24 Laboratory and Field Applications of Ribosomal RNA Probes to Aid the Detection and Monitoring of Harmful Algae

K. Metfies, K. Töbe, C. Scholin, and L.K. Medlin

24.1 Introduction

As discussed elsewhere, harmful algal blooms are steadily increasing worldwide. Given the numerous threats posed by them, early warning of harmful blooms and rapid detection of the species that cause them is highly desirable. Many countries have launched monitoring programs to serve as warning systems. Samples are typically transported to a centralized laboratory and examined for harmful or toxic species using traditional methods based on microscopy (e.g. Todd 2003). This approach has proven very successful, but becomes more difficult to manage as the number of samples and frequency of their collection increases, reaching a point where analysis of a sample can take days. This time lag can increase further when more elaborate methods are required to identify positively species that cannot be resolved using light microscopy alone. Such time lags impair our ability to provide an early warning of bloom events. For this reason, on-site, near real-time water analysis is desirable. This requirement can be met in part by equipping trained observers (paid staff or volunteers) with field microscopes to speed up assessments concerning the relative abundance and distribution of numerous species (Conrad et al. 2003), and that information can be used in an adaptive fashion to "flag" specific samples for expert examination. Whereas this can help alleviate some time lag associated with identifying problem species, there still remains a need to quantify them and accommodate the need for detailed analyses when required. Towards that end, we consider here application of ribosomal RNA (rRNA) targeted probes in fluorescent in situ hybridization (FISH) and DNA probe array formats. Reviews of other techniques that also have proven useful for identifying harmful algae in cultured and natural samples as well as the prospects of using those methods aboard in-water autonomous sensor systems are found elsewhere (Scholin et al. 2003).

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24.2 Ribosomal RNA Sequences as Markers for Phylogenetic Studies and Species Identification

Molecular biological techniques have greatly enhanced our ability to understand phylogenetic relationships among organisms and to develop means to detect specific species, genes and gene products. Although a number of genetic markers are used for this purpose (Paul et al. 1999), ribosomal RNA (rRNA) genes have historically figured most prominently in studies of harmful algae. Ribosomal RNA molecules have a number of attributes that make them excellent molecular markers (Woese 1987). The conserved and variable regions of the molecule can be used to develop oligonucleotide probes of varying specificity, making it possible to identify phytoplankton at various taxonomic levels from classes down to species or strains using whole-cell and cell-free formats (Scholin et al. 2003; John et al. 2003, 2005; Metfies and Medlin 2004).

24.3 Fluorescent In Situ Hybridization (FISH) for Identifying Intact Cells

Fluorescence hybridization (FISH) allows the rapid detection of algal groups and even the separation of closely related, morphologically similar species or strains (Lim et al. 1993; Miller and Scholin 1996, 2000; Simon et al. 1997, 2000; Peperzak et al. 2001; Rhodes et al. 2001; John et al. 2003, 2005; Groben et al. 2004; Groben and Medlin 2005; Anderson et al. 2005). FISH techniques rest on hybridization of fluorescently labelled oligonucleotide probes to rRNA within intact cells, thus “labelling” target species when appropriate reaction conditions are met.

The FISH technique begins with a fixed sample to preserve overall cell morphology, reduce autofluorescence, and permeabilise the cell wall to exchange probes and hybridization solutions. Several FISH protocols are in use, but the diversity of organisms targeted makes it difficult to find a method that works well for all or at least most species. Many different fixation and sample processing protocols have emerged in recent years. For example, Anderson et al. (2005) have used a two-step fixation where cells are initially treated with formaldehyde, and within 36 h resuspended in cold methanol and then stored refrigerated. For *Alexandrium*, preserved cells were stored for at least 1 year without signal loss. Others have explored alternative means of preserving samples for FISH. Medlin and co-workers compared different fixation protocols and found that saline ethanol treatment (Scholin et al. 1996) gave good results with most species tested, but it must be made fresh and is stable only for a few hours. ”Modified saline ethanol fixative“ has a reduced ethanol concentration and is stable for several months at room temperature, Cells preserved using this solution can be stored for at least 1 month at room temperature without signal loss (Miller and Scholin 1998, 2000). Its ease of preparation, low toxicity, and stability make it an attractive choice when working outside of a laboratory, but samples must be processed within several weeks after collection. Some species will autofluoresce even with prolonged exposure to it. Here 50 % dimethylformamide (DMF) treatment can help (Groben and Medlin 2005).

FITC (fluorescein-5- isothiocyanate) is often used to label probes in FISH experiments. With an excitation maximum of 494 nm and an emission maximum of 517 nm labelled algal cells are coloured light green and are normally easily distinguished from non-labelled and autofluorescent cells appearing orange to red in colour depending on the filter set (Scholin et al. 2003). Negative controls are always recommended in FISH experiments, e.g. "no probe" (hybridization buffer only) and/or application of a labelled probe that does not react with the target species (NON-EUK 1209R; Amann et al. 1990; Scholin et al. 2003). Another common FISH fluorochrome is Cy5 (Cyanin5), a derivative from indodicarbocyanine (Shapiro 2003), which has an excitation maximum at 649 nm and emission maximum at 670 nm in the far red/close infrared region. Visualizing Cy5 requires use of infrared sensitive cameras connected to the microscope.

Hybridization reactions for phytoplankton are often performed on polycarbonate filter membranes with one or two fluorescently labelled probes. In addition to flow-through methods where a sample may be captured in a filtration apparatus with an entire hybridization process following (e.g. Miller and Scholin 1998, 2000; Anderson et al. 2005), filters can also be cut into pieces and treated individually for the detection of more algal species (e.g. Miller and Scholin 1998; John et al. 2003). Where target and non-target sequences are very similar, stringent hybridization conditions must be used. Groben and Medlin (2005) found that addition of formamide in the hybridization buffer (up to 20 %) and salt reduction in washing steps permitted discrimination of single-base mismatches. Sodium dodecylsulfate (SDS), the detergent commonly used in FISH hybridization buffers (Amann 1995) can destroy fragile cells like unarmoured dinoflagellates. In contrast, IGEPAL-CA630 (or the chemically identical NONIDET-P40) maintains cell integrity while giving a good probe penetration into the cell. Fading of the fluorescence signal is reduced by applying an antifade mounting solution to the filter before sealing it with a coverslip on a slide (e.g. SlowFade Lite™, Molecular Probes, Inc.). The sealed filter can be examined microscopically directly or stored at –20 °C for several days without a loss of the fluorescence signal. Finally, DAPI (4′, 6-diamidino-2-phenylindole) counterstains cells' DNA (Shapiro 2003). DAPI is excited with UV light at 365 nm and stains DNA bright blue.

24.3.1 TSA-FISH for Flow Cytometry

Enumerating labelled phytoplankton cells manually using epifluorescence microscopy has proven a viable means of conducting a variety of research and monitoring programs (e.g. Rhodes et al. 2004; Scholin et al. 2003; Anderson et al. 2005), but the approach is time consuming and demanding when dealing with large numbers of samples. For this reason a number of workers have explored the use of flow cytometry. Flow cytometry (FCM) detects microalgal cells in liquid suspension based on their optical characteristics. Phytoplankton can be rapidly counted and sized with FCM by analyzing cells autofluorescence (Veldhuis and Kraay 2000) but limited discrimination of taxonomic groups and species is possible (e.g. Jonker et al. 2000; Marie et al. 2005). Application of the FISH technique enhances resolution afforded by FCM. Most flow cytometers equipped with a single light source use an argon laser emitting blue-green (excitation 488 nm), and dual-laser instruments possess a red (excitation 633–640 nm) helium-neon or diode laser as well. Larger benchtop and sorting cytometers often contain a UV laser (excitation 325–365 nm). Fluorescein and Cy5 have been used in FCM protocols (Shapiro 2003) and dual labelling of phytoplankton in one sample is possible. FISH for flow cytometry is typically performed in suspension. The cells are processed in a tube with reagent exchange generally achieved by centrifugation. Careful and precise removal of the supernatant is required to minimize the cell loss. Additionally, the adhesion of cells to the tube surface can lead to cell loss. Treating the tubes with surfactants, adding surfactants to the cell suspension and sonification can remedy this problem (Biegala et al. 2003). High autofluorescence, low rRNA content and poor accessibility of probe target sites can result in weakly labelled cells using a traditional FISH technique (Fuchs et al. 2001). The tyramide signal amplification (TSA), or synonymously called catalyzed reporter deposition (CARD), method can overcome this problem and can be used with FCM (Biegala et al. 2003). The enzyme horseradish peroxidase (HRP) is linked to the 5’- end of the oligonucleotide probe, and in the presence of small amounts of hydrogen peroxide it converts its labelled substrate, tyramide, into short-lived, extremely reactive intermediates that covalently link to electron rich regions of adjacent proteins, such as tyrosine residues. This reaction only occurs adjacent to the probe target sites (Schönhuber et al. 1997, 1999; Pernthaler et al. 2002). The fluorochrome that is bound to the tyramide can be FITC, Cy5 or Alexa fluor conjugates (Shapiro 2003). This method greatly enhances signal intensity relative to a fluorescent label directly attached to a probe. However, naturally occurring peroxidases can lead to non-specific binding of the tyramide and therefore must be quenched (Pougnard et al. 2002). Addition of dextran sulphate to the fluorescence tyramide substrate solution improves localization of the fluorescently labelled tyramide and prevents its free diffusion before it is immobilized (Schönhuber et al. 1999). Hybridization reactions with TSA must be performed between 35 and 37 °C to prevent HRP denaturation, so higher formamide concentrations in the hybridization buffer are required to ensure probe specify. Negative control reactions also include a "no probe" treatment with the addition of the labelled tyramide. When proper care is taken, the TSA method greatly improves the signal-to-noise ratio, particularly for bacteria (Schönhuber et al. 1997), cyanobacteria (Schönhuber et al. 1999; West et al., 2001), picoplankton (Not et al. 2002, 2004; Biegala et al. 2003) and bacteria associated with microalgae (Biegala et al. 2002; Alverca et al. 2002).

24.3.2 TSA-FISH for Solid-Phase Cytometry

Solid-phase cytometry (SPC) combines the advantages of FCM with image analysis (Kamentsky 2001; Lemarchand et al. 2001). In SPC, a laser is moved over cells immobilised on a solid support (Vives-Rego et al. 2000). SPC allows the rapid enumeration of several thousand cells with similar accuracy to FCM and is reported useful for the detection of rare events as compared to epifluorescence microscopy and flow cytometry (Lemarchand et al. 2001). The Chem*Scan*™ (Chemunex, France) is a SPC (Mignon-Godefroy 1997; Reynolds and Fricker 1999), initially developed for industrial and environmental microbiology (Vives-Rego et al. 2000). Recently, it was adapted for the detection of toxic microalgae using antibodies (West et al. 2006) and oligonucleotides probes (Töbe et al. 2006). The Chem*Scan* ™ with a 488-nm argon-ion laser is suited for FITC labelled probes. Samples are collected by filtration onto membranes, treated with the probe, and subsequently scanned. The optical system records emissions at three different wavelengths: green (500–530 nm), green-yellow (540–570 nm) and yellow-amber (570–585 nm) (Bauer et al. 1996; Roubin et al. 2002). Fluorescent particles are detected and the Chem*Scan* computer program applies discrimination criteria to discriminate between "true" and "false" events (Roubin et al. 2002), while calculating the signal ratios of the positive signals detected at the three wave-length intervals. The oligonucleotide probe-labelled cells ("true" signals) can automatically be distinguished from particles or auto-fluorescent cells ("false signals"). Expected cell size and shape can be defined for more discrimination. Positively identified cells are coloured spots on a scan map, a display of the membrane. The cells are visualized by transferring the membrane holder to an epifluorescence microscope equipped with a computer controlled motorized stage connected to the Chem*Scan*. Each positive point is validated by the user as desired (Reynolds and Fricker 1999; Roubin et al. 2002). TSA enhancement is strongly recommended for reliable detection of target cells with the Chem*Scan* (Fig. 24.1), because FITC-labelled cells give insufficient intensity for sufficient discrimination between labelled and non-labelled cells. Although promising, this method is only adequate for counting spherical microalgae, not long filamentous cells. However, improved software should eliminate this problem. Thus, validation of the positive counted cells, at least for a subset of the filter, is recommended.

24.4 Detecting Many Species Simultaneously Using DNA Probe Arrays

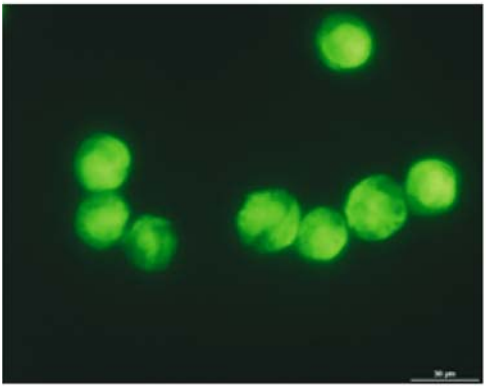
FISH and TSA-FISH only allow the identification of one or a few organisms at a time (Metfies and Medlin 2004; Groben et al. 2004). Cells must remain intact throughout those procedures, so careful sample handling and efforts to minimize cell loss are needed. Thus, many researchers changed to cell-free methods, such as those that rely on inferring presence of organisms based on detecting sequences of nucleic acid in solution. DNA probe arrays offer more possibilities to identify numerous “signature sequences” simultaneously in a single sample (DeRisi et al. 1997; Lockhart et al. 1996; Brown and Botstein 1999). According to the “Taxonomic Reference List of Toxic Plankton Algae” from 2002 (IOC 2002), there are approximately 85 algal species that can form blooms and produce potent toxins. Many are cosmopolitan. Many areas are threatened by multiple toxic algal species. Thus, DNA probe arrays could be developed to monitor harmful species, as well as many other organisms of interest. In this section we present several methods based on this concept wherein probes are attached to a solid support and target sequences are detected using fluorescent, electrochemical and chemiluminescent reporting.

24.4.1 Microarrays on Glass Slides and Fluorescence Detection

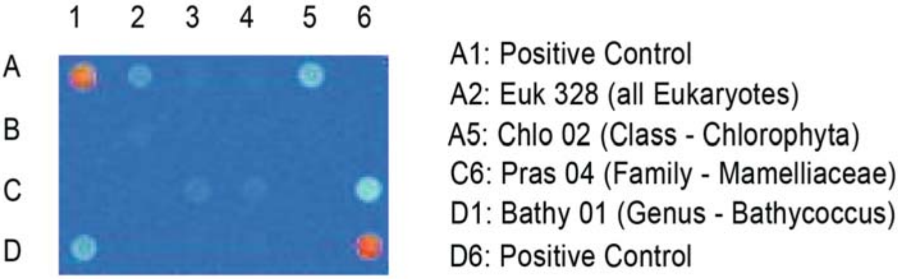
DNA microarray technology is based on an ordered array of probes attached to a solid support (Lockhart and Winzeler 2000; Shena 2000; Rampal 2001). Deposition of probes onto “DNA-microchips” is achieved in two ways: direct synthesis on the chip-surface (Singh-Gasson et al. 1999) or deposition on the surface with a high precision robot. Glass slides are the most commonly used solid support for DNA-microchips because they have low auto fluorescence.

Traditional microarray experiments employ a step to label fluorescently the target DNA/RNA prior to hybridization with the chip. The labelling step can either happen directly by an incorporation of a fluorescent dye into the target or indirectly by the incorporation of some other moiety (e.g. biotin) that is detected with another fluorescent label (Southern et al. 1999; Cheung et al. 1999). Once the hybridizations are completed, the chip is scanned with a device containing a laser or a polychromatic light source, and the fluorescence pattern recorded (DeRisi et al. 1997).

Probe development for arrays is based on known sequences. Although the number of ribosomal RNA sequences is continually growing (Maidak et al.



24.1. Whole-cell hybridization coupled with tyramide signal amplification and *Alexandrium fundyense* (CA 28) TSA-FISH with HRP-labelled probe NA 1 (Miller and Scholin 1998). Bar = 30 :m



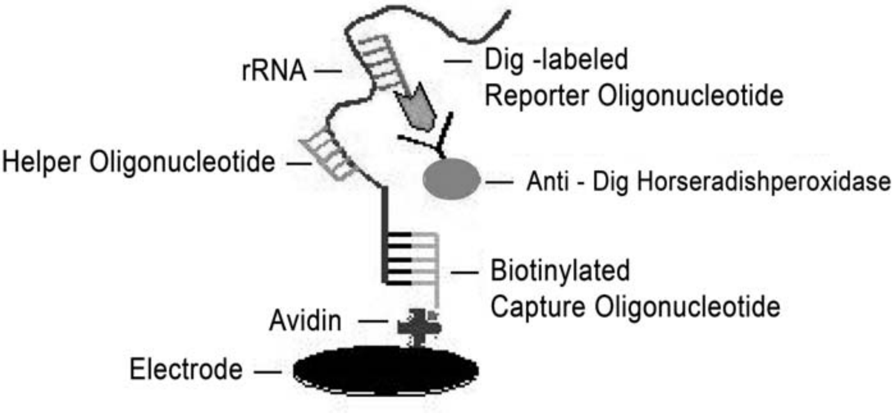
24.2. DNA-microarray that contains a hierarchical set of probes for identification of the genus *Bathycoccus*. The microarray was hybridized to a PCR-product that was amplified from a clone assigned to the genus *Bathycoccus*. The *dots* result from a hybridization of the immobilized probe with the target DNA

2001), it is widely accepted that the majority of microbes are unknown at the rRNA level. Consequently, the probe specificity has to be updated continually with respect to new sequences and results of empirical tests. Cross-reactions are always possible, particularly when dealing with environmental samples. The application of hierarchical probes, ones that detect that target species at different taxonomic levels, is possible given the large number of probes that can be spotted on a single chip. This approach makes identification of harmful species more accurate because a "positive" detection would depend on multiple probes all reacting with each sample. Hierarchical probes were implemented on a preliminary DNA-microarray dedicated to the assessment of phytoplankton composition (Fig. 24.2). That array contained probes limited to higher taxonomic levels of algae. Environmental samples taken in the North Sea at Helgoland were analysed using that chip. The data obtained were consistent with clone library results from the same samples (Medlin et al. 2006).

Despite the obvious potential of DNA-microarrays to facilitate monitoring and identification of microbes and harmful algae (Guschin et al. 1997; Loy et al. 2002; Call et al. 2003; Metfies and Medlin 2004), some limitations and pitfalls should be noted. Developing and evaluating DNA-microarrays is time-consuming and costly. Cross-reactions are always possible. Second, current hybridization reactions require all probes to have the same optimal hybridization temperature. This is not a trivial task when dealing with many probes, and intensive optimisation experiments are required to find a comprehensive set of probes that work well under the same conditions (Feriotto et al. 2002; Boireau et al. 2005). Third, whereas it is possible to quantify the amount of target bound to probes on the DNA-chip, it remains to be determined whether those signals can be correlated reliably to cell numbers in natural samples (Anderson et al. 2005). Commercial DNA microarray processors and scanners are currently designed for use in the laboratory.

24.4.2 Handheld Array Device That Uses Electro-Chemical Detection

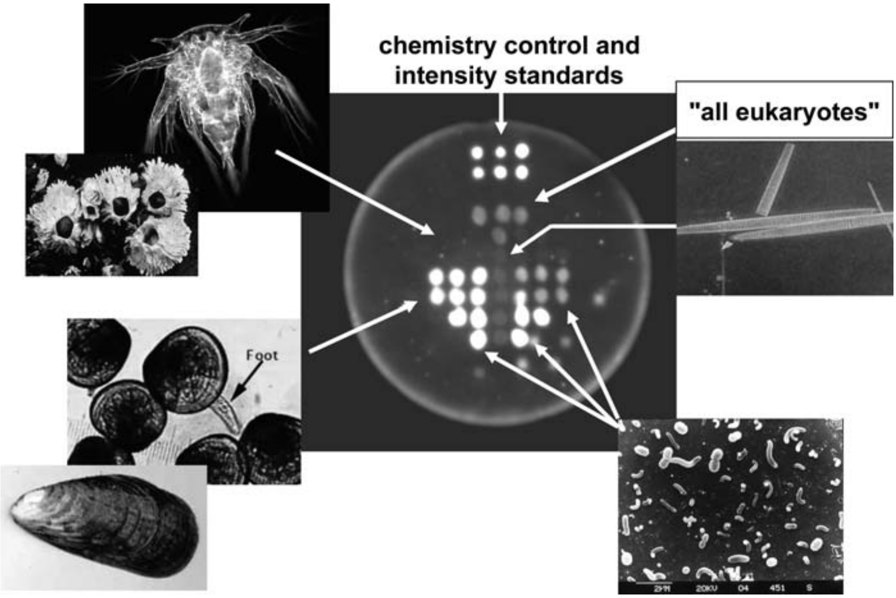
Electrochemical detection of DNA probe/target hybrids offers an alternative to fluorescence-based systems (Azek et al. 2000; Litaker et al. 2001; Baeumner et al. 2003; Metfies et al. 2005). Such applications have been explored at the AWI (Bremerhaven, Germany) for the detection of *Alexandrium ostenfeldii* (Metfies et al. 2005). The sensor consists of two major parts: a disposable sensor chip and a portable handheld device in which the chip is inserted for the measurement of the electrochemical signals. Detection of target sequences utilizes a sandwich-hybridization method that takes place on a carbon electrode of the chip (Zammatteo et al. 1995; Rautio et al. 2003, Fig. 24.3). Sand-



