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# *Identification of Algae Species Using Advanced Molecular Techniques*

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## ABSTRACT

*Abstract: Molecular techniques for the detection of organisms, such as algae species in aquatic environments, have become the most attractive tools for typical laboratory approaches. These techniques provide more accurate and faster ways of identifying species compared to conventional methods based on microscopic counting and culture tools. The techniques of molecular biology are mostly used when numerous algal species that are present in very low amounts require identification. These tools use either entire cells or nucleic acid cell-free formats. For the entire cells, several molecular methods could be used, for example, fluorescence in situ hybridization (FISH) and microscope-based enumeration assays. For the nucleic acid cell-free formats, the most commonly used tools are the sandwich hybridization assay (SHA), biosensors, quantitative PCR, real-time PCR, and microarrays. These techniques can be used individually or in combination with one or more functional laboratory tools, such as lab-on-a-chip (namely, a single incorporated system) or next-generation sequencing (NGS) to create a much higher data output. Moreover, this review integrates additional methods that promote the performance of molecular techniques. These approaches provide a high capability for identifying algal species, such as platforms and nano-bioengineered probes, magnetic systems for separation molecules, and solid-phase hybridization. Additionally, hybridization PCR and isothermal amplification tools can improve the hybridization of probes with DNA to enhance the amplification of nucleic acids. Lastly, this review discusses a field case study considered as one of the few examples of monitoring harmful algal blooms (HABs) and closes the discussion with concluding remarks and future directions.*

**Key words:***Molecular approaches; algae species; aquatic ecosystems; FISH assay; RT-qPCR; next-generation sequencing; nanotechnology.*

## INTRODUCTION

The identification of organisms is quicker and more accurate when performed by molecular techniques compared to conventional methods including microscopy or culture techniques. They are the favored strategies for organism detection in view of the expanding acknowledgment of the plenitude of species containing identical individuals as well as the enormous number of microorganisms that cannot be isolated for in vitro study. In this manner, these species and microorganisms go undetected and molecular tools are needed for their identification. Hence, most organisms, including plants, vertebrates, and viruses with very high diversity profiles, can be easily identified using molecular assays [1, 2].

The identification and classification of algae species using specific probes for antibodies (including monoclonal and polyclonal antibodies) and ribosomal RNA, as well as qPCR detection assays, are some of the modern tools being considered in favor of conventional tools as they can also be used for quantitation [3, 4].

The most proficient and viable approach to identify microorganisms or complex microbial samples is to utilize ribosomal RNA (rRNA) genes containing large and small subunits [4, 5], which have turned into the best standard tools on the ground as they give a more extensive perspective regarding the profile of the population.

Hence, the composition of the samples can be easily acquired by the propagation of the DNA through cloning and then identifying the nucleic acid structures by sequencing the target genes from the collected samples of an organism [6, 7].

These strategies enabled the revelation of an enormous quantity of unknown biodiversity [8]. DNA barcodes or probes, which can be designed using specific databases of DNA and RNA as well as higher taxa sequences, might be used to identify numerous unknown species [9, 10].

Moreover, databases of ribosomal RNA are considered to be the most important tool for the recognition and detection of a huge number of organisms.

Specific probes designed against the ribosomal RNA genes of specific species are utilized for the detection of any species community and can be used for whole-cell techniques (i.e., undamaged cells, such as in the FISH method). Furthermore, cell-free or morphological methods, in which probes are employed directly onto DNA (e.g., microarrays, SHA, and biosensors), can also be used. For certain organism communities, for example, marine phytoplankton and algae, precise protocols can be used following Karlsen et al. [11] and Lewis et al. [12] for microarray assays.

The majority of the techniques introduced below have a restriction in that although they are utilized to identify a lot of unknown species, they are unable to predict the harmful effects of these species. The eukaryotic algae species include toxic and non-toxic species, and many bacterial strains have non-harmful and toxic variants, so the use of such techniques to detect and identify algae species cannot necessarily determine which are harmful. However, a few molecular techniques could be used to identify toxic genes, confirming the presence of specific species of algae and, consequently, recognizing whether the aquatic environment is safe or harmful.

Several studies have identified the DNA from single algal cells using phylogenetic trees after culturing of the collected algae species from the environment or selecting specimens collected from plankton samples living in an aquatic environment [13, 14]. In the last method, the algal cells are extracted within a short period of time (from 1 to 24 h) from the originally collected samples under a light microscope using capillary tubes. However, this method is not always appropriate because the isolation of single-cell algae requires high skill and taxonomic expertise. Therefore, this becomes a problem when the collected algal samples have taken a long time to arrive at the laboratory for identification, or when they are used for other purposes such as molecular or genetic analyses. Hence, this problem has encouraged biologists to find better methods to keep the algal cells healthy and preserve their DNA from being damaged so it can be used as a good template for molecular analyses. Thus, the molecular methods require healthy and intact algal cells to avoid mismatching molecular results and to subsequently provide correct identification of algae species collected from ecosystems.

The current review discusses advance molecular markers techniques that are used to determine different algae species. Likewise, the present status and future prospects in the breeding of algal species will also be discussed.

## MOLECULAR METHODS FOR WHOLE CELLS

### Fluorescence in situ Hybridization (FISH)

FISH alludes to the utilization of a fluorescent marker of an oligonucleotide probe that enters a cell membrane and hybridizes with specific molecules, such as the ribosomes of cells. A bright fluorescent color emanates from the whole-cell as an outcome of the great number of target ribosomes inside the cells. Hence, co-existing species containing intact cells can be differentiated while being counter-stained with a general DNA dye, for example, 4′, 6-diamidino-2-phenylindole stain.

