Detecting harmful algal blooms with isothermal molecular strategies

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Abstract: The use of isothermal nucleic acid amplification strategies to detect harmful algal blooms is in its infancy. We describe recent advances in these systems and highlight the challenges for the achievement of simple, low-cost, compact and portable devices for field applications.

Keywords: nucleic acid isothermal amplification; microalgae; monitoring; environment; *in-situ* analysis.

Why isothermal molecular strategies?

Monitoring of harmful algal blooms (HABs) (Box 1) is vital to guarantee the safety and health of coastal environments. Identification and quantification of HAB species has conventionally relied on the observation of morphological characters using a microscope, which is laborious and time-consuming. Additionally, microalgae identification is sometimes difficult due to morphological similarities among species. Molecular (nucleic acid) techniques have the principle advantage of high specific recognition and discrimination of the target species. However, traditional techniques such as PCR still require samples to be sent to centralized laboratories, resulting in a time lag between sample procurement and analysis, increasing the risk of extended contamination. Accordingly, there is an increasing demand for portable and autonomous systems able to perform accurate and rapid analysis in the field. Isothermal amplification techniques can contribute to achieving this objective. Unlike typical thermal cycling amplification, isothermal techniques are carried out at a constant temperature, which reduces the power needed and makes them more compatible for integration into miniaturized systems. Although widely used in clinical diagnostics, the application of isothermal molecular strategies (including isothermal amplification and detection steps) to detect microalgae is still at its preliminary stage.

Isothermal molecular strategies for HABs

Among all isothermal techniques described to date, loop mediated isothermal amplification (LAMP), hyperbranched rolling circle amplification (HRCA), recombinase polymerase amplification (RPA) and nucleic acid sequence-based amplification (NASBA), coupled with different detection strategies, have been successfully used to detect microalgae, with LAMP being the most widely reported technique. 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 visualized by a colour change upon addition of a fluorescent intercalating dye, as reported for the detection of *Prorocentrum minimum, Karenia mikimotoi* and *Alexandrium* species [1]. Another strategy to detect LAMP products relies on lateral flow (LF) dipsticks, recently described for *Karlodinium veneficum* [2], *Prymnesium parvum* [3] and *Amphidinium carterae* [4]. In these dipsticks, the use of a biotin-labelled primer results in biotinylated LAMP products that, after immobilisation on the strip, hybridize with a FITC-labelled probe, and the addition of gold-labelled anti-FITC antibodies generates the characteristic red band (Fig 1A). Such LAMP strategies achieve around 10-fold improvement in sensitivity as compared to standard PCR.

Because of its excellent amplification power, HRCA strategies usually show better sensitivities than PCR and LAMP. HRCA 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 hybridizes to the target, circularizes and amplification occurs. As with LAMP, HRCA products have been detected with fluorescent dyes (for *Heterosigma akashiwo* [5] and *A. carterae* [6]) and LF (for *K. veneficum* [7]). Additionally, a dot blot DNA array has been developed to simultaneously detect multiple microalgae species (Fig 1B) by using different capture probes immobilized on a nylon membrane and an enzyme-streptavidin reporter conjugate [8]. 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 hybridization with the PLP and the probe, respectively.

To avoid the need to denature amplified products before their detection, and to simplify assay cost and time, an innovative approach has been proposed, which relies on RPA. RPA is one of the most rapid amplification techniques, often completed in just 20 min. Target dsDNA is amplified using three enzymes (recombinase, ssDNA-binding protein and strand-displacing DNA polymerase) and two primers, at a constant temperature (37-40 °C). The innovation lies on the use of "tailed primers" (primers modified with short oligonucleotide tails), which results in dsDNA products flanked by ssDNA tails. Colorimetric detection of RPA products is achieved via a sandwich hybridization assay (SHA) using probes complementary to the tails: a capture probe immobilised on maleimide-microtitre plates and an enzyme-labelled reporter probe. This strategy has been used to detect and discriminate *K. veneficum* and *K. armiger* [9] as well as *Ostreopsis* cf. *ovata* and *O*. cf. *siamensis* [10]. Additionally, a biosensor that exploits a SHA on maleimide-coated magnetic beads immobilised on an electrode array has recently been developed for *O.* cf. *ovata* (Fig 1C), offering simplicity and easy integration into portable systems [11].