24.3. Principle of electrochemical detection of nucleic acids using sandwich hybridization wich-hybridization reactions use a set of two probes that bind in close proximity to the target nucleic acid. In the current assay one probe is immobilised via biotin on a carbon electrode coated with avidin. The second probe signals the captured molecules via an antibody-HRP reaction that, in turn, catalyses the reduction of H_2O_2 to water. The resulting redox electron-transfer is measured as current and is proportional to the amount of target applied to the sensor. The device is currently limited to the detection of *A. ostenfeldii* and *A. tamarense* but is being expanded in the EU ALGADEC project to regional chips for 14 species. Manual isolation of the RNA is done prior to analysis; fully automated sample processing is planned.

24.4.3 DNA Probe Arrays for Autonomous Detection of Species Using the Environmental Sample Processor (ESP)

The ESP is an electromechanical/fluidic instrument system designed to collect discrete water samples from the ocean subsurface, concentrate cells (par-



24.4. Example of a custom 25-mm DNA probe array developed automatically in the ESP using a natural seawater sample. Printed for demonstration purposes are (*top center*) probes for chemistry controls and array intensity standards, and (*clockwise, right to left*) small-subunit rRNA-targeted, probes for "universal eukaryote" "pennate diatoms," specific groups of marine bacteria, *Roseobacter* and *Cytophaga* , mussel larvae and barnacle larvae . This array illustrates the simultaneous detection of mussel larvae, marine bacteria including *Roseobacter* (weak) and *Cytophaga* (strong), and pennate diatoms (weak); barnacle larvae were not present in this sample. ©2005 MBARI ticulates), and automate application of rRNA-targeted probes. In addition, the ESP archives discrete samples for a variety of nucleic acid analyses, microscopy and other types of analytical procedures after the instrument is recovered. The ESP has been applied to detect a broad range of marine planktonic organisms (Fig. 24.4). "First generation" ESP prototypes were deployed in Monterey Bay, CA, and Gulf of Maine, ME, USA (Goffredi et al. 2005; Scholin et al. 2005).

To develop a probe array, the ESP first collects a sample and removes seawater, then homogenizes material retained using a chaotrop and heat. A crude homogenate is applied to the array, followed by a sequence of reagents that reveal target molecules retained at specific locations on the array grid using sandwich hybridization and chemiluminescence (Scholin et al. 2005). An array image is captured by a CCD camera and transmitted to a remote location for interpretation (Fig. 24.4). The entire process, from collection of a live sample to broadcast of the imaged array takes about 2 h and occurs sub-surface. The reagents employed in the ESP assays are stable for extended periods (none require refrigeration), and the chemical reactions are amenable to microfluidic scaling. Different arrays can be tailored to specific groups of organisms. The ESP can support detection of many different rRNA target sequences using a common methodology, suite of reagents and core sample processing instrumentation.

24.5 Conclusions

Molecular techniques can be used to unequivocally and rapidly identify, and in some cases quantify, particular species and strains of harmful algae. Integrated instrument systems that are designed to automate sample preparation and distribution, as well as to employ molecular probe technologies for detecting a variety of cell markers and processing raw data to speed up and aid interpretation of assay results, are becoming increasingly common, although limited to use in a research laboratory. Much of that technological revolution is driven by the biomedical research and diagnostics industries, but parallel applications in environmental science have also firmly taken root. For example, "portable" sample preparation and analysis systems designed for use outside of a laboratory are gaining attention, but at this time are not routine in environmental research and monitoring practices. Prototypes of autonomous, in-water sensors that utilize molecular probe technology are also emerging (Scholin et al. 2005), but like the portable devices are still in the experimental stage and their use largely restricted to a handful of researchers studying a restricted set of target organisms. Nonetheless, the prospects of developing both the field portable and in-water systems to the point where they are robust and available commercially are bright, and it is well within the foreseeable future for such tools to be part of an "early warning system" in some areas and for certain species as an aid to invoke mitigation strategies to minimize the effects of harmful blooms. Numerous issues remain as to how such techniques and instruments will be tested, calibrated, validated, made available commercially, and ultimately used routinely in concert with the present-day, accepted methods for identifying and quantifying harmful species and their toxins (Scholin et al. 2005). In the meantime, those involved with harmful algae research and monitoring can expect a future that includes vigorous debate and intense innovation on both the analytical and instrumentation development fronts. The remaining issue is having these rapid techniques validated and accepted by the monitoring agencies worldwide to replace the more traditional methods.

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