DETECTION AND QUANTIFICATION OF HAB SPECIES USING ANTIBODY AND DNA PROBES: PROGRESS TO DATE AND FUTURE RESEARCH OBJECTIVES

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ABSTRACT

Routine monitoring for harmful algal bloom (HAB) species is severely hampered by the lack of simple, sensitive, rapid diagnostic tests to identify and quantify particular species as they occur in natural assemblages. Over the past few years new molecular probe technologies designed to meet this challenge have emerged from a number of laboratories around the world. In all cases, the objective of these tests is to detect molecules that serve to reveal a particular species, or group of species, specifically, A "probe" is any one of a number of molecular tools to accomplish this task. This contribution summarizes some recent advances in the development and application of lectin, antibody and nucleic acid probes for HAB species. While the development of probes themselves has been rapid and the list of available probes is extensive, relatively little work has been done to explore probe application methodology for routine analyses of field samples. When progressing from culture models to natural samples, many adjustments to the probe application protocol may be required. Changes in the physiological state of the targeted organism may also alter probe label intensity, and the extent of this change may depend on the type of probe in question and the format in which it is applied. Given the data collected to date, there appears to be no one type of probe that is "best". The optimal type of probe and application strategy will likely vary depending on the species in question and required lower limit of detection.

INTRODUCTION

Identification of harmful algal bloom (HAB) species in discrete water samples has relied traditionally on microscopy. Microscope-based methods are proven, powerful techniques in this regard, but they are time-consuming and require expertise to recognize morphological characters on which species identifications are based. In many instances these restrictions are not important relative to the benefits a microscope offers. However, when one must examine many samples for a variety of species routinely and when the results are required in as near real-time as possible, alternate means of species detection are desirable. Considerable advantages are to be gained by developing novel techniques to increase the rate of detecting and quantifying HAB organisms as they occur in natural populations, and by making it possible to conduct such measurements in the field, in real-time.

Molecular probes assays offer one possible means of accomplishing this goal. The basis of this approach stems from well-established methods in biomedicine for detection of pathogenic organisms or defective genes, with the further assumption that such technology would transfer to HAB research easily. The first formal progress report on this effort was given at the Sixth International Conference on Harmful Algae [1]. Now, four years later, we provide an update to that last presentation and look ahead to the challenges for future investigation. For more detailed information concerning the development of probes for specific organisms, the reader is referred to other contributions in this volume and references herein.

MOLECULAR PROBES FOR HAB SPECIES

Lectins, antibodies and nucleic acid probes have all been evaluated as tools to discriminate among HAB species. These probes detect molecules that serve as unique identifiers of a species or group of species (Table 1). Lectins are proteins that have a known, high affinity for specific sugar residues; they are used most often for detecting cell surface glycoproteins and glycolipids [e.g., 15]. Antibodies bind to specific molecules generically referred to as antigens. An antigen may be a protein, carbohydrate, nucleic acid, toxin, etc., essentially a "molecular shape" that is recognized by a mamalian immune system [e.g., 5]. Probes that detect specific sequences of nucleic acids (DNA or RNA) come in variety of forms [e.g., 3], but for the purposes of this article we consider those that recognize their target specifically through the pairing of nucleotides in one strand with their complements in another to form double stranded helixes.

Antibodies are the most common type of probe used in HAB research, with nucleic acid and lectin probes following thereafter. Many of the antibody probes and all of the lectin probes target molecules associated with the cell surface. A number of antibodies are also directed against toxins. Nucleic acid probes for HAB species focus extensively on ribosomal RNAs (rRNAs), the operon that encodes those molecules (rDNA) or the spacer regions that separate genes within the rDNA operon. What is perhaps most impressive about the data shown in Table 1 is the number of probes that exist for HAB species, the variety of organisms they detect, the range of cellular compartments being targeted successfully, and the speed at which the list of available probes is growing. Over the last decade numerous investigators have shown that it is possible to use molecular probes to identify a specific microalgal species. However, in many cases is not yet possible for a wide variety of workers to use those probes routinely, especially for field research.

PROBE APPLICATION STRATEGIES

In order to track the whereabouts of a probe, and the substance it binds to, it must be "labeled." Probe labels can consist of electron-dense material, radioisotopes, fluorescent molecules, or enzymes that drive colorimetric

Table 1. List of molecular probes for harmful algal species, and one strain of bacteria thought to produce PSP toxins

Organism	Probes types	Reference
Bacillariophyceae		
Pseudonitzschia spp.	Ab, DNA, lectin	2-7
Chrysophyceae		
Aureococcus anophagefferens		Ab 8
Dinophyceae		
Alexandrium spp.	Ab, DNA, lectin	9-15
Dinophysis spp.	DNA	16, 17
*Gymnodinium*spp.	Ab, DNA, lectin	15, 18-22
Gyrodinium spp.	Ab	22
Pfiesteria piscicida	DNA	23
Prorocentrum spp.	Ab, lectin	15, 24, 25
Prymnesiophyceae		
Chrysochromulina spp.	DNA	26
Prymnesium spp.	DNA	26, 27
Phaeocystis spp.	DNA	27
Pelagophyceae		
undescribed (Texas "brown tide")		Ab 28
Raphidophyceae		
Chatonella spp.	Ab	29, 30
Heterosigma spp.	DