and management. Annual review of marine science 2012; 4: 143-176.

Anderson DM, Kulis D, Erdner D, Ahn S, Walt D. Fibre optic microarrays for the detection and enumeration of harmful algal bloom species. African Journal of Marine Science 2006; 28: 231-235.

Antonella P, Luca G. The quantitative real-time PCR applications in the monitoring of marine harmful algal bloom (HAB) species. Environmental Science and Pollution Research 2013; 20: 6851-6862.

Barra L, Ruggiero MV, Chen JX, Kooistra W. Specificity of LSU rRNA-targeted oligonucleotide probes for *Pseudo-nitzschia* species tested through dot-blot hybridisation. Environmental Science and Pollution Research 2014; 21: 548-557.

Bragg WA, Garrett A, Hamelin EI, Coleman RM, Campbell K, Elliott CT, et al. Quantitation of saxitoxin in human urine using immunocapture extraction and LC-MS. Bioanalysis 2018; 10: 229-239.

Casper ET, Patterson SS, Bhanushali P, Farmer A, Smith M, Fries DP, et al. A handheld NASBA analyzer for the field detection and quantification of *Karenia brevis*. Harmful Algae 2007; 6: 112-118.

Casper ET, Paul JH, Smith MC, Gray M. Detection and quantification of the red tide dinoflagellate *Karenia brevis* by real-time nucleic acid sequence-based amplification. Applied and Environmental Microbiology 2004; 70: 4727-4732.

Chen GF, Cai PP, Zhang CY, Wang YY, Zhang SB, Guo CL, et al. Hyperbranched rolling circle amplification as a novel method for rapid and sensitive detection of *Amphidinium carterae*. Harmful Algae 2015a; 47: 66-74.

Chen GF, Ma CS, Zhang CY, Zhou J, Wang YY, Wang GC, et al. A rapid and sensitive method for field detection of *Prorocentrum donghaiense* using reverse transcription-coupled loop-mediated isothermal amplification. Harmful Algae 2013; 29: 31-39.

Chen GF, Zhang CY, Wang YY, Chen W. Application of reverse dot blot hybridization to simultaneous detection and identification of harmful algae. Environmental Science and Pollution Research 2015b; 22: 10516-10528.

Chu FJ, Wan TJ, Chen H, Wu CH, Kao PM. Magnetophoretic harvesting of *Nannochloropsis oculata* using iron oxide immobilized beads. Water 2020; 12: 10.

Compton J. Nucleic-acid sequence-based amplification. Nature 1991; 350: 91-92.

Deng HM, Gao ZQ. Bioanalytical applications of isothermal nucleic acid amplification techniques. Analytica Chimica Acta 2015; 853: 30-45.

Diaz MR, Jacobson JW, Goodwin KD, Dunbar SA, Fell JW. Molecular detection of harmful algal blooms (HABs) using locked nucleic acids and bead array technology. Limnology and Oceanography-Methods 2010; 8: 269-284.

Diercks-Horn S, Metfies K, Jackel S, Medlin LK. The ALGADEC device: A semi-automated rRNA biosensor for the detection of toxic algae. Harmful Algae 2011; 10: 395-401.

Fire A, Xu SQ. Rolling replication of short DNA circles. Proceedings of the National Academy of Sciences of the United States of America 1995; 92: 4641-4645.

Fu MQ, Chen GF, Zhang CY, Wang YY, Sun R, Zhou J. Rapid and sensitive detection method for *Karlodinium veneficum* by recombinase polymerase amplification coupled with lateral flow dipstick. Harmful Algae 2019; 84: 1-9.

Galluzzi L, Bertozzini E, Penna A, Perini F, Garces E, Magnani M. Analysis of rRNA gene content in the Mediterranean dinoflagellate *Alexandrium catenella* and *Alexandrium taylori*: implications for the quantitative real-time PCR-based monitoring methods. Journal of Applied Phycology 2010; 22: 1-9.

Galluzzi L, Cegna A, Casabianca S, Penna A, Saunders N, Magnani M. Development of an oligonucleotide microarray for the detection and monitoring of marine dinoflagellates. Journal of Microbiological Methods 2011; 84: 234-242.

Galluzzi L, Penna A, Bertozzini E, Vila M, Garces E, Magnani M. Development of a real-time PCR assay for rapid detection and quantification of *Alexandrium minutum* (a dinoflagellate). Applied and Environmental Microbiology 2004; 70: 1199-1206.

Gescher C, Metfies K, Medlin LK. The ALEX CHIP - Development of a DNA chip for identification and monitoring of *Alexandrium*. Harmful Algae 2008; 7: 485-494.

