

Review

Towards standardized molecular monitoring of HABs: A consensus guide to qPCR

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ABSTRACT

Harmful algal blooms (HABs) are a growing concern in marine and freshwater ecosystems due to their adverse impacts on human health, aquatic ecosystem functioning, and economic activities. Conventional HAB monitoring based on morphological identification remains important but is often limited by time, resolution, and reliance on

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expert taxonomists. Quantitative PCR (qPCR) and digital PCR (dPCR) techniques have emerged as powerful molecular tools for the specific, sensitive, high throughput and rapid detection of HAB species. This review, based on a consensus among experts in the field, aims to provide an accessible, practical guide for integrating qPCR and dPCR for current and future HAB monitoring frameworks. Building upon previously published literature reviews with a broader focus, we present the details and specifics of step-by-step approaches of assay design, validation, application in global and regional monitoring programmes, and recommendations for method standardization and future development. This consensus guide provides valuable information to benefit laboratory users, regulatory and monitoring agencies. Detailed information is provided for ~175 qPCR assays directed at >70 marine and freshwater HAB taxa. Our goal is to bridge the gap between research and operational monitoring, provide a unique, shared perspective of the use of quantitative species-specific molecular methods across applications and world regions.

1. Introduction

Marine harmful algal blooms (HABs) are occurring worldwide and cause problems for fisheries, aquaculture, human health and tourism, due to food safety concerns and socio-economic losses. Ocean climate change, comprising temperature increases, changes in weather and ocean circulation patterns, ocean acidification and deoxygenation, as well as local coastal eutrophication trends, can cause an increase in HAB events (Dai et al., 2023), although trends may vary locally (Brandenburg et al., 2025). Global increases in HAB events may also be attributed in part to increased monitoring efforts in relation to increased aquaculture operations worldwide (Mardones et al., 2021). HABs unequivocally form a threat to human health, as many HAB organisms produce phytoxins that accumulate in seafood species. Consuming such contaminated shellfish can lead to different human poisoning, including Paralytic Shellfish Poisoning (PSP), Neurotoxic Shellfish Poisoning (NSP), Diarrhetic Shellfish Poisoning (DSP), Azaspiracid Shellfish Poisoning (AZP) and Amnesic Shellfish Poisoning (ASP) (Hallegraeff and Bolch, 2016; Sommer and Meyer, 1937). Important taxa responsible for marine biotoxin production include the dinoflagellates *Alexandrium* spp., *Gymnodinium catenatum*, *Centrodinium punctatum*, *Pyrodinium bahamense* (saxitoxins - associated with PSP), *Dinophysis* spp. and *Prorocentrum* spp. (okadaic acid and dinophysistoxins- associated with DSP), *Azadinium* spp. and *Amphidoma languida* (azaspiracids - associated with AZP), and the diatoms *Pseudo-nitzschia* spp. and *Nitzschia navis-varingica* (domoic acid - associated with ASP), *Karenia brevis* and most recently, *K. cristata* (Murray et al., 2025) (brevetoxins - associated with NSP). Bivalve molluscs, such as mussels, oysters, cockles, clams and scallops are key toxin vectors, though other organisms such as crabs, lobster and abalone can also accumulate marine biotoxins (Castberg et al., 2004). Additionally, Ciguatera Poisoning (CP) occurs in tropical and sub-tropical areas, where benthic epiphytic microalgae such as *Gambierdiscus* spp. and *Fukuyoa* spp. produce ciguatoxins which accumulate in fish and marine invertebrates. Furthermore, aerosolized phytoxins from species such as *Karenia brevis* and *K. cristata* (Murray et al., 2025) (brevetoxins, PbTx) and *Ostreopsis* spp. (Palytoxins analogues, PLTX; Ovatoxins, OVTX) can affect the human respiratory system (Berdalet et al., 2022; Lim et al., 2023; Fleming et al., 2007), and *Vulcanodinium rugosum* (Portimine A) that causes severe acute dermatitis in humans (Gorse et al., 2025). HABs can also affect the health of wild and farmed aquatic species (John et al., 2022) and may result in mass mortalities through marine biotoxin production, oxygen depletion upon the collapse of blooms (e.g., associated with *Karenia mikimotoi* blooms) or through the toxic metabolites released to the water. Animal species of higher trophic levels, (i.e. fish, seabirds, mammals) are susceptible to accumulation of algal toxins via consumption of toxic prey species (e.g. Lefebvre et al., 2016), and can encounter disruption of thermal regulation due to mucilaginous covering (Jessup et al., 2009).

Freshwater HABs are due to cyanobacteria which are ubiquitous in most terrestrial water systems. Toxic cyanobacteria can impose a major threat to human health as the water is used for drinking, irrigation and recreation. These blooms consist of potentially toxin producing genera, such as *Microcystis* spp., *Planktothrix* spp., *Dolichospermum* spp.,

Aphanizomenon spp., *Raphidiopsis* spp., *Microseira* spp., *Microcoleus* spp., and *Cylindrospermopsis* spp., where the risk is notably associated with their toxins, including microcystins, saxitoxins, anatoxins, and cylindrospermopsins.

To minimise the health effects of HABs on humans and to ensure food resource sustainability, accurate and early detection is essential. Conventional approaches entail morphological identification via light microscopy (Edler and Elbrächter, 2010; Utermöhl, 1958, 1931). Although these methods are relatively low-cost to implement (except for qualified human resources), they require substantial taxonomic expertise and are often time-consuming. Many methods of light microscope-based identification and counting of HAB species have been devised, with differing levels of detection, precision and accuracy. Differences in HAB species detection and identification using light microscopy depend on factors like the use of particular fixatives, collection techniques (including sampled water volume), the filtration or settling method, the use of fluorochromes targeting cellular components (e.g., thecal plates, chloroplast number and distribution) and the number of cells identified and counted per sample (establishing the detection limit, Godhe et al., 2007; Karlson et al., 2010). However, most importantly, light microscopy-based HAB detection is dependent on the skill and experience of the taxonomist. Nevertheless, it is considered the standard method for identification and quantification of phytoplankton for both freshwater and marine water samples also transposed, per example, to the European Norm EN 15,204: (2006), (BSI, 2006) that is used in accredited laboratories and in intercomparison proficiency tests. Furthermore, many HAB species (especially those associated with fish mortalities) are small (<15 µm), lack cell walls that preserve their distinctive features making them non-distinguishable by light microscopy, or generally lack distinguishing features. Automated optical image instruments and recognition systems, such as the Imaging Flow Cytometers (Imaging FlowCytobot, FlowCam, Planktoscope, Cytosense), offer a higher-throughput, morphology-based alternative (Clayton et al., 2022; Sosik and Olson, 2007) that is less dependent on taxonomic skills. However, there is considerable expensive upfront effort needed to construct an accurate database of reference images and classifiers for automatic species detection and quantification (Sosik and Olson, 2007). These methods also cannot discriminate cryptic phytoplankton species from one another. Cryptic microalgae species are common and are defined by a complete lack of discriminating morphological features, despite important differences in factors such as ecology, physiology, and toxicology.

Given these considerations, molecular genetic tools such as fluorescent *in situ* hybridization (FISH) (Groben et al., 2004; John et al., 2003) and polymerase chain reaction (PCR)-based assays and have been shown to offer increased sensitivity and specificity for detecting HAB taxa. Molecular methods also have the ability to discriminate toxin producers based on the utilisation of specific gene targets involved for the biosynthesis of these toxins. During the COVID pandemic, the application of quantitative PCR (qPCR) became a society-accepted standard tool for pathogen/virus detection (Smith et al., 2024). The instruments and utility of molecular approaches, in particular qPCR, targeting pathogens are nowadays well accepted. The instruments, reagents, and technical

knowledge are widely available and accessible, resulting in a relatively low-cost, rapid and high-throughput method.