Addressing systems that can be deployed to the point of need, an integrated microfluidic platform has been developed for the detection of *Karenia brevis* using NASBA [12]. NASBA is specifically designed for the detection of RNA targets owing to the combination of reverse transcription with amplification processes. The system uses two primers and three enzymes (reverse transcriptase, RNase and RNA polymerase), which amplifies target ssRNA at 41 °C for 90 min. In contrast to the previous strategies, which are end-point, NASBA products from *K. brevis* were monitored in real time (during amplification) using molecular beacons and a portable fluorescence reader. Real-time detection can speed up decision-making in surveillance activities (e.g. closure of fishing and seafood harvesting areas, banning swimming and other recreational activities or moving research vessels or unmanned vehicles to next location).

Challenges and future perspectives

Ribosomal DNA genes are the preferred target in molecular strategies because they are sufficiently diverse to discriminate among many (although not all) species. For these genes, the number of target copies per microalgae cell may vary depending on the genus, species, strain, growth phase and/or environmental factors.

Such variability may compromise the correlation with microscopy counts in the analysis of environmental samples. Thereby, to evaluate their applicability, results obtained with isothermal strategies are usually compared to conventional molecular techniques. However, current thresholds for HAB species are expressed in cell abundances. We must consider whether it is crucial to quantify cells rather than DNA in molecular methods. Perhaps DNA can provide additional and/or more convenient information than cell abundances.

Regarding sensitivity, although efforts have focused on developing highly sensitive methods, and single cell detection has been achieved, the key point in monitoring of HABs is the ability to detect cell abundances close to the thresholds established from risk assessment studies. Consequently, it is also important to consider if quantitative approaches are required in the analysis of microalgae or if qualitative or semi-quantitative approaches could be adequate.

Another important parameter is specificity. Given that assays are eventually applied to the analysis of environmental samples containing DNA from a wide range of microorganisms, the specificity of the primers used in the molecular methods is critical. However, designing reliable primers is challenging due to the scarcity of DNA sequences available in public databases. Next-generation sequencing offers a great potential to construct genomic databases for microalgae.

To move molecular diagnostics from the laboratory to the field, not only amplification and detection steps need to be compatible with miniaturization, but also other steps such as DNA extraction. Efforts in this area have already resulted in innovative solutions, such as portable nucleic acid extraction kits. With the advances in microfluidics and nanotechnology, the integration of these steps into compact devices will lead to hand-held battery-operated analysis tools. These miniaturized systems are also more amenable to be incorporated into autonomous in-water platforms including fixed-location (moorings and buoys) and mobile platforms (buoyancy gliders, wave gliders and underwater autonomous vehicles), which are able to acquire water samples, conduct sample pre-treatment and apply the molecular methods [13].

Nowadays, the use of isothermal molecular strategies requires a certain degree of expertise and is still limited to research activities. Nevertheless, straightforward and standard protocols can be defined with the aim of being marketed. Additionally, because of the high throughput analysis capability, the cost per sample is affordable. The integration of these strategies into portable or autonomous systems will make them even simpler and user-friendly, thus suitable for fish/shellfish producers, coastal managers and regulators. The future of these nucleic acid-based alternative methods looks bright, especially when miniaturization and field deployment will be achieved.