Glibert PM, Berdalet E, Burford MA, Pitcher GC, Zhou MJ. Harmful algal blooms and the importance of understanding their ecology and oceanography. In: Glibert PM, Berdalet E, Burford MA, Pitcher GC, Zhou M, editors. Global Ecology and Oceanography of Harmful Algal Blooms. 232. Springer, New York, 2018, pp. 9-25.

Greco M, Saez CA, Brown MT, Bitonti MB. A aimple and effective method for high quality co-extraction of genomic DNA and total RNA from low biomass *Ectocarpus siliculosus*, the model brown alga. Plos One 2014; 9: 13.

Greenfield DI, Marin R, Jensen S, Massion E, Roman B, Feldman J, et al. Application of environmental sample processor (ESP) methodology for quantifying *Pseudo-nitzschia australis* using ribosomal RNA-targeted probes in sandwich and fluorescent *in situ* hybridization formats. Limnology and Oceanography-Methods 2006; 4: 426-435.

Huang HL, Gao WF, Zhu P, Zhou CX, Qiao LL, Dang CY, et al. Molecular method for rapid detection of the red tide dinoflagellate *Karenia mikimotoi* in the coastal region of Xiangshan Bay, China. Journal of Microbiological Methods 2020; 168: 7.

Huang HL, Zhu P, Zhou CX, He S, Yan XJ. The development of loop-mediated isothermal amplification combined with lateral flow dipstick for detection of *Karlodinium veneficum*. Harmful Algae 2017a; 62: 20-29.

Huang HL, Zhu P, Zhou CX, Yan XJ, Zou YX, Lv PW. Detection of *Skeletonema costatum* based on loop-mediated isothermal amplification combined with lateral flow dipstick. Molecular and Cellular Probes 2017b; 36: 36-42.

Joda H, Beni V, Willems A, Frank R, Hoth J, Lind K, et al. Modified primers for rapid and direct electrochemical analysis of coeliac disease associated HLA alleles. Biosensors & Bioelectronics 2015; 73: 64-70.

Ki JS, Han MS. A low-density oligonucleotide array study for parallel detection of harmful algal species using hybridization of consensus PCR products of LSU rDNA D2 domain. Biosensors & Bioelectronics 2006; 21: 1812-1821.

Kon NF, Lau WLS, Law IK, Lim PT, Leaw CP. On-site rapid detection of toxic *Alexandrium tamiyavanichii*: integrating the species-specific hydrolysis probe in insulated isothermal polymerase chain reaction (iiPCR). Journal of Applied Phycology 2016; 28: 2815-2820.

LaGier MJ, Jack WFB, Goodwin KD. Electrochemical detection of harmful algae and other microbial contaminants in coastal waters using hand-held biosensors. Marine Pollution Bulletin 2007; 54: 757-770.

Litaker RW, Vandersea MW, Kibler SR, Reece KS, Stokes NA, Steidinger KA, et al. Identification of *Pfiesteria piscicida* (Dinophyceae) and Pfiesteria-like organisms using internal transcribed spacer-specific PCR assays. Journal of Phycology 2003; 39: 754-761.

Liu FG, Chen GF, Zhang CY, Wang YY, Zhou J. Exponential rolling circle amplification coupled with lateral flow dipstick strips as a rapid and sensitive method for the field detection of *Karlodinium veneficum*. Journal of Applied Phycology 2019; 31: 2423-2436.

Loukas CM, McQuillan JS, Laouenan F, Tsaloglou MN, Ruano-Lopez JM, Mowlem MC. Detection and quantification of the toxic microalgae *Karenia brevis* using lab on a chip mRNA sequence-based amplification. Journal of Microbiological Methods 2017; 139: 189-195.

Lyu Y, Richlen ML, Sehein TR, Chinain M, Adachi M, Nishimura T, et al. LSU rDNA based RFLP assays for the routine identification of *Gambierdiscus* species. Harmful Algae 2017; 66: 20-28.

Magriñá I, Toldrà A, Campàs M, Ortiz M, Simonova A, Katakis I, et al. Electrochemical genosensor for the direct detection of tailed PCR amplicons incorporating ferrocene labelled dATP. Biosensors & Bioelectronics 2019; 134: 76-82.

Mayboroda O, Katakis I, O'Sullivan CK. Multiplexed isothermal nucleic acid amplification. Analytical Biochemistry 2018; 545: 20-30.

Miller PE, Scholin CA. On detection of *Pseudo-nitzschia* (Bacillariophyceae) species using whole cell hybridization: Sample fixation and stability. Journal of Phycology 2000; 36: 238-250.