Therefore, many organisms can be identified using this tool in conjunction with flow cytometry or fluorescent microscopy. Along this line, Amann [15] was one of the first researchers who identified many species as being either close to each other or separated based on morphological patterns. Although this tool can be used for the detection of many species, it is challenging if applied in plants due to the presence of chlorophyll, which is already fluorescent. However, some fluorescent dyes have been discovered that overcome this problem. Of these dyes, fluorescein isothiocyanate (FITC) can be used as a fluorochrome. This dye is attached to probes, and emits fluorescent green, and can be used as a barcode for staining plant chlorophyll, which has natural orange autofluorescence. However, CY5-labeled probes with red fluorescence are considered to be distinguishing from the orange fluorescence of chlorophyll. Therefore, scientists are using dimethylformamide to remove the chlorophyll from plant samples [16]. Additionally, FISH has been effectively used for the identification of many algae [9, 16–20] as well as bacterial [13] species.

After hybridization with the FISH technique, the quest to characterize target cell arrangement using specific microscopy "epifluorescence" can be tedious and can lead to scientific error due to the inconsistencies in the amount of RNA, which can produce a very low signal, or due to masking of the target cells underneath. Hence, using a robotized counting machine, such as flow cytometry (FCM), including phase and liquid, is the favored method.

The success of FISH techniques in identifying target species depends on the number of species that need to be recognized for a set of given experimental conditions and methods. FITC and CY5 are two different types of fluorochromes that are routinely applied for sample recognition (Figure 1). (Standard fog collectors (SFC) and large fog collectors (LFC) were costly instruments for regular identification, similar to analyses involving HRP-labeled probes. Methods involving poly- and monoclonal antibodies could be used to identify grouped cells, antigens of the target cell surface, and cultured cells [21]. Hence, immunomagnetic beads coupled to either poly- or monoclonal antibodies can be used to separate target cells from mixtures [22].

Hybridoma cell lines are required for the production of monoclonal antibodies. These cell lines are delivered by fusing mouse spleen cells that have been inoculated with a target antigen complex with myeloma cells, which is considered to be functional for binding to polyclonal antibodies compared to monoclonal antibodies. Therefore, monoclonal antibodies can provide unlimited support for generating material required for the specific recognition and hybridization process of FISH. The permeabilization of the cell is essential for the FISH method, which through the cell membrane, will be dissolved in lipids and be permeable to the antibodies. Therefore, the resulting fluorescence emissions during this process are much greater than those resulting from the binding of probes to DNA molecules, and the cell is not affected by physiological conditions [23].

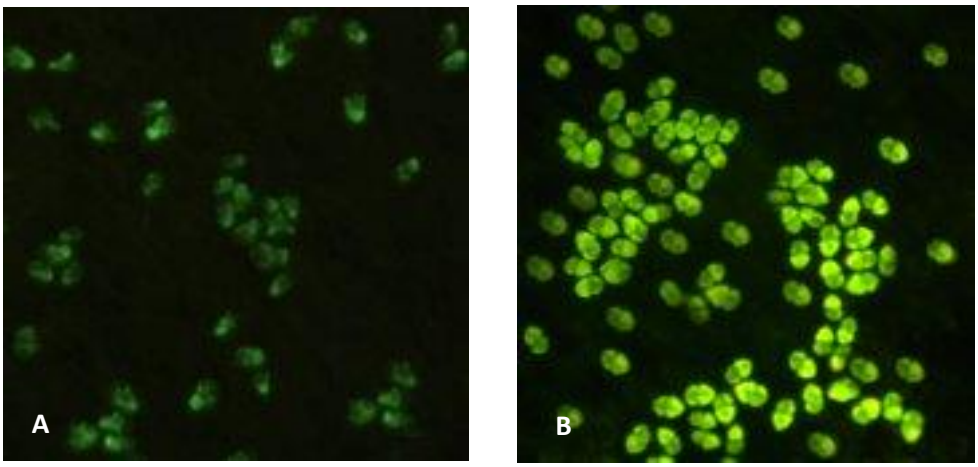


**Figure 1.** Use of (FISH) combined with a fluorescein isothiocyanate (FITC) label (**A**) and CARD-FISH (**B**) for evaluating the relationship between FISH and CARD-FISH for the detection of toxic algal species such as *(Azadinium* spp.). CARD-FISH seems to be more accurate for species detection compared to FISH combined with an FITC label.

## NUCLEIC ACID CELL-FREE FORMATS

This section discusses the extraction of nucleic acids from plant cells, because, when nucleic acids whether DNA or RNA are isolated from live cells, the morphology of the cells will be disrupted. The same applies to nucleic acids isolated from dead cells [24]. Therefore, in this section, we discuss how different molecular methods rely on the quantification of nucleic acids isolated from samples collected from the environment for identifying different aquatic organisms, including algae species.

### Sandwich Hybridization Assay (SHA)

SHA method uses the capture of DNA and one specific probe for targeting RNA or DNA in a form called sandwich hybridization (Figure 2), so two structures are hybridized together. Through this assay, the captured probe is first bound with the target sequence, and second, the probe sends a signal when it links with the specific molecule [25, 26] corresponding to a successful hybridization event. The probe types are digoxigenin (DIG) or fluorochrome that bind to a hybridized complex and help its identification. To identify and detect different types

and species of algae, one of these specific probes is needed through immobilization on electrode or membrane or microtiter plate containing the target DNA sequences of the samples [27–30]. Additionally, for the digoxigenin probe, an additional enzyme, namely horseradish peroxidase, is needed to make a complex consisting of the anti-digoxigenin antibody and the enzyme with the probe against the target DNA sequence. The horseradish peroxidase enzyme is helpful for detecting the target sequences through colorimetric or electrochemical reactions. This mechanism of SHA is time-consuming and represents a cheap reaction that can be used to detect specific algae species [31].

The advantages of the SHA method are that (a) it is an ultra-sensitive tool for detecting the target molecules; (b) it increases the recognition of the target sequences over nonspecific sequences; (c) it does not require a high level of purification of the target sequences (DNA or RNA) [32].

The SHA protocol has been generally utilized for the discovery of lethal algae [32–34] and has been designed for a robotized automated assay called UAP (Saigene Biotech, Inc., Denver, CO, USA), which gives researchers adaptability and power over different measured parameters [35].

**Biosensors**

The Biosensor-based techniques are easy, quick, and could be made as reasonably compact devices [36–39]. They can overcome some of the obstacles encountered during conventional identification and subsequent measurement. Among the identification techniques pertinent to biosensors, electrochemical identification requires high selectivity, sensitivity, and fast response. Thus, it has a much smaller energy prerequisite than other techniques. The characteristics of this assay make it increasingly adaptable and convenient to use outside of the laboratory. Electrochemical sensors are used for the legitimate detection of nucleic acids whether DNA or RNA, even if target samples are in a complex form. Hence, these tools are considered to represent a profitable, preferred method compared to other molecular techniques, such as those that need complicated processes, for example, purification, amplification, and enzymatic inhibitors for detecting the target samples [40].

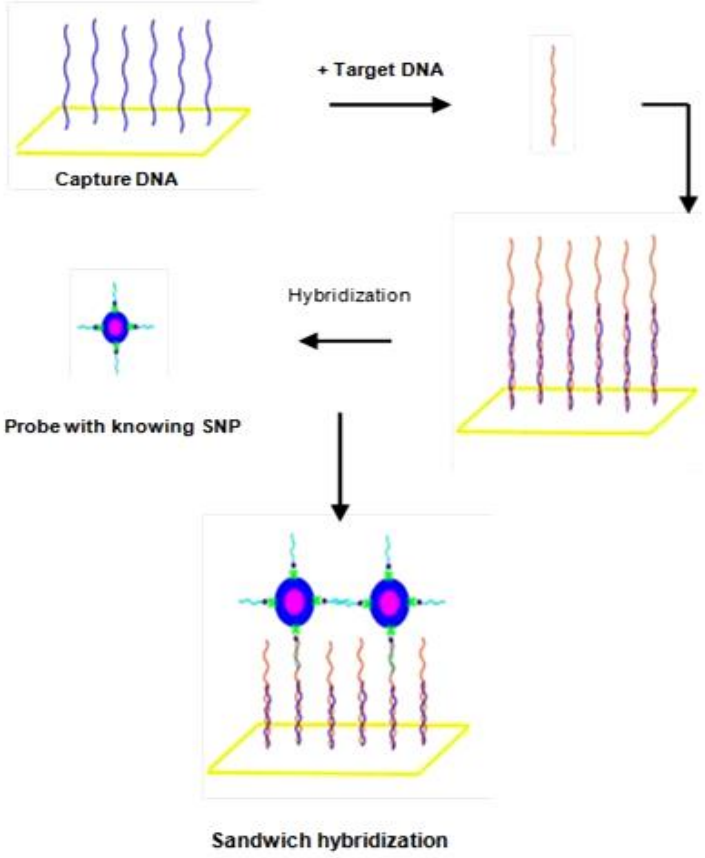


**Figure 2.** Design for sandwich hybridization assessment (SHA). This method describes the capture of DNA and aspecific probe for targeting the RNA or DNA of the tested samples.

Nowadays, biosensors have become important and useful assets in the detection of species. The work process of biosensors is dependent on the strategy of SHA, which aims to electrochemically identify the DNA or RNA

molecules in the best way [36–38]. In this regard, Diercks et al. [39] constructed a framework of the target samples adjusted to biosensors with a number of probes utilized in a half-automated device for detecting many toxic algae species (n=14). The steps of this strategy were predicated to explain the various steps of the process of biosensor manufacturing from the electrochemical perspective using evidence of various algal species and the assessment of the impact of the material on biosensor performance and transducer platform geometry [33, 36]. At the end of the process, the correction curves of the 14 identified species of poisonous algae were constructed through the advancement of the SHA biosensor scheme [30].

The use of fiber-optic genosensors to detect toxic dinoflagellates, which is presented as other biosensor technical methods [41, 42], utilizes an SHA identification procedure. As part of this method, the capture probes are linked to the microspheres to catch the rRNA of the HAB species in the last part of every optical fiber. Once hybridization, the microarray is plunged into formamide to remove the signal probe and capture RNA for reusing. Hence, this mechanism can detect at least five cells in unknown phytoplankton or algae samples. The SHA device biosensor could likewise be utilized with colorimeter identification.

The detection mechanism in an algae sample is that the HRP is linked with an anti-digoxigenin antibody in the presence of an alternate substrate. Hence, this conjugation results in a colored component in which the concentration could be measured through spectrophotometer or by using a camera with a specific biosensor.

**Microarrays**

The tools of the microarray include a sequence of DNA molecules, which are linked to the unique surface glass of a specific slide arranged to cluster like a dot blot [43–46]. This tool is considered one of the most important accessible approaches because of the capacity of the microarray to conceivably identify a large number of unknown samples in only one experiment. In this tool, some biological processing steps are needed, such as extraction of the DNA or RNA, purification and hybridization, as well as statistical analysis. The sample DNA or RNA molecules are labeled with a fluorescent dye and immediately combined with nucleic acids or other labeled target compounds [45–47]. At that point, the microarray will carry the targets hybridized with the labeled probes (Figure 3).

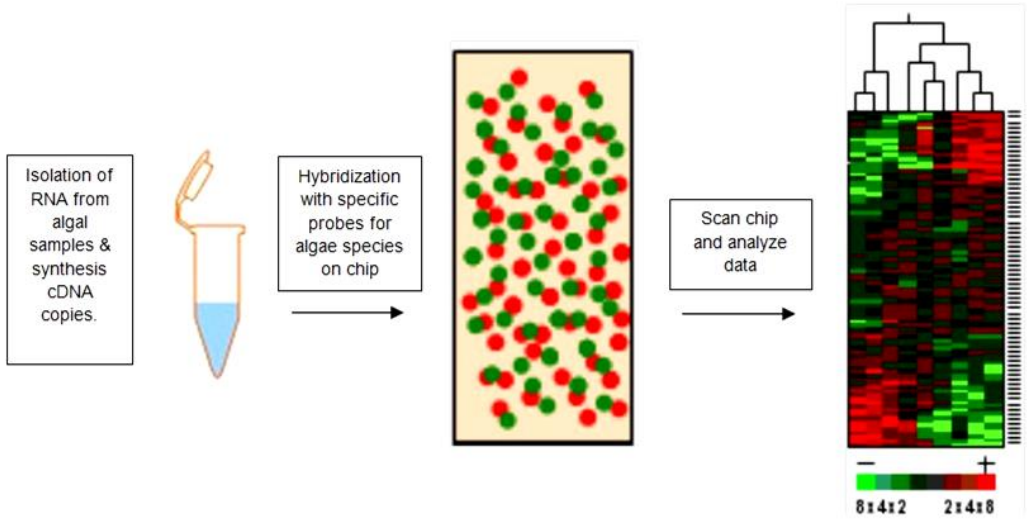


**Figure 3.** An example of a scanned image of a cDNA microarray from algal samples to identify different field samples. Each cluster group explains an amplified probe that is specific for the target sequences of species loaded on the slide to hybridize with labeled ribonucleic acids isolated from the samples collected from the field.

The laser scanner scans the hybridization pattern of a microarray that is captured on the slides by means of fluorescent emission. The fluorescent emission demonstrates which unknown species are detected [44]. Nucleic acid PhyloChips or microarrays, which are strain-specific have been utilized to distinguish aquatic organisms [47], poisonous algal organisms [48–61], bacteria [62–68], and larvae and eggs of fish species [69]. PhyloChip®, an all-inclusive microarray for every single prokaryotic organism, is commercially available and identifies the extended examination moment to achieve prokaryote population investigation using various molecular tools.

In this regard, the dedicated EU collaborative enterprise [70] was established for the development of a common microarray to identify poisonous algal strains. The MIDTAL project delivered an institutionalized technique for analysis and hybridization as well as adjustment [12] the signs, such as cell numbers, for poisonous algae monitoring. This is fundamental to check in light of the fact that almost all decisions regarding fishery closure depend on cell numbers that trigger toxicity testing.

The successive EU project μAQUA was focused on building a universal microarray to detect aquatic microorganisms and develop new techniques for cyanobacteria poisoning identification. This project was also field-tested in 8 EU countries over two years, and the outcomes of these surveys have been issued [71, 72]. There are many novel techniques that are used to detect poisons, the toxin array was assigned to identify the messenger RNA from cyanobacterial toxic genes.

Processing of microarray integrating a barcode, namely, elongation reverse transcriptase probe, was utilized to confine the mRNA emitted from target toxin DNA, also this elongation probe integrated labeled nucleotides that worked sometimes above the background level to increase the signal on the microarray [73]. This made it now possible to detect messenger RNA expressed in very small amounts below the detection limit of conventional HPLC techniques. This technique may serve as a warning system that indicates any waterbody's potential for poisoning. The Luminex scheme, a distinct type of multiplex scheme, also utilizes measurable fluorescent microscopy or fluorescent flow cytometry principles to allow concurrent detection. With a distinct proportion of two fluorophores, each distinctive population of coded beads is internally colored and functionalized through a capture probe specific for connecting with the host nucleic acids.

A reporting molecule (e.g., phycoerythrin combined with streptavidin) recognizes the target's hybridization. A microarray for different species of toxic algae was created with the use of multiplexed, bead-based ranges using flow cytometric detection for color-coded fluorescent bead populations [74, 75]. The attempted development of confirmatory technology remains under consideration for Luminex-based tracking approaches.

**Quantitative PCR (qPCR)**

qPCR refers to use the DNA as a starting material in a PCR experiment. One of the strongest techniques using in molecular biology is the polymerase chain reaction (PCR) [76]. However, there is no exact quantitative information of the initial substance in the analyzed samples when using traditional qualitative "endpoint" PCR.

By contrast, qPCR can overcome the low accuracy of conventional PCR, because, in quantitative PCR, data are available regarding the amount of initial substance. The available qPCR data allows for this estimation due to the presence of fluorescent markers which are integrated into every PCR product as the DNA sequences are amplified. As a result of the changing signal of the fluorescent dye, the level of labeled, amplified PCR products can be calculated at each cycle. The estimated PCR data can be monitored by an automated detector integrated into the PCR machine. Furthermore, the amount of beginning material can be computed in qPCR since data can be obtained for each PCR round using labeled fluorescent probes. During the linear exponential stage of the PCR, this information is tracked through an embedded detection scheme [76].

The fact that qPCR can detect base pair differences means that it can distinguish genetically comparable species or populations [77]. External requirements should be calculated in ecological samples for the measurement of amplified DNA. This can be done through using DNA or plasmid dilution from cultural communities in the laboratory by assessment of the given template density. For each targeted species, a standard curve should be established to deduce its quantities in an unidentified sample due to changes in the amount of the DNA in each cell [77]. The number of genes encoding rDNA could differ between strains of the organism/species and must be taken into consideration when estimating the starting volume of an unknown target sample [78]. Hence, the copy number of estimated genes can be calculated from the qPCR analysis.