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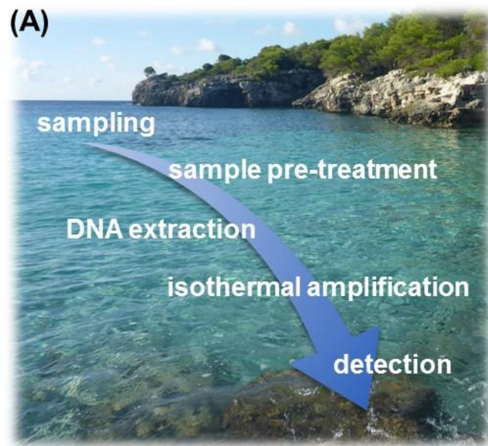
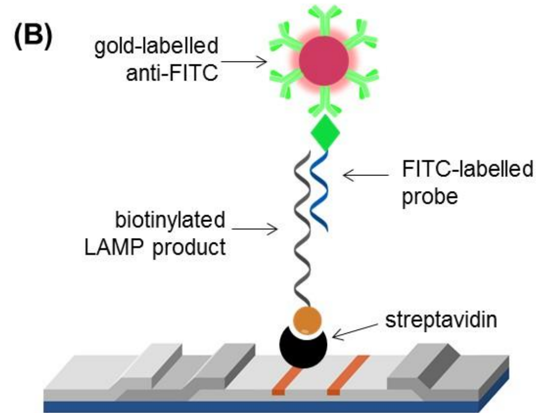
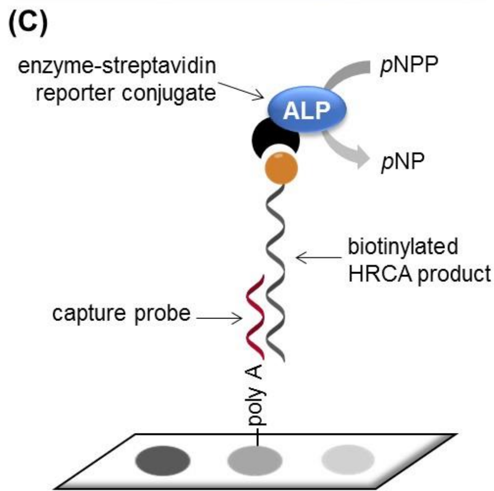
Box 1. Harmful algal blooms (HABs)

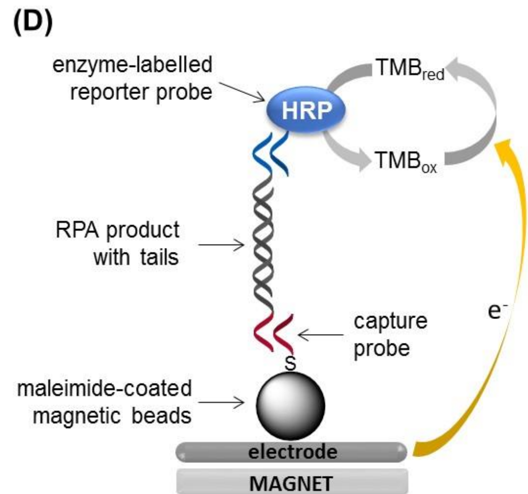
HABs are common natural phenomena caused by a proliferation of microalgae, macroalgae or cyanobacteria, with negative impacts on human health, ecosystems and/or socioeconomic activities (e.g. aquaculture and tourism). HABs are increasing in frequency, intensity and distribution worldwide possibly due to globalisation and climate change. Most HABs are caused by harmful marine microalgae, which are associated with ocean animal mortality or human health risks. One group of harmful algae includes microalgae that after reaching high concentrations cause physicochemical damage by oxygen depletion, as well as microalgae responsible for fish mortality due to the physical obstruction of the gills or the production of toxins (e.g. *Karlodinium, Prymnesium* and *Heterosigma*). Another group includes microalgae that produce toxins that bioaccumulate in shellfish and fish causing foodborne diseases in humans (e.g. *Alexandrium*, *Prorocentrum*, *Pseudo-nitzschia, Karenia* and *Gambierdiscus*). It also includes microalgae associated with respiratory and skin irritations due to the production of toxins or to their physical presence (e.g. *Ostreopsis*). Some HABs have multiple adverse effects.

Figure 1. General isothermal molecular strategy for HAB detection (A) and examples: LAMP followed by a colorimetric lateral flow assay (B), HRCA followed by a colorimetric dot blot assay (C) and RPA followed by an electrochemical biosensor (D).









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