Nagai S, Itakura S. Specific detection of the toxic dinoflagellates *Alexandrium tamarense* and *Alexandrium catenella* from single vegetative cells by a loop-mediated isothermal amplification method. Marine Genomics 2012; 7: 43-49.

Nagai S, Miyamoto S, Ino K, Tajimi S, Nishi H, Tomono J. Easy detection of multiple *Alexandrium* species using DNA chromatography chip. Harmful Algae 2016; 51: 97-106.

Nie XL, Zhang CY, Wang YY, Guo CL, Zhou J, Chen GF. Application of hyper-branched rolling circle amplification (HRCA) and HRCA-based strip test for the detection of *Chattonella marina*. Environmental Science and Pollution Research 2017; 24: 15678-15688.

Nishimura T, Hariganeya N, Tawong W, Sakanari H, Yamaguchi H, Adachi M. Quantitative PCR assay for detection and enumeration of ciguatera-causing dinoflagellate *Gambierdiscus* spp. (Gonyaulacales) in coastal areas of Japan. Harmful Algae 2016; 52: 11-22.

Notomi T, Okayama H, Masubuchi H, Yonekawa T, Watanabe K, Amino N, et al. Loop-mediated isothermal amplification of DNA. Nucleic Acids Research 2000; 28: 7.

Noyer C, Abot A, Trouilh L, Leberre VA, Dreanno C. Phytochip: Development of a DNA-microarray for rapid and accurate identification of *Pseudo-nitzschia* spp and other harmful algal species. Journal of Microbiological Methods 2015; 112: 55-66.

O'Sullivan CK, Beni V, Willems A, Rainer F, Hansen-Hagge T, Joda H. Special polymerase chain reaction primers. World Patent: WO2013113965A1, 2013.

Orozco J, Villa E, Manes CL, Medlin LK, Guillebault D. Electrochemical RNA genosensors for toxic algal species: enhancing selectivity and sensitivity. Talanta 2016; 161: 560-566.

Penna A, Battocchi C, Capellacci S, Fraga S, Aligizaki K, Lemée R, et al. Mitochondrial, but not rDNA, genes fail to discriminate dinoflagellate species in the genus *Ostreopsis*. Harmful Algae 2014; 40: 40-50.

Penna A, Magnani M. Identification of *Alexandrium* (Dinophyceae) species using PCR and rDNA-targeted probes. Journal of Phycology 1999; 35: 615-621.

Perini F, Casabianca A, Battocchi C, Accoroni S, Totti C, Penna A. New approach using the real-time PCR method for estimation of the toxic marine dinoflagellate *Ostreopsis* cf. *ovata* in marine environment. Plos One 2011; 6: 9.

Pham QN, Trinh KTL, Jung SW, Lee NY. Microdevice-based solid-phase polymerase chain reaction for rapid detection of pathogenic microorganisms. Biotechnology and Bioengineering 2018; 115: 2194-2204.

Piepenburg O, Williams CH, Stemple DL, Armes NA. DNA detection using recombination proteins. Plos Biology 2006; 4: 1115-1121.

Qin Y, Chen GF, Zhang CY, Wang YY, Zhou J. Development of loop-mediated isothermal amplification combined with a chromatographic lateral-flow dipstick for rapid detection of *Chattonella marina*. Harmful Algae 2019; 89: 10.

Ronkainen NJ, Halsall HB, Heineman WR. Electrochemical biosensors. Chemical Society Reviews 2010; 39: 1747-1763.

Schena M, Shalon D, Davis RW, Brown PO. Quantitative monitoring of gene-expression patterns with a complementary-DNA microarray. Science 1995; 270: 467-470.

Scholin CA, Birch J, Jensen S, Marin R, Massion E, Pargett D, et al. The quest to develop ecogenomic sensors: a 25-year history of the Environmental Sample Processor (ESP) as a case study. Oceanography 2017; 30: 100-113.

Scorzetti G, Brand LE, Hitchcock GL, Rein KS, Sinigalliano CD, Fell JW. Multiple simultaneous detection of Harmful Algal Blooms (HABs) through a high throughput bead array technology, with potential use in phytoplankton community analysis. Harmful Algae 2009; 8: 196-211.

Simon N, Campbell L, Ornolfsdottir E, Groben R, Guillou L, Lange M, et al. Oligonucleotide probes for the identification of three algal groups by dot blot and fluorescent whole-cell hybridization. Journal of Eukaryotic Microbiology 2000; 47: 76-84.

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

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

Toldrà A, Andree KB, Bertomeu E, Roque A, Carrasco N, Gairín I, et al. Rapid capture and detection of ostreid herpesvirus-1 from Pacific oyster *Crassostrea gigas* and seawater using magnetic beads. Plos One 2018a; 13: 15.