Regarding the strategy of using fluorescent dyes, the SYBR Green dye is designed to bind with small amounts of DNA, specifically double-stranded DNA (dsDNA). During each reaction, the increase in dye emission is equivalent to the increase in the resulting copy number of the dsDNA. However, qPCR has a disadvantage in that the dimers of the primer are measured as a result of amplification of the DNA due to nonspecific binding of SYBR Green to all dsDNA produced throughout all PCR cycles. Hence, good primer design is critical in preventing primer-dimer artifacts, which can be tested for by the analysis of the melting temperature of the target sequence relative to the target amplicons (Figure 4) [79].

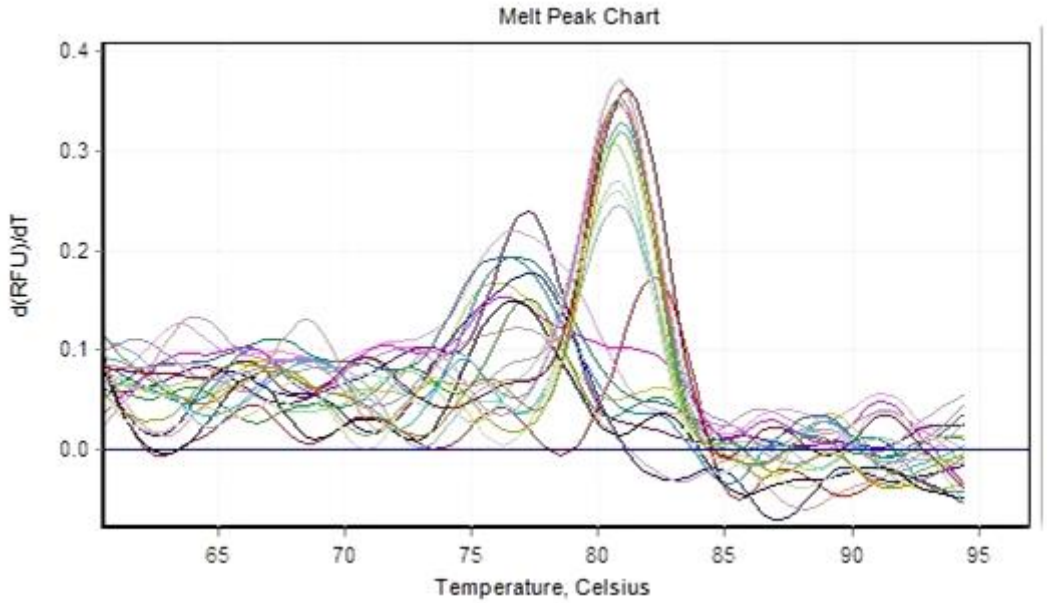


**Figure 4.** qPCR melting curves of algal samples. The melting curves are normally constructed to examine the efficiency of the specific primers used for identifying the tested species or samples.

Using the TaqMan kit for qPCR, amplification signals resulting from the hybridization process with a particular labeled probe are used with particular or non-particular primers. The transition of energy from those in the elevated fluorophore, the donor, to the next fluorophore, the acceptor, occurs when the particular probe connects to its target [80]. This improved fluorescence is referred to as the fluorescence resonance energy transfer FRET.

Fast identification of many unknown species in a single target sample could be molecularly analyzed by using particular primers and fluorescent-labeled probes with varying signal wavelengths. The number of measurable target sequences of the unknown sample is restricted by the number of accessible signal reporters for distinct labeled molecules. Hence, one of the important aims of qPCR is the identification of more than one species (possible n=6) in a single target sample. Therefore, qPCR tests such as the multiplex test involve detailed modifications and must be closely optimized [81].

Robotic (dPCR) is another technique that has gained popularity [82]. In this technique, the sample is distributed as a suspension pipetted to a microwell plate in which one well contains a droplet that includes <1 target DNA molecule. Hence, every well can contain numerous droplets of target DNA, and a single sample can be allocated to thousands of accessible droplets.

Fragmentation of a target sample allows for the assessment of many template molecules throughout claiming the molecular population is based on Poisson distribution. Each fraction will, therefore, include a low number of molecules (0 to 1) giving a positive or adverse response. The DNA of the genome is digested using DNase I to produce DNA fragments (2–4 kb) as a reference. The DNA fragment reference is divided into small droplets, which bind to the specific primer pairs. Hence, every segment of the microfluidic chip is bound by at least several thousand sample droplets. Usually, the amount of specific primer pairs is low compared to the droplets of the DNA template, and a certain number of cycles are required for contact, as well as droplets of the previous reference.

The coalescence of the field sample-containing droplets is achieved by two particles combining with one droplet of PCR were gathered and treated as an emulsion PCR reaction [83]. The DNA molecules are measured in the PCR reaction by measurement of the content of the wells containing the PCR amplicons.

In order to enhance the variety of the assay, distinct primer mixtures can be assigned to different wells of the plate. Presently, there are approximately 6 independent places where digital PCR is being performed, which can be classified into two groups: droplet- and chip-based forms [84]. A single microchip with dsPCR can contain several hundred partitions for every plate, while the droplet-based dsPCR plate generally has many more partitions (20,000), resulting in a high number of reactions—up to 10million. Hence, the complete amount of partitioning carried out and the quantity of partitions relies on the dPCR platform used in the measurement. To

estimate the concurrent quantification of *Microcystis* [85] comparing between qPCR and dPCR. The former was discovered to be simpler to use, but the latter was more delicate and therefore more precise.