Previous reviews in this field have covered a broader spectrum and wider focus (Coyne et al., 2022; Penna et al., 2013). This review, which compliments and significantly builds upon previously published literature, comes from a specifically assembled group of experts in qPCR, dPCR, and molecular methods who aim to provide a consensus-based and unified vision of the possible applications of qPCR and dPCR for monitoring HABs worldwide. This idea was developed from a joint Scientific Committee on Oceanic Research (SCOR) and the Intergovernmental Oceanographic Commission (IOC) of UNESCO- GlobalHAB endorsed workshop held in Hiroshima, Japan in 2023. With the aim of promoting qPCR application, this review provides a standardised consensus guide for using qPCR and digital PCR (dPCR) in HAB detection for their implementation in an operational capacity in routine monitoring and research programmes worldwide as a complement to traditional monitoring methods.

2. Molecular methods for HAB detection: from morphology to molecules

Traditional morphology-based detection using light microscopy remains the most used technique for phytoplankton and HAB species monitoring. However, some species are difficult to identify due to their small size, a lack of any distinguishing morphological features, or cell deformation during preservation (Liu et al., 2022; Medlin and Orozco, 2017). In addition, in many countries, few people are trained with a

sufficiently high level of skill on the taxonomy of marine microalgae. As a response to this, fluorescence *in-situ* hybridization, monoclonal and polyclonal antibodies, sandwich hybridization assays, microarrays, biosensor chips, and various PCR-based techniques have all been developed for HAB detection (Ahn et al., 2006; Bowers et al., 2017, 2000, 2006; Carrera et al., 2010; Galluzzi et al., 2005; Groben et al., 2004; John et al., 2005; Pitz et al., 2021; Toebe et al., 2013).

Among these, qPCR has been widely adopted for the detection of HAB species in environmental samples due to its attributes of high sensitivity, specificity, reproducibility and data release-speed (Fig. 1). qPCR tracks the amplification of a target gene in real-time by measuring the fluorescence response of a DNA- or RNA-specific intercalating dye (as a function of the bound copies of the nucleic acids), with reaction times typically less than one hour (see also Section 3.2). The cost per sample ranges from 1 to 30USD depending on assay design and choice or commercial kit used (excluding personnel and capital equipment). Cost and availability of reagents and consumables can vary across the globe and is dependent on regional availability and supply. Each qPCR assay requires calibration using standard curves and knowledge of gene copy numbers per cell (Penna and Galluzzi, 2013; Pearson et al., 2021; Toebe et al., 2013). dPCR, a more recently developed technique, partitions the extracted pool of DNA from a sample into thousands of reactions, thereby providing absolute quantification without standard curves (Fig. 2). This considerably reduces the potential effect of sample inhibitors that can impact qPCR assay performance. The dPCR platform offers improved sensitivity and robustness but at a higher cost and with slightly longer sample preparation and detection times (Medlin and

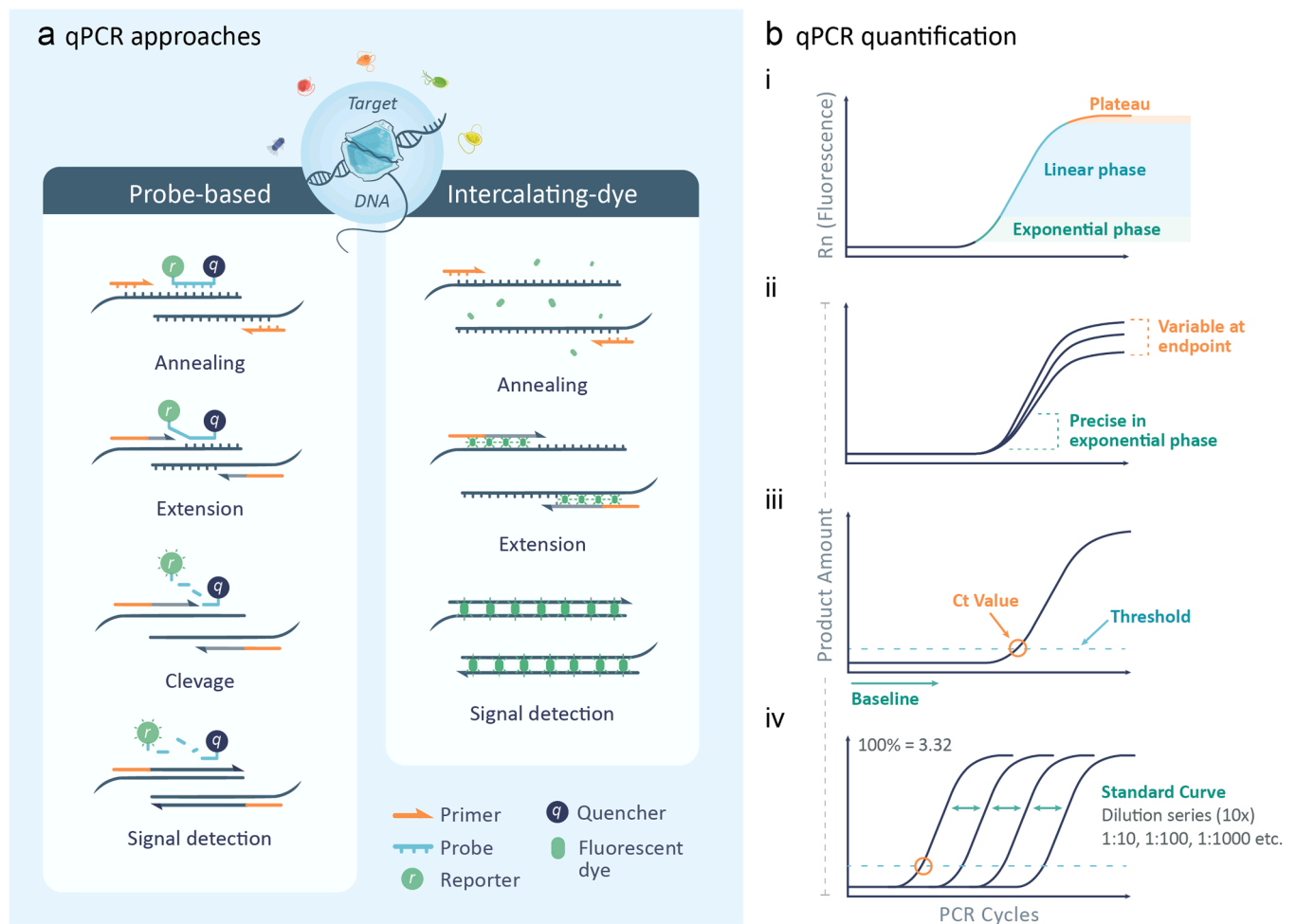


Fig. 1. Comparison of two quantitative PCR (qPCR) detection approaches. (a) probe- and intercalating-dye-based assays and (b) qPCR quantification procedure. (i) Typical amplification curve. (ii) Quantification is in the exponential phase. (iii) The cycle threshold (Ct) value determination. (iv) Standard-curve method.

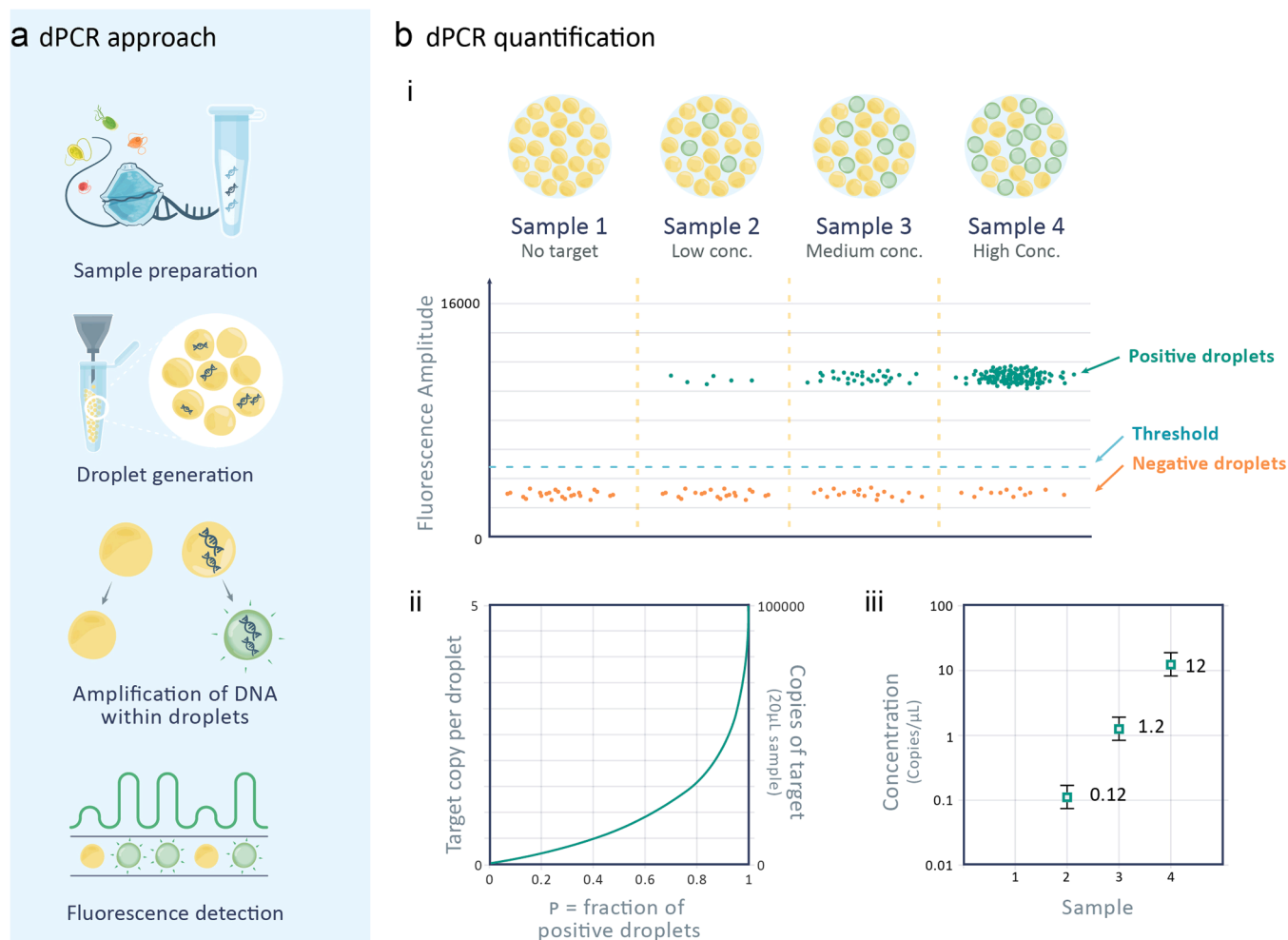


Fig. 2. Schematic representation of digital PCR approach (a) and quantification procedures (b).

Orozco, 2017).

The assays of qPCR and dPCR rely on the same biochemistry and provide specific and quantitative detection in real-time, with assay transferability between these two techniques being relative straightforward and requiring limited optimisation (Weinstock, et al., 2026).

Molecular approaches represent a novel paradigm in HAB monitoring (Jacobs-Palmer et al., 2021; Medlin and Orozco, 2017; Smith et al., 2024.), whereby the focus shifts from the detection of specific organisms to the detection of genes linked to harmful algal species and, in some cases specifically to the synthesis of their toxins (Penna et al., 2015; Ruvindey et al., 2023; Al-Tebrineh et al., 2012). To accurately detect these genes, the assays utilized need to be both sensitive (to avoid false negatives) and specific (to avoid false positives). In the context of HABs, the most prevalent genetic target used for qPCR/dPCR is the ribosomal DNA (rDNA) region. Several genes in this region are ubiquitous and high in copy number per cell and have therefore become common targets for species detection. For marine HAB testing these include the small subunit (SSU, 18S), the internal transcribed spacer regions (ITS1, ITS2 inclusive of 5.8S), and the large subunit (LSU, 28S) (Fig. 3). This rDNA operon generally has high genetic variability to enable the resolution of species and ecotype discrimination. In addition to their frequent use in species identification and differentiation, they are utilised for phylogenetic analysis to determine lineage and placement of HAB species in relation to each other and within the context of all phytoplankton (e.g. Jacobs-Palmer et al., 2021; John et al., 2014). qPCR assays often target different parts of the rDNA operon, depending on the species of interest (see Table S1. Supplementary Material) and

references therein). One potential drawback in the use of qPCR based on regions of the rRNA array is that the variability in copy number of rRNA genes per cell between species and strains can make accurate quantification difficult, especially in dinoflagellates due to their large genomes and repetitive genomic regions (Dyhrman et al., 2006; Perini et al., 2011; Ruvindey et al., 2023). For this reason, it is necessary to ensure that standards such as locally isolated strains are used for quantification.

In recent years, due to the accumulation of further genomic information and a deeper understanding of toxin biosynthetic pathways in cyanobacteria and of some phycotoxins, several qPCR assays have been developed that directly target genes involved in toxin synthesis pathways. Consequently, specific genes responsible for toxin production, such as those encoding microcystin (mcy), cylindrospermospin (cyr), saxitoxins (sxt) and domoic acid (dab), can be targeted to identify and quantify toxigenic populations *in situ*, providing a direct tool to assess potential risks of harmful bloom development (Al-Tebrineh et al., 2012; Gao et al., 2015; Murray et al., 2011; Penna et al., 2015; Savelle et al., 2016; Weinstock et al., 2026). Some analysed marine biotoxin synthesis genes also appear to have more stable copy numbers than rRNA between strains and samples, which may assist in more consistent quantification of blooms temporally and spatially. Toxin biosynthesis genes are generally present in lower genomic copy numbers than rRNA genes, as they are functional genes (Penna et al., 2015; Ruvindey et al., 2023). This means that maybe higher sample volumes are necessary as compared to sample volumes for the use of rRNA based assays to reach the necessary levels of detection sensitivity (Ruvindey et al., 2024). Assays targeting functional toxin genes can be more informative, as they

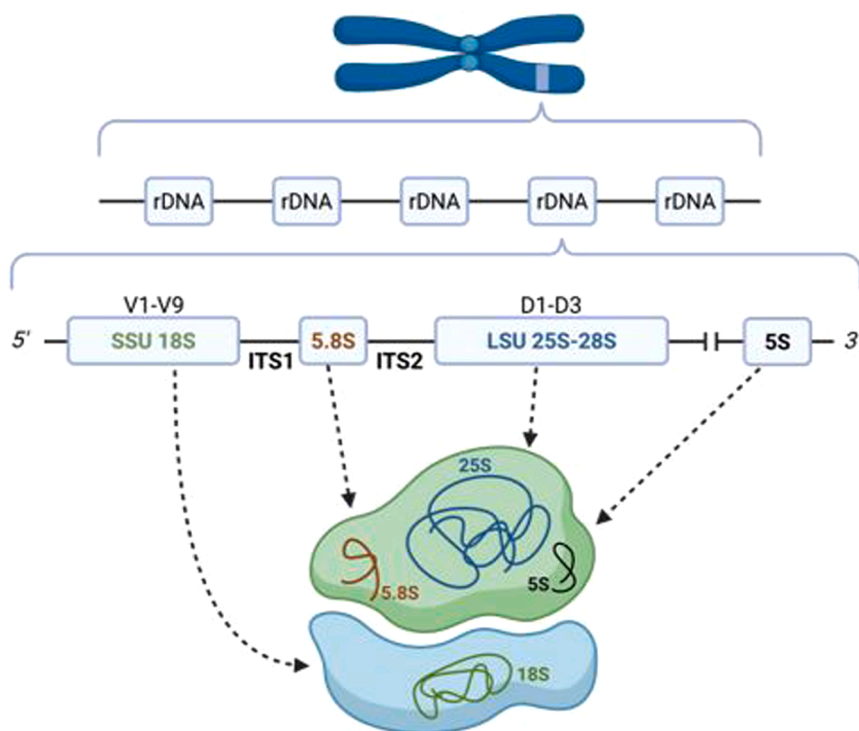


Fig. 3. Schematic representation of the ribosome configuration and common regions for species detection (ribosomal DNA (rDNA), small subunit (SSU), internal transcribed spacer (ITS), and large subunit (LSU)).

enable monitoring of HABs to be based on toxin risk rather than taxonomic identification (Murray et al., 2011), especially since toxin production often varies by strain within the same species (e.g. Bowers et al., 2018; Trainer et al., 2012).

3. From sample to signal: workflow and assay design

This chapter focuses on the key steps and important focal aspects for fully robust and quality controlled procedures to be employed for analysis and result interpretation. These workflow steps are briefly summarised as follows and graphically illustrated in Fig. 4.

- Analyse in laboratories with controls in place to mitigate against contamination.
- Correct sample collection and preservation.
- Validated assay (probe or dye) design specific to target DNA.
- Assessment of DNA extraction efficiency per assay.
- Use of internal and external controls throughout the analysis (Table 1).
- Use of standard curves for quantification of target DNA for qPCR methods.

The general workflow starts with samples being collected (preserved or unpreserved) and concentrated in some way (filtration, centrifugation) prior to extraction of genetic material (DNA and/or RNA) (Fig. 4). Validated assays (including positive and negative controls) are used to quantify a specific genetic target within the extracted pool by comparing results to a standard curve assessing amplification of genes with known concentrations. Throughout each of these steps sterile/aseptic techniques must be maintained to prevent potential and reduce contamination as to not compromise samples/results. It is highly recommended that dedicated separate laboratories and work areas are assigned for each of the processes, e.g. separate clean room, DNA extraction laboratory and a PCR amplification laboratory, each with their own specific equipment, work processes and flows. New or acid-washed containers (bottle, carboy) should be used, the working surfaces for extraction and

qPCR/dPCR reaction set-up should be decontaminated (e.g. 10% bleach), gloves must be worn (and recommended to change frequently) during the whole process, and the use of aerosol barrier pipette tips (changed between samples) is crucial.

3.1. Environmental sampling and nucleic acid extraction

Samples for the detection of HAB species can be collected from various environmental matrices, including water, sediment, aerosol, and biological tissue. The volume/weight collected will depend on the species of interest and the chosen matrix. Gently mix the sample to ensure a homogenous mixture before the concentration step (this is crucial when only a subset of the sample is being processed). Filtration or centrifugation is used to concentrate material and needs to occur as soon as possible after sample collection if samples are not preserved. For transport to the laboratory, samples should be stored in a cool, dark environment, thereby eliminating deteriorating effects from light and temperature changes, as well as impacts from predator/prey grazing or bacterial degradation if samples are live. Cell pellets or filters are then stored at either -80°C or in liquid nitrogen for RNA extraction or at -20°C for DNA extraction. DNA (or RNA for gene expression studies) is extracted using a commercial kit or published protocol (e.g., CTAB or phenol-based). The extraction method selected must remove/limit the potential presence of PCR inhibitors (e.g., humic acids, polyphenols, polysaccharides) found in environmental samples to ensure sensitivity and accurate quantification (see Section 3.4 for assessment via controls). Extracted DNA is stored at either -20°C or -80°C and extracted RNA at -80°C . Repeated freeze-thawing should be avoided as this may degrade DNA/RNA quality and yield.

3.2. Assay development and validation

There are several factors related to adopting and implementing qPCR/dPCR assays, including choice of extraction methodology (Section 3.1), probe chemistry (assay design), instrument type, and use of appropriate standards and controls. Groundwork guidance for adopting

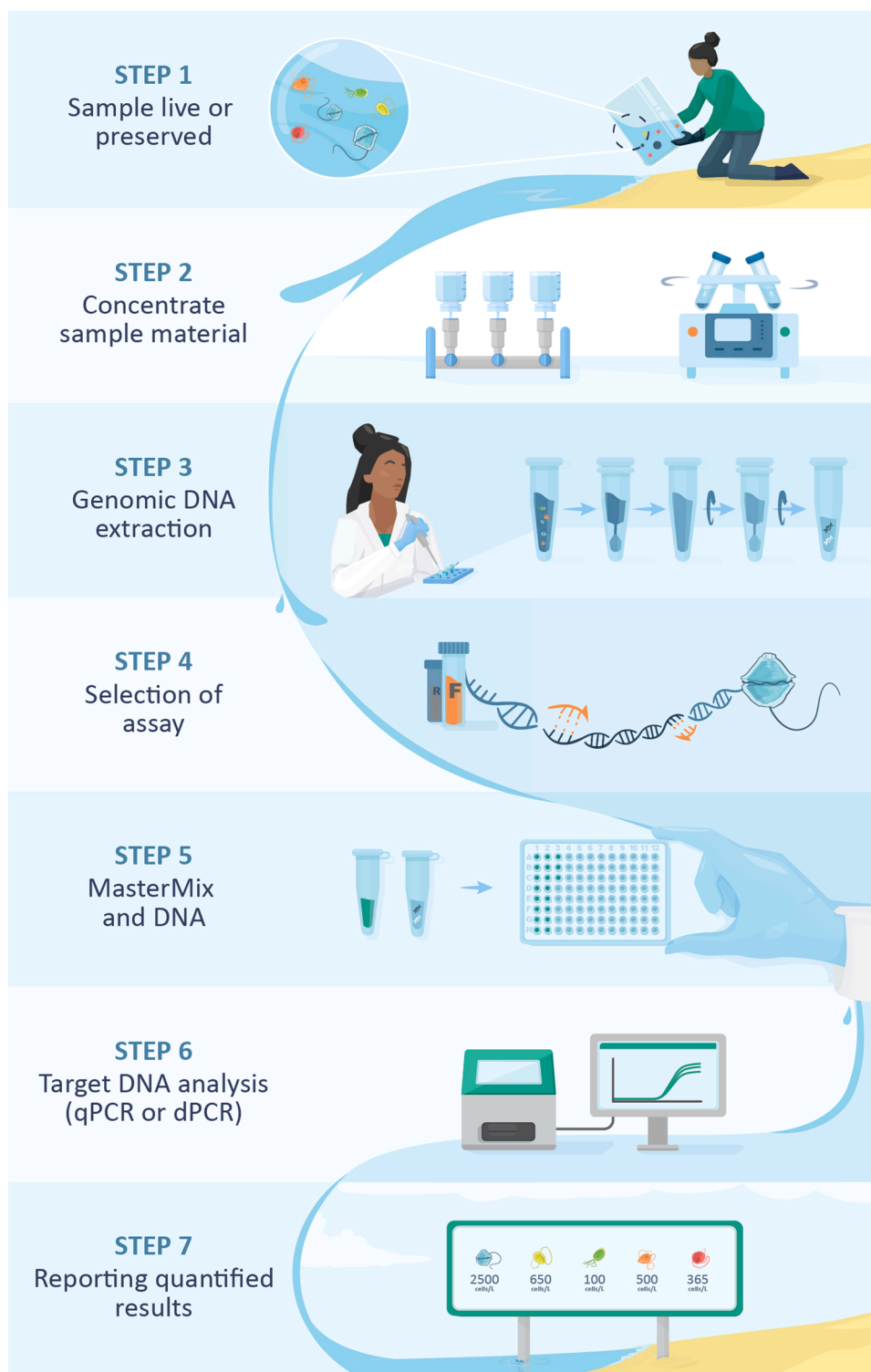


Fig. 4. Schematic overview of the key steps from field sampling to molecular analysis and target quantification.

and implementing an assay includes consulting the literature for published assays of the target species, downloading available sequence data from a public database (e.g. GenBank, PR2), and constructing sequence alignments for the target species and closely related taxa. These initial steps will aid in the selection of a suitable species-specific (typically 18S rDNA, ITS, or 28S rDNA) or biosynthesis toxin gene target (typically *mcy*, *sxt*, *dab* or *cyr*) for primer/probe design. Software packages (e.g. Geneious, primer3 or other non-commercial or open-access) can design bespoke primers/probes based on user input of sequence data (*in silico*),

while assay developers are highly encouraged to simultaneously consult the MIQE guidelines (Bustin et al., 2009) and technical guides available on vendor websites for important design parameters adequate to the reagents to be used.

There are two general types of assay chemistries used in qPCR and dPCR (Fig. 1a).

Intercalating-dye (e.g., SYBR Green) assays: a DNA-binding dye intercalates into double-stranded DNA. Fluorescence increases in proportion to amplicon accumulation during PCR. Probe-based detection

Table 1

Positive and negative controls in qPCR: types, applications, and expected outcomes. Ct: Cycles threshold.

Control Type	Base Used	Purpose / Expected Outcome
Positive PCR control	Defined stock (e.g., plasmid, gBlocks, purified PCR product) containing the target gene used in PCR amplification	Confirms PCR reagents and cycling conditions are functional. Should amplify at a known Ct (ideally just above the detection limit).
Positive extraction control	Sample of similar matrix spiked with a known amount of target DNA (e.g., filtered seawater + target) used in DNA extraction and PCR	Verifies DNA recovery and detects inhibitors. Should amplify at the expected Ct; delayed or absent amplification suggests inhibition or loss.
External amplification control	Exogenous (non-target with own probe/primers) DNA control added to PCR reaction used in PCR amplification	Confirms the assay is functioning as expected. Should consistently amplify at a known Ct. Can detect inhibition or issues in sample processing and extraction efficiency.
Exogenous process control	Known amount of unrelated DNA sequence spiked into the sample used in PCR amplification	Detects PCR inhibitors in the sample matrix. Should amplify consistently; failure indicates inhibition.
Endogenous process control	Endogenous reference (housekeeping) gene co-extracted with target DNA used in DNA extraction and PCR	Assesses DNA extraction quality and sample integrity. Should always amplify if extraction was successful.
Negative PCR control (No Template Control)	Molecular biology grade (nuclease-free) water instead of sample DNA used in PCR amplification	Detects contamination or primer-dimer formation. Should show no amplification.
Negative extraction control	Sample of similar matrix without target sequences (e.g., filtered seawater) used in DNA extraction and PCR	Detects contamination introduced during extraction or amplification. Should show no amplification.
No amplification control	Reaction with master mix lacking DNA polymerase used in PCR amplification	Confirms no unintended amplification without enzyme activity. Should show no amplification.

provides higher specificity and enables multiplexing, whereas dye-based assays require melt-curve analysis to verify specificity.

Intercalating-dye (e.g., SYBR Green-based) assays might be sensitive to non-specific-amplification generating non-specific fluorescence, this can be examined with additional melting curve analysis to determine the specific melting temperature related to the target amplicon. Therefore, we recommend to initially test a variety of primer combinations and subsequently screen monocultures of target and closely related species (preferably isolated from the geographical region where assays are to be deployed) to assess specificity *in vivo*.

Probe-based (e.g., TaqMan 5'-nuclease) assays: a dual-labelled probe carrying a reporter and quencher hybridises within the target between the primers. During extension, the polymerase's 5'→3' exonuclease activity cleaves the probe, separating reporter from quencher and generating fluorescence proportional to product formation. Probe-based assays, allow for greater specificity, sensitivity, and multiplexing options (detecting more than one species/gene in a reaction). Probe assays are generally preferred for species-level detection. Probes and primers should be extensively validated (*in silico* and *in vivo* where possible) to ensure specific amplification of target DNA with no demonstrated cross-reactivity with non-target species. An assay validation scale has previously been developed, entailing necessary validation steps to ensure accurate species-specific detection for routine molecular monitoring.

3.3. Optimization and standard curves

For qPCR, assay efficiency and sensitivity are evaluated using standard curves based on dilution series (minimum of five points) of known templates (e.g., plasmid containing the target amplicon; e.g. Bowers et al., 2006), synthetic oligos (IDT gBlocks; e.g. Kon et al., 2015), or extracted DNA from monocultures (Fig. 1b). Acceptable reaction efficiency is typically between 90 and 110% with an $R^2 > 0.98$ (Bustin et al., 2009). Technical replicates of samples and controls should be employed to determine assay precision and upper/lower limits of detection. Establishing the copy number of the target gene region per cell is useful (Ben Amor et al., 2022; Hariganeya et al., 2013; Hii et al., 2024; Murray et al., 2019; Penna and Galluzzi, 2013; Yarimizu et al., 2021), in particular for monitoring programmes based on HAB cell concentrations in water samples.

3.4. Controls

For a qPCR/dPCR assay to be valid it must include a set of controls to ensure that no inhibition or contamination has occurred, to assess performance (efficiency) and sensitivity (lower limit of detection), and to provide quantification of the target DNA. A range of positive and negative controls options is listed in Table 1.

3.5. Ensuring accuracy in qPCR: validation and troubleshooting tips

There are a variety of issues that can arise along the PCR pipeline. Table 2 outlines some common problems and solutions, while many qPCR reagent vendors provide more detailed troubleshooting guides on their websites and product information.

4. Global applications and monitoring practices for marine HABs

qPCR methods for marine HAB species have been applied globally in both research and operational HAB monitoring programmes, while dPCR adoption remains a newer approach. The degree of integration varies by region depending on local expertise, infrastructure, budgetary resources, regulatory frameworks, and historical bloom dynamics. Below, we give examples of the way in which molecular genetic methods and specifically qPCR is currently being used in monitoring programs worldwide.

4.1. Asia

In Southeast Asia, HAB monitoring focuses heavily on *Pyrodinium bahamense*, identified relatively easily using light microscopy due to its large size and clear morphology. While molecular assays have been developed for *Alexandrium tamiyavanichii* (Kon et al., 2015, 2016; Hii et al., 2024) and *Karlodinium australe* (Kon et al., 2017), these are not yet routinely implemented. More specifically, Japan relies on morphology-based monitoring via regional fisheries offices, though qPCR assays have been developed for Multiple species, including *Alexandrium catenella*, *A. pacificum*, *Chattonella* spp., and *Karenia mikimotoi* (Hosoi-Tanabe and Sako, 2005; Kamikawa et al., 2005, 2007). South Korea integrates qPCR into a national HAB monitoring framework, especially for early detection of *Margalefidinium polykrikoides* and *Alexandrium* spp. The qPCR method is used at low cell concentrations (<10 cells mL^{-1}), switching to light microscopy at higher levels. National guidelines support the use of qPCR in sampling design and data interpretation (Park et al., 2014). China has applied qPCR for mapping the East Asian ribotype of *M. polykrikoides* along the coast, providing baseline data for future monitoring system development and also mapping the four main harmful algal species, *Karenia mikimotoi*, *Margalefidinium polykrikoides*, *Prorocentrum donghaiense*, and *Heterosigma akashiwo* in the East China Sea (Yangtze River estuary) via qPCR methods and analysed

Table 2
Overview of common qPCR problems and troubleshooting tips.

Observation	Possible cause	Recommended action
Amplification in No Template Control (NTC)	Contamination or primer-dimers	- For SYBR Green assays: Examine melting temperature (T_m) on melt curve, which will correspond to primer-dimers (low T_m), target amplicon (same T_m) or non-specific target (different T_m). - An electrophoresis gel can be used to verify amplicon size from a SYBR Green or probe-based assay. - Repeat reaction with new aliquot of qPCR enzyme mix and new primer aliquots.
No amplification in positive control.	PCR instrument, reagent, or positive control issues (degradation, omission).	Repeat the reaction using a freshly prepared positive control.
Inaccurate amplification of Internal Reference or Housekeeping Gene.	Possible presence of PCR inhibitors.	Dilute the DNA template (e.g. 1:10, 1:100) with molecular biology-grade water and repeat PCR.
Poor precision in the cycle threshold (C _t) of technical replicates.	Incorrect volumes of reagents were pipetted into the PCR reaction.	- Verify pipettor calibrations. - Repeat the experiment with careful addition of components.
Poor precision in biological replicates.	- Non-homogeneous sample. - Natural variation in template amount.	- Repeat extraction. - Provide sufficient replication to overcome variation.
Unexpected low or no amplification of the target gene.	Complexity of the target (e.g., GC-rich regions or secondary structures).	- Choose DNA polymerases with high affinity for DNA templates and more suitable for amplifying difficult targets. - Use additives to enhance PCR (e.g. DMSO, BSA). - Use internal positive control to check the accuracy of the PCR reaction. - May need to re-design primer(s) and/or probe.
No amplification of the target gene in experimental samples that should contain the target, with correct amplification in the positive control.	- No target or very low abundance of target present in samples. - Pipetting errors. - Cycling conditions are not optimal for primer annealing and extension.	- Repeat extraction or concentrate DNA samples (e.g. ethanol precipitation, commercially available kits). - Repeat the experiment with careful addition of components. - Reduce the annealing temperature. - Increase time for annealing and/or extension (for SYBR Green Assays). - Repeat sample extraction to exclude contamination. - Verify specificity of primers. - Redesign primer(s).
Double peak in the melt curve.	Non-specific amplification of contaminating DNA in the sample.	- Repeat sample extraction to exclude contamination. - Verify specificity of primers. - Redesign primer(s).

their possible response to the main ecological status under the global climate change (Hu et al., 2022, 2024).

4.2. Oceania

New Zealand's Marine Biotoxin Monitoring Programme integrates qPCR assays alongside microscopy to rapidly confirm the identity of potentially toxic taxa, especially *Alexandrium* spp., *Karenia* spp., and benthic taxa such as *Gambierdiscus* spp. (Ruvindy et al., 2018; Smith et al., 2017, 2014, 2016). The qPCR results support biotoxin monitoring decisions and have improved toxin management. In **Australia**, for marine HABs, qPCR has been used to detect *Karenia* species during a widespread HAB in 2025 (Murray et al., 2025), as well as many HABs of *Pseudo-nitzschia*, *Dinophysis* and *Alexandrium* species (Ajani et al., 2022, 2021; Ruvindy et al., 2018). qPCR-based HAB detection was directly used on a large mussel farm in Tasmania, by farmers themselves, over a period of 18 months and was able to detect two large-scale HAB events producing Paralytic Shellfish Toxins (PST), at least two weeks before PST toxins were detected in shellfish tissue (Ruvindy et al., 2024). In **French Polynesia**, qPCR assays are coupled with passive artificial substrate samplers to target epiphytic and benthic HAB species. These methods are applied during field surveys to determine the abundance of *Gambierdiscus* species to correlate with ciguatera fish poisoning risk assessments (Darius et al., 2017).

4.3. Americas

Chile has implemented large-scale microscopy-based monitoring through the Fisheries Development Institute (IFOP). Recently, molecular tools, particularly qPCR assays for *Alexandrium catenella*, have been incorporated to improve resolution and early warning capacity (Espinoza et al., 2021). In the **United States**, several coastal states operate long-term monitoring programmes (e.g., SoundToxins, ORHAB, CalHABMAP) that employ a combination of light microscopy, imaging, and molecular tools, although wide adoption of qPCR has not come to fruition. Along the mid-Atlantic coast, qPCR has been employed as a monitoring tool in support of aquaculture (Pease et al., 2021) and shellfish industries (Wolny et al., 2020) for *Margalefidinium polykrikoides*, *Alexandrium monilatum*, *Karlodinium veneficum*, *Prorocentrum cordatum* (minimum) and *Chattonella subsalsa*. Although **Canada** does not have a phytoplankton monitoring program for harmful algae, a research project has recently been funded by ArcticNet (<https://arcticnet.ca/project/sustaining-inuit-led-assessment-and-risk-management-of-harmful-algal-blooms-using-omics/>) to study HABs and food web linkages in the Canadian Arctic. One of the objectives of this project is to empower Inuit communities to detect HABs (*Alexandrium* and *Pseudo-nitzschia* species) using on-site genomic-based tools for early detection.

4.4. Europe

Within the EU official-controls framework for shellfish safety (which mandates monitoring of toxin-producing phytoplankton in classified harvesting areas and the use of validated methods), qPCR has moved from research into supplementary, operational use in several national programs alongside light microscopy and LC-MS/MS. **Ireland** has been active in designing and developing, and also implementing existing published molecular assays since 2007 using a combination of several assay types, including SYBR Green, TaqMan and Fluorescence Resonance Energy Transfer (FRET) hybridization assays. These assays, some developed through research projects i.e. PHYTOTEST, ASTOX II, PSPSafe have been used to detect and quantify specific known toxin-producing species in field samples of the genera *Alexandrium*, *Amphidoma*, *Azadinium*, *Dinophysis* and *Pseudo-nitzschia* in Irish coastal waters. **Scotland** has likewise developed and field-evaluated species-specific TaqMan qPCR assays for *Alexandrium* (e.g., *A. tamarense* groups, *A. minutum*, *A. ostenfeldii*) to support harmful-algal risk management and

targeted screening during official-control investigations (Catherine et al., 2009). In **France** the long-term monitoring systems REPHY based of morphological identification of HAB species is supported by qPCR identification for specific and problematic cases. In parallel qPCR assays both in water, superficial and ancient sediments (ancient eDNA) have been developed and applied for research purposes for *Alexandrium minutum* (Klouch et al., 2016a, 2016b) and *Ostreopsis* spp. (Drouet et al., 2021). A qPCR-based monitoring system is in under development across the French Spanish Basque countries to follow *Ostreopsis* cf. *ovata* and *Ostreopsis* sp. 9 blooms. This specifically adapted qPCR monitoring will provide local stakeholders with a practical, coast-effective, and rapid tools to prevent problems to beachgoers during summertime when toxic aerosol occur in the region (project Ostreobila). In **Spain** (Galicia), the regional reference laboratory INTECMAR has piloted molecular screening on routine monitoring samples (e.g., microarray/phylochip approaches) to complement conventional counts during toxic events, illustrating the region's broader shift toward DNA-based tools (Medlin, 2013). In **Portugal**, complementary to the monitoring, qPCR methods are being developed for detection and differentiation between species, which is performed as required by conventional PCR for *Pseudo-nitzschia*, *Karlodinium*, *Azadinium* and *Gymnodinium*. qPCR methods are currently being developed.

More broadly, multiple European programs and research consortia have deployed genus, species, and toxin gene-targeted qPCR assays across Baltic, Atlantic, and Mediterranean coasts for taxa including *Alexandrium*, *Azadinium*, *Gambierdiscus*, *Ostreopsis* and *Pseudo-nitzschia*, indicating that qPCR is increasingly adopted and technically consolidated in EU monitoring practice (Casabianca et al., 2014; Penna et al., 2013).

4.5. Africa

There is currently limited documented use of qPCR/dPCR for HABs in African coastal regions. In **Tunisia**, groups have developed and applied TaqMan qPCR assays (e.g., *Karenia* spp. Multiplex, *Gymnodinium catenatum*) with field applications, illustrating research-level use rather than established national PCR-based control (Ben Amor et al., 2022; Elleuch et al., 2020). In **Namibia**, training and capacity enhancement in implementing molecular methods and tools, capacity-enhancement and technology transfer have commenced to support their current HAB monitoring programmes.

5. qPCR assays for marine harmful algal bloom species

More than 150 different qPCR assays have been developed and published in the peer-reviewed literature for detecting marine HAB species (Table S1, Supplementary Material). These assays target 78 different marine HAB species, representing ~35 assays for PST producers, 5 assays for DST producers, 24 assays for *Gambierdiscus*/*Fukuyoa* species potentially involved in CTX or MTX production, 4 assays for YTX producers, 5 assays for AZP producers, 6 assays for PTX or related toxin producers, 10 assays for *Pseudo-nitzschia* species potentially producing DA, 25 assays for fish-killing dinoflagellates (e.g., *Karenia*, *Karlodinium* etc.), 20 assays for fish-killing raphidophytes, 8 assays for fish-killing haptophytes, and 2 assays for pelagophytes. Of these, about 50% were designed for SYBR green chemistry, while most of the remainder apply TaqMan or similar probes. Almost all assays are species-specific and target regions of the rRNA array (Table S1, Supplementary Material), while 7 assays target functional genes related to PST genes (sxt). In addition, several assays have been commercialized and are available for purchase in a dried format. In studies in which the genomic copy number of rRNA has been determined, this has varied greatly, from 10^1 to 10^7 copies per cell (Table S1 Supplementary Material).

6. qPCR Assays for freshwater Harmful Algal Blooms

Over the last three decades, genes targeting cyanobacterial genera and/or species, as well as those involved in toxin synthesis have been developed (Ouellette and Wilhelm, 2003). For example, genes targeting cyanobacterial genera and/or species include the phycocyanin operon (*rpoC1*) and rRNA 16S genes. Specific genes for toxins include those involved in the synthesis of microcystins (*mcyA*, *mcyB*, *mcyD*, *mcyE*, *mcyF*, *mcyG* and *mcyJ*), saxitoxins (*sxtA*), anatoxins (*anaC*) and cylindrospermopsins (*cyrA*, *cyrJ*) (Dittmann and Börner, 2005; Kurmayer and Christiansen, 2009; Yuan and Yoon, 2021) have been studied extensively. They have become the principal targets for the development and standardisation of qPCR assays in detecting toxic cyanobacteria (Table S2, Supplementary Material). Standardised toxin gene-based assays developed from these targets are now accepted and have been accredited for use in risk assessment and general monitoring programs by a number of utilities and associations and are used in the risk assessment of cyanotoxins in both drinking and recreational waters.

Since 2018, the state of Ohio in the USA has used qPCR in their public water system response strategy (DeWine and Logue, 2025). The state utilises 16 s rRNA gene to monitor for the presence of cyanobacteria and toxin producing genes (*mcyE*, *sxtA*, & *cyrA*) to detect the presence of toxin producing species which triggers a sampling requirement for toxin analysis. Specifically, if either of the *cyrA* or *sxtA* genes are above the stated detection limit (<0.18 gene copy/L) there is an immediate requirement for toxin analysis, whereas for *mcyE*, a threshold of 5 gene copies/L is used before toxin testing is initiated to confirm a HAB event in the raw water source. In their validation of the method the 5 copy/L threshold detected all cases where the microcystin levels was over 5 $\mu\text{g/L}$ and 90% of the cases where it was over 1.6 $\mu\text{g/L}$, the US EPA guidance level for adults.

A critical component of the Ohio's EPA response strategy guidance, is the mandate that certified laboratories follow an accredited SOP validated by the State "Ohio EPA DES 705.0 Ver 1.0 2018" when performing analyses for the State's Public Water Systems.

The use of qPCR to assess HAB risk using toxin gene copy number is supported by several studies, including one by the US EPA (Lu et al., 2020) where the authors determined a threshold criteria of 4.46 *mcyE* gene copy/L for microcystin levels above 1.6 $\mu\text{g/L}$, and a recent test of bathing waters in the Netherlands, showed that *mcyE* copy numbers overall corresponded to microcystin concentrations in the water (Schürmann et al., 2024). These findings show that a microcystin concentration of $12 \mu\text{g} \times \text{L}^{-1}$ (i.e., the highest WHO alert level; Chorus and Welker, 2021) corresponds to about 4.3×10^6 *mcyE* copy number/L.

Many US utilities have now integrated a molecular approach into their HAB monitoring programs. As an example, Wichita Falls, Texas (Adams et al., 2022) highlight the use of image flow cytometry, qPCR, ELISA and LC-MS as tools they utilise in an integrated approach for cyanobacteria monitoring.

Given the development and availability of both cyanobacterial DNA reference material and a standardised qPCR assay format, a recent study (Pinheiro et al., 2025) demonstrated through an interlaboratory performance study that a qPCR based DNA quantification method can reach levels of accuracy and reproducibility comparable to those achieved with digital PCR technology. Specifically, of the 55 scored study results obtained from 14 different laboratories using a qPCR-based technique, 62% were within the 8% relative expanded uncertainty based on dPCR measurements, while 100% of the study results returned satisfactory z scores calculated using a set performance coefficient of variation equivalent to one Ct value.

Standard Methods for the Examination of Water and Wastewater, a voluntary consensus standards body sponsored by the American Public Health Association (APHA), American Water Works Association (AWWA) and the Water Environment Federation (WEF) provide guidance on standard methods for their stakeholders. These stakeholders submit methods that they feel have reached utility to drive uptake. As a

measure of developing acceptance for molecular methods for cyanobacteria HABs the Board of the organisation were asked by their stakeholders to consider reviewing and publishing a Standard Method. A Task Group reviewed the data published in the qPCR interlaboratory performance study (Pinheiro et al., 2025), along with real world applications and recommended its publication as a standard method (10,120 quantitative PCR for cyanobacteria and cyanotoxin-producing genes 2025).

In Australia molecular tools have increasingly been used routinely to complement traditional identification. qPCR methods are very commonly used in Australia for freshwater monitoring and are applied by most drinking water utilities to understand the presence of cyanobacteria, often using assays based on cyanobacterial toxin genes. In a recent draft of new Australian Recreational Water Quality Guidelines published for public consultation, qPCR for toxin species detection is being proposed as an option to determine whether the recreational water sources can maintain normal surveillance or move to an alert level that mandates further laboratory analyses (Australian Recreational Water Quality Guidelines (the draft Guidelines). The National Health and Medical Research Council NHMRC (2026).

Worldwide there are multiple studies underway to evaluate the utility of molecular methods, specifically qPCR assays targeting toxin pathway genes, including a qPCR assay for cyanotoxins (Al-Tebrineh et al., 2012) in the Baltic Sea (Malin Olofsson, pers. comm. to Bengt Karlsson) and the Water Research Foundation, Project 5266 which is trying to determine the role of spectral imaging as an early warning system for the presence /significance of algal bloom, qPCR is being used as a reference measure.

7. Challenges of qPCR-based quantification and application

Accurate qPCR-based quantification of HAB species remains difficult because of: (1) the natural copy-number variability of the target gene and (2) the properties of the standard curve used for calibration. Gene dosage (the number of copies of a region of genomic DNA) can differ by orders of magnitude among isolates of the same species, so a single threshold cycle (Ct) is not a fixed proxy for cells mL⁻¹. In dinoflagellates such as *Alexandrium*, Ruvindey et al. (2023) analysed 51 strains and recorded 7- to 20-fold differences in both rRNA operon and *sxtA4* gene copy number. Thus, two field samples that yield identical Ct values may have cell densities that differ by a full log₁₀ if those per-cell copy numbers are not normalized. Comparable variability has been reported in diatoms: Andr  e et al. (2011) showed that ITS copy number among *Pseudo-nitzschia* spp. varies sufficiently to shift qPCR-derived abundances by a factor of three to five.

Even when the gene dosage is known, standard-curve construction introduces additional error. Plasmid standards often amplify more efficiently than genomic DNA (gDNA) because they lack chromatin and secondary structure, artificially steepening the slope and inflating calculated copy numbers (Lin et al., 2011). Linearized gDNA from a well-characterised reference strain (or better, a synthetic gBlock that mimics the natural amplicon length) more closely resembles environmental templates and therefore produces slopes (-3.3 ± 0.1) and efficiencies (95–105%) that match MIQE guidelines (Bustin et al., 2009).

To mitigate these issues, best practices now recommend using linearized genomic DNA standards derived from the target organism (or empirically calibrated by digital PCR), constructing fresh standard curves for each run, and incorporating inter-assay controls. Ideally, these should be complemented by standards from several locally isolated strains of the target organism, or a dilution series of the HAB to be measured, as compared to microscopy cell counts from that HAB. dPCR can be used to determine actual copy numbers per cell, providing ‘gold-standard’ calibration that improves qPCR accuracy and reproducibility in HAB monitoring (Kim et al., 2025; Lee et al., 2017; Pinheiro et al., 2025). Another key step forward is to use single- or low-copy marker genes, which may provide more reliable quantification than multi-copy

targets like ribosomal DNA. However, current known single/low copy genes frequently lack the taxonomic resolution required to distinguish closely related species, for example, within genera such as *Alexandrium* and *Pseudo-nitzschia*. Based on the fast-increasing genomic data resources the development of novel, species-specific molecular markers in the future may help overcome this limitation and offer consistent, high-resolution detection. Hence to date we recommend the use of ribosomal markers, but regional genomic variability must be considered when utilizing ribosomal DNA data, since rDNA copy numbers and sequences can vary greatly even amongst populations of the same species from the different regions of the world.

7.1. Recommendations for incorporating qPCR/dPCR into monitoring frameworks

1) **Assay Selection and Validation:** Laboratories should begin by identifying assays relevant to HAB species in their region, using those published and validated in at least one laboratory, such as those listed in Tables S1 or S2, Supplementary Material as a guide. Where possible, monocultures of regional strains, including non-target taxa, should be used to assess assay performance. As copy numbers of rRNA genes will vary among strains of the same species, if assays targeting these genes are used, rRNA gene copy number per cell must be validated using locally isolated strains or consider using functional gene assays to avoid this source of variability. Equipment and reagents used should be validated before use.

2) **Sample Preparation Standardization:** Use of consistent filtration volumes (>1 L but more is obviously better), extraction kits, PCR reagents, positive controls and inhibition controls will improve the reproducibility and comparability of results. Kits and reagents that have been specifically developed for environmental samples will reduce issues associated with PCR inhibition.

3) **Capacity Building:** Investment in equipment and personnel training is essential for routine applications. Organization and participation in regional workshops, development of a long lasting, multi edition qPCR school, inter-laboratory comparisons, and open-source publication of assays is highly encouraged. There are a variety of qPCR/dPCR platforms on the market, with new ones always in development. When evaluating which platform to purchase, consider the following parameters related to user needs: portability, sample capacity, number of available fluorescence channels, and reagent and consumable requirements (proprietary versus off-the-shelf).

4) **Data Integration:** Molecular results should be integrated into broader HAB surveillance systems alongside microscopy and toxin analysis for comprehensive risk assessment. The development of international databases on HABs would further facilitate the integration of qPCR-based data.

5) **Digital PCR Expansion:** While still more expensive, dPCR offers advantages in precision and inhibition resistance. Future monitoring should evaluate the integration of dPCR where absolute quantification is required.

6) **Global Collaboration:** A coordinated international database of validated qPCR/dPCR assays and protocols would improve data sharing, reduce duplication, and accelerate capacity development, and help to identifying troubles (challenges) and finding corrective measures.

7) **Complementary eDNA approaches:** qPCR and dPCR provide accurate quantification of known HAB species but are limited to taxa with available assays. To capture emerging or unexpected species, these methods should be complemented by other molecular techniques, such as eDNA metabarcoding, using universal markers. This would enable a more comprehensive and adaptive monitoring framework and providing additional data about the biodiversity and potential shifts and appearance of potential invasive HAB species.

8. Conclusion

This review states an international consensus view suggesting qPCR and dPCR as the most effective powerful tools for advancing HAB monitoring. These approaches provide more specific, sensitive, and scalable detection methods that can complement traditional techniques based on morphological identification. This user guide aims to support agencies and researchers in adopting molecular methods by providing practical advice and global case studies towards fostering international coordination. With increased access, harmonization, and collaboration, these tools will significantly contribute to early warning and response systems for HAB events worldwide.

This consensus guide highlights a number of areas within qPCR/dPCR monitoring approaches, which could be standardised from a global perspective, including sample collection, preservation and storage, laboratory procedures and workflows to reduce/mitigate for contamination, DNA extraction protocols, use of internal and external controls to validate the assay and assess potential inhibition and extraction efficiency, the use of standard curves for quantification and reporting units. From a regional perspective, it is also highlighted that before implementing molecular assays in a region, they should be designed, tested and validated to ensure high specificity to the target by using local species and cultures representative of the monitoring region based on the toxin profiles observed and known.

Among the advantages, provision of sensitive and precise quantification of targeted species through high-throughput platforms enable laboratories to screen large numbers of samples more quickly and cost-effectively than with light microscopy alone. Molecular assays reduce the risk of human error, particularly when target species occur at low concentrations or are morphologically similar, and when designed against toxin-synthesis genes, can distinguish toxic from non-toxic strains in real time. Unlike microscopy, these methods require less specialized training, making them accessible to a broader range of technical staff. As programs shift from reporting cell counts to reporting molecule abundance, correlating gene-copy estimates with toxin accumulation in shellfish will help regulators and industry stakeholders embrace molecular data. Positive qPCR/dPCR results can still be confirmed by microscopy or chemical analyses, ensuring a layered, robust approach to HAB surveillance (Fig. 5).

Looking ahead, one of the strengths of qPCR and dPCR monitoring programmes are best realised when combined with complementary novel and traditional approaches and methodologies, including standard traditional microscopy and metabarcoding, automated imaging systems with morphological and trait-based classifiers for species identification and quantification, and high-throughput sequencing (HTS) for broad taxonomic surveillance and also functional insights through metagenomics and metatranscriptomics. Together, these integrated workflows could enable and improve both the discrimination and quantification of known HAB taxa and the early detection of emerging novel or unexpected species, supporting more comprehensive, adaptive, and proactive HAB monitoring and management.

Regions with diverse algal threats may need multiple assays or multiplexed formats, highlighting the need for rapid, high-capacity testing pipelines and a detailed understanding of regional HAB species. Furthermore, natural variation in target-gene copy number can affect quantitative accuracy unless calibration standards (ideally derived from linearized genomic DNA or digital-PCR-verified copy numbers) are rigorously maintained.

Through continued harmonization of protocols, data sharing, and collaborative training, qPCR and dPCR will become increasingly integral to effective, timely responses to harmful algal bloom events.

CRedit authorship contribution statement

Uwe John: Writing – review & editing, Writing – original draft, Supervision, Resources, Investigation, Formal analysis,

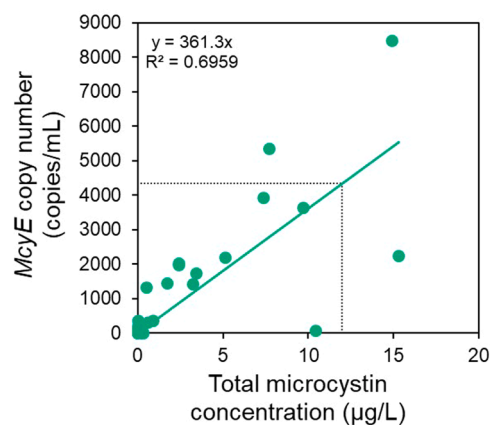


Fig. 5. Relationship between *mcyE* copy numbers and microcystin concentration in the water. Data represent field data from 11 lakes in the Netherlands at various time points in the period May–October 2019. Modified from Schürmann et al. (2024).

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Declaration of competing interest

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Supplementary materials

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Data availability

all data and links are in the manuscript

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