Toldrà A, Andree KB, Fernández-Tejedor M, Diogène J, Campàs M. 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 2018b; 30: 2435-2445.

Toldrà A, Jauset-Rubio M, Andree KB, Fernández-Tejedor M, Diogène J, Katakis I, et al. 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 2018c; 1039: 140-148.

Trinh TND, Lee NY. A rapid and eco-friendly isothermal amplification microdevice for multiplex detection of foodborne pathogens. Lab on a Chip 2018; 18: 10.

Tsaloglou MN, Laouenan F, Loukas CM, Monsalve LG, Thanner C, Morgan H, et al. Real-time isothermal RNA amplification of toxic marine microalgae using preserved reagents on an integrated microfluidic platform. Analyst 2013; 138: 593-602.

Utermöhl H. Zur Vervollkommung der quantitativen phytoplankton-methodik. Mitt Int. Ver Limnol. 1958; 9: 38.

Wang L, Chen GF, Zhang CY, Wang YY, Sun R. Rapid and sensitive detection of *Amphidinium carterae* by loop-mediated isothermal amplification combined with a chromatographic lateral-flow dipstick. Molecular and Cellular Probes 2019; 43: 72-79.

Wang L, Chen GF, Zhang CY, Wang YY, Sun R. Application of loop-mediated isothermal amplification combined with lateral flow dipstick to rapid and sensitive detection of *Alexandrium catenella*. Environmental Science and Pollution Research 2020; 27: 4246-4257.

Wang L, Li L, Alam MJ, Geng YH, Li ZY, Yamasaki SJ, et al. Loop-mediated isothermal amplification method for rapid detection of the toxic dinoflagellate *Alexandrium*, which causes algal blooms and poisoning of shellfish. Fems Microbiology Letters 2008; 282: 15-21.

Zhang CY, Chen GF, Liu Y, Wang YY, Xu Z, Zhang BY, et al. Simultaneous detection of harmful algae by multiple polymerase chain reaction coupled with reverse dot blot hybridization. Harmful Algae 2014a; 35: 9-19.

Zhang CY, Chen GF, Wang YY, Sun R, Nie XL, Zhou J. MHBMDAA: Membrane-based DNA array with high resolution and sensitivity for toxic microalgae monitoring. Harmful Algae 2018a; 80: 107-116.

Zhang CY, Chen GF, Wang YY, Zhou J, Li CH. Establishment and application of hyperbranched rolling circle amplification coupled with lateral flow dipstick for the sensitive detection of *Karenia mikimotoi*. Harmful Algae 2019a; 84: 151-160.

Zhang CY, Chen GF, Zhou J, Wang YY, Lu DD. Development of a quantitative PCR for detection and quantification of *Prorocentrum donghaiense*. Journal of Applied Phycology 2016; 28: 1683-1693.

Zhang CY, Sun R, Wang YY, Chen GF, Guo CL, Zhou J. Comparative detection of *Karenia mikimotoi* by exponential rolling circle amplification (E-RCA) and double-ligation E-RCA. Journal of Applied Phycology 2019b; 31: 505-518.

Zhang CY, Wang YY, Guo CL, Chen GF, Kan GF, Cai PP, et al. Comparison of loop-mediated isothermal amplification with hyperbranched rolling circle amplification as a simple detection method for *Heterosigma akashiwo*. Harmful Algae 2018b; 73: 1-11.

Zhang DY, Brandwein M, Hsuih T, Li HB. Ramification amplification: A novel isothermal DNA amplification method. Molecular Diagnosis 2001; 6: 141-150.

Zhang FY, Ma LB, Xu ZL, Zheng JB, Shi YH, Lu YA, et al. Sensitive and rapid detection of *Karenia mikimotoi* (Dinophyceae) by loop-mediated isothermal amplification. Harmful Algae 2009; 8: 839-842.

Zhang FY, Shi YH, Jiang KJ, Song W, Ma CY, Xu ZL, et al. Rapid detection and quantification of *Prorocentrum minimum* by loop-mediated isothermal amplification and real-time fluorescence quantitative PCR. Journal of Applied Phycology 2014b; 26: 1379-1388.

Zhang FY, Shi YH, Jiang KJ, Xu ZL, Ma LB. Sensitive and rapid detection of two toxic microalgae *Alexandrium* by loop-mediated isothermal amplification. Acta Oceanologica Sinica 2012; 31: 139-146.

Zhu P, Huang HL, Zhou CX, Xu JL, Qiao LL, Dang CY, et al. Sensitive and rapid detection of *Prymnesium parvum* (Haptophyceae) by loop-mediated isothermal amplification combined with a lateral flow dipstick. Aquaculture 2019; 505: 199-205.