Depending on the extraction technique used, distinct DNA extractions methods are known to produce distinct quantities. It is also known that humic substances inhibit PCR reaction. To use a good-quality of DNA isolation technique for example TRI Reagent could be solved or minimize these drawbacks and constraints of qPCR issues. qPCR could be readily conducted during in situ samplings onboard a ship. Maintained samples may also be used, but the inhibition of PCR may result from the preservatives used.

Either no conservation or conservation with ethanol, combined with freezing, are the preferred strategies. In this regard, Hosoi-Tanabe and Sako [86] reported that they could identify and quantify target cells a long time after processing of the target samples.

The sensitivity of qPCR is markedly reduced when the specimens are contaminated with glutaraldehyde or formalin. Additionally, the use of several fixative solutions, such as Lugol's iodine, affects the sensitivity of qPCR, which is clearly reduced [86], and while others have been effectively implemented in identifying toxic algae [87, 88]. Multiplex qPCR tests involve comprehensive optimization to work together on a single PCR experiment with distinct primers and/or samples. Regarding the efficiency of PCR, Handy et al. [77] investigated the use of either conventional or multiplexing PCR on a sample and the and found that although the two assays were competent, multiplexing PCR was performed much better in identification once it was optimized.

**RT-qPCR**

RT-qPCR differs from qPCR in that the used work material in the quantitative PCR is ribonucleic acid (RNA). However, the materials used in the qPCR in the form of mRNA or total RNA are first reverse-transcribed into which called complementary DNA cDNA. The cDNA is then used as a template in the qPCR reaction. It is possible to perform RT-qPCR in either only one step or in two steps [89]. Through the assay with one step, all steps of the PCR can be merged, i.e., the reverse transcription and quantitative amplification can be merged using buffers required for both reactions in a single tube. In one-step RT-qPCR, target-specific primers are used. The RT and qPCR steps are conducted individually in two-step assays with distinct optimized buffer environments for reaction and strategies for priming. Optimizing the one-step technique is almost impossible due to the fact that both reactions are very distinct from each other, requiring distinct temperatures. The one-step technique has the advantage that it is much cheaper than the two-step assay and also has very low susceptibility to mistakes during pipetting. Moreover, the produced cDNA in the two-step technique can be reused at a subsequent date, if necessary. The two-step technique can be used to address more distinct types of objectives.

**Lab-on-a-Chip (LOC)**

This assay is a technique that combines many research laboratory tasks to a single integrating system with only millimeters to several square centimeters in length for robotics and high-throughput monitoring or single or multiple targets [90, 91]. LOCs readily handle extremely tiny quantities of liquid down to less than a few picoliters. Fluorometric, colorimetric, or electrochemical identification can be accomplished. Usually were intended for single-using and expendable, and its applications span a broad range of disciplines [90]. This method is an evolving technology providing custom-designed LOCs for many businesses. Some instances of created LOCs include those used medically in point of care for the identification of numerous pathogens [91, 92].

**Next-Generation Sequencing (NGS)**

The tool is also called high-throughput sequencing (HTS). Ebenezer et al. [93] explained the accessibility of HTS and NGS technology and their main characteristics. HTS and NGS systems have been used in science research by molecular methods, for example, complete genome sequencing. These pyrosequencing methods have much greater performance in producing information, such that, the simultaneous sequencing of millions to billions of sequences can be derived using small amounts of material.

For infield sample research, HTS and NGS techniques collect DNA information from environmentally-friendly DNA or PCR goods amplified by environmental DNA. To produce clonal representatives, the DNA templates will bound to substrates and amplified by PCR however the cloning of DNA templates into vectors of bacterial will not be required for HTS and NGS techniques. The number of sequencing reads by HTS and NGS

techniques is constantly increasing for a read of greater than 500 bp in size, and HTS and NGS are rapidly becoming the preferred tools for identifying and detecting microbes from natural samples [93]. The long-term processing of information, however, remains a significant problem and this makes to use the microarray technique more appealing because it allows analyzing a huge number of samples, especially if the targets are known.

Oxford Nanopore is a superior method of Illumina NGS sequencing [94]. There is now a novel kind of DNA sequence, nanopore, or MinION which analyzes DNA by dragging the molecules in near-real-time through a small, sensitive pore. Each mixture of the genetic bases A, G, C, and T causes the electrical current to shift as the DNA passes through the pore, enabling the molecule to be read (sequenced).

## ADVANCED METHODS BASED ON NANO-BIOENGINEERED PLATFORMS

Various approaches, for instance, microarrays, multiplexed biosensors, and NGS, as stated in the previous section, may possibly solve the issue of broad kinds of species cohabiting in the same aquatic ecosystem. Moreover, the decisive factor determining the feasibility of their surveillance is sometimes the decrease in abundance of specific species in the given setting. In this context, this section of the review describes several methods used to improve the efficiency of molecular techniques based on Nano-Bioengineered Platforms.

### Nano-Bioengineered Probes and Platforms

The biosensors technique, as well as those techniques already listed in the prior section, have gained significance for species-specific detection tasks due to their simplicity, portability, and miniaturization opportunities. Molecular methods for tracking aquatic organisms that rely based on electrochemical biosensors. The system has the ability to quickly track and convert the produced signal into an electrochemical signal. Electrochemical identification can be direct, either through inherent nucleic acid electroactivity and DNA duplex electroactive intercalators or, alternatively, through electroactive probes [95]. The resulting signal can be amplified by a mixture of enzymes, inorganic nanoparticles, fluorescent labels or a mixture of them [96].

By regulating the surface chemistry and surface coverage, the stability, sensitivity, and hybridization effectiveness can be modulated and the particular adsorption can be minimized. Many of the components that operate as transducers are semiconductor materials and noble metals, for instance, polymers and carbonaceous materials. Moreover, nanomaterial construction of transducers, such as nanoparticles, graphene and carbon nanotubes has resulted in the huge development of biosensor devices [97]. The resulting nano-bioengineered constructions are notable for their excellent improvements in the sensitivity as shown by the latest genosensor-based methods. Their greater sensitivity is associated with their expanded surface area and improved catalytic characteristics, including enhanced nanostructure characteristics. For example, immobilized bioreceptors in an uneven nanostructured surface enhance target molecule availability and thus encourage more effective mechanisms of electron transfer and quicker reaction kinetics.

Nano-bioengineered samples are whichever combined or anchored with reporters at the transducer. The first reasonable assembly of nanoparticles into a macroscopic fabric using DNA strands emerged in Prof. Mirkin's groundbreaking work. Such a notable finding made it possible to tailor the characteristics of the nanoparticles via the specificity interaction of the DNA [98]. Recently, Prof. Bard applied Pt nanoparticles with a single labeling reaction to electrochemically amplify the signal for DNA analysis [99].

These are, by far, the most popular materials chosen to amplify the DNA-based electrochemical biosensors thanks to the inherent electrochemical characteristics in AuNPs. Pairing DNA sequences with nanoparticles enable greatly enhanced efficiency to be developed as genosensors. Target content could be inferred either after AuNPs are dissolved in acidic media, subsequently determined by anodic stripping voltammetry [100, 101], or by linking them to electroactive complexes with more chronocoulometry interrogation [102].

Gold tracer "amplification" by silver coating onto the Au surface was also introduced to increase sensitivity and decrease the identification limits of such biosensor types [103]. Regarding the surveillance of aquatic ecosystems, an extremely delicate and specific electrochemical genosensor relying on gold nanoparticles was shown to be appropriate as a diagnostic instrument in the aquaculture sector for the detection of 18S rRNA and internal transcribed spacer (ITS) regions of the fish pathogen *Aphanomyces invadans* [104].

Organic quantum dots (QDs) and inorganic and have proven to be an option for enzyme-based amplification, overcoming its long-term flexibility and storage constraints and its inhibition in hostile environments. For

instance, an electrochemical biosensor protocol with CdS, ZnS, and PbSQDs as markers has been extremely suitable and sensitive for concurrent identification of *Vibrio cholerae*, *Salmonella* sp. non-protein coding RNA sequences, and *Shigella* sp. with elevated environmental sample tracking potential for multiple pathogens [105].
Hybrid nanocomposites have demonstrated improved characteristics with regard to their respective polymers acting alone in the growth of molecular biosensors. For instance, for the subfemtomolar identification of the *Aeromonas hydrophila* DNA sequence in fishpond water, a new DNA biosensor based on decreased graphene oxide-decorated ceria nanoparticles was designed [106]. A graphene oxide (GO)–cerium nanocomposite-selective capture probe was immobilized, and fast Fourier transform square wave voltammetry (FFT-SWV) has been used to determine the existence of a redox marker to discriminate among changes in target analyte levels using a GO, thionine (Thi), and Au nanoparticle-covered $SiO_2$ nanocomposite (GO–Thi–Au@$SiO_2$)-based tag [107].

**Preconcentration and Magnetic Separation Systems**

Several preconcentration schemes have been suggested for addressing the restriction in aquatic ecosystems of a little abundance of microorganisms. However, the most popular practice is to preconcentrate the species by handling huge quantities of water. But the disadvantage of this method is power- and time-consuming and it is not suitable for outdoor surveillance. However, in the EU μAQUA project, >50 L of water was filtered by using hollow fiber filters, which filter efficiently concentrated all living things in 30 minutes [70, 71].
Magnetic micro- and nanocarriers have received great attention and have become the preconcentration systems due to their speed, practicality, cost-effectiveness, high velocity, high-throughput automation, and low amount of work required [108]. The probe-modified magnetic particles will be added to the destination DNA or RNA comprising the sample, which has been selectively captured. A magnet may readily contain the captured target at a particular location and be washed off from the rest of the sample.
The resulting focused goal is therefore free of interventions that may finally occur in the original sample. An establishment of the microfluidic PCR system allowed for the preconcentration of microbial pathogens through magnetic isolation before PCR amplification of DNA [109]. Delicate and specific genomagnetic assay relying on in situ DNA amplification with magnetic primers created by double-hybridizing DNA [110]. The use of a DNA-based genosening approach has been demonstrated for the electrochemical identification of food pathogens. It has also been important for detecting a multitude of species.
Magnetic beads have been implemented in diagnostic microbiology for more than two centuries as a separation method. However, their implementation in microorganism tracking in the environment is scarce [111]. Sequencing of nucleic acids could be in the solid phase, where biotin-labeled amplicons are connected to streptavidin-coated magnetic beads from the target sequences. Denaturation can be accomplished through NaOH or heat treatment, as well as linked to magnetic beads, and the loose dissolved strands isolated by a magnet. For sequencing, all strands could be retrieved.

**Solid-Phase Hybridization**

By connecting a probe to solid-phase support to capture low-abundance target DNA sequences, the target DNA can be immobilized. In being able to identify the existence of small quantities of target genetic material, it has been demonstrated to be a high productivity and performance technique. For instance, femtograms of ribosomal RNA were sufficient for detecting bacterial fish pathogens such as *Tenacibaculum maritimum*, *Aeromonas salmonicida, Yersinia ruckeri*, and *Lactococcus garvieae* using solid-phase PCR. The method was focused on an RT-PCR–enzyme hybridization method for liquid-and solid-phase PCR in one tube using NucleoLink (TM) strips [112].

**Isothermal Amplification**

Apart from PCR, isothermal amplification is a method for the amplification of nucleic acid so all the reactions occur at a stable temperature, therefore the requirement of the thermal cycler reduces and allows amplification to be done in outdoor environments. One of the many isothermal amplification methods, loop-mediated isothermal amplification (LAMP), is a version wherever two or three sets of primers are used to amplify the target sequence, as well as a polymerase with elevated strand displacement and replication.
In addition, the reaction can be further accelerated by a couple of “loop primers”. LAMP is regarded as an improved amplification method because the quantity of DNA generated by ordinary PCR-based amplification is

significantly greater. For the extremely delicate and specific identification of *Eriocheir sinensis* reovirus, a pathogen causing elevated death-rate in crab aquaculture, a fast RT-LAMP assay has been established [113].
The method is delicate, specific, and quick with the possibility for concurrent pathogen screening in a broad range of samples. For the visual identification of *Streptococcus iniae* and *Streptococcus agalactiae* in tilapia, a colorimetric LAMP test was also evaluated [114]. The technique showed usefulness in the fish farming sector for monitoring fish health in grow-out ponds. LAMP has also identified other aquatic pathogens such as *Edwardsiella tarda* [115], *Vibrio anguillarum* [116], and *Vibrio vulnificus* [117] as well as *Lactococcus garvieae* [118].

**Hybridization Chain Reaction**

The HCR is a method depending on a chain reaction of recognition and hybridization events around 2 groups of stable DNA hairpin molecules and store potential energy and provides an enzyme-free option for fast identification of particular DNA sequences [119]. In HCR, different from ordinary PCR and binding DNA to a substratum accomplishes not only identification but also linear amplification of the signal at room temperature without any external input. The DNA hairpin monomers coexist in this method in the solution until the target, functioning as initiator strand, causes a cascade of hybridization occurrences that produce double helices similar to the alternating copolymers [119]. The method has the ability to amplify brief sequence oligonucleotides with high efficiency. For instance, concurrent in situ DNA-HCR to discover many Archaea, Methanosaetaceae, and Bacteria [120].
Similarly, an enhanced in situ DNA HCR technique called fast HCR-FISH was tested for the fast and critical discovery of low rRNA for marine bacteria in marine water and sediment [121]. HCR has recently been used for non-enzymatic amplification, label-free, isothermal, and ultrasensitive electrochemical detection of nucleic acids in tandem with a DNA-fueled target recycling reaction [122]. Although it was only a proof of concept, it was possible to exploit the tandem technique for aquatic ecosystems in the environmental analysis of pathogens.

## INSITU REMOTE SENSING: HAB MONITORING IN BUOYS AS A STUDY CASE

The Environmental Sample Processor is a completely independent, electromechanical fluidic system that was initially intended for collecting separated samples from water, concentrating particulates, and automating the use of molecular analytical methods [123].
It obtains and procedures milliliter sample quantities at the depths of up to 50 m in up to several liters. It is also suitable for subsurface deployment, though it is usually co-deployed with subjective sensors that provide physicochemical and biological data based on the tool's place and depth. The ESP has also been implemented at a fixed depth on a drifter to provide a degree of passive mobility and is suitable for shore-based/pier deployments.
It is publicly accessible from McLane Research Laboratories, Inc. (East Falmouth, MA, USA), but it costs more than $300,000 with the development of its third generation. The chemistry for identification involves membrane-based DNA samples and protein arrays. The qPCR capacity for addressing microbial objectives was demonstrated [124]. DNA probe arrays aim to identify HAB species using rRNA sequence probes in SHA format to detect various organisms simultaneously in a sample with chemiluminescent identification [119].
The protein arrays comprise a competitive ELISA method for the identification of toxins generated by HAB species [125]. "The significance of immobilizing HAB detection on independent platforms that can intelligently target sample acquisition as a function of environmental circumstances and biological patch encounter"[126, 127] is an overriding objective for all toxic algal bloom employees.

## FUTURE DIRECTIONS AND CONCLUDING REMARKS

In practical applications, conventional techniques for pathogen assessment in aquatic ecosystems suffer from constraints and disadvantages. In this regard, the step-by-step profiling of molecular techniques is promising for tracking microorganisms in nature [128]. The practicality of molecular-based methods has been shown to be restricted by both species diversity and presence in environmental matrixes at very low levels. At the same time, multiplexed biosensors, microarray formats, and NGS/HTS have helped cover various types of species assessment.

Since the advent of nanomaterials, the growth of nano-bioengineered samples and platforms has led to the development of biosensors with immense sensitivity, selectivity, and detection limits. Magnetic isolation technologies provide an encouraging solution to low-abundance genetic resource preconcentration. Similarly, the hybridization of the solid phase is an alternative for capturing sequences of low-abundance target DNA.

## CONCLUSIONS

The detection of algae species using morphological examination is still an important method for registration and certification of the biodiversity of different algae species. Moreover, it is considered to be at an early stage regarding the molecular detection of species, such as for taxonomists using fluorescence in situ hybridization (FISH) and microscope-based enumeration techniques. Algae species have large genomes with repetitive sequences and a remarkable ability to survive under the difficult conditions of their surrounding environments. Therefore, these physical properties encourage scientists to discover and document algal biodiversity for different biological applications to bioassessments. Hence, the sequencing of algal genomes will be a regular area of study in the near future for complete algae mapping. Additionally, for algal communities, the use of biosensors, SHA, qPCR, RT-PCR, and microarrays, as well as NGS systems, will create a wealth of beneficial data for the identification of algae species. Moreover, recent tools, such as bioinformatic tools, are greatly needed for the prediction and analysis of complex environmental samples using specific computing software. Such tools, in association with NGS, will help to identify and detect species biodiversity with much higher accuracy and generate inventories of automatic taxonomies of different environmental populations identified through algal bioassessment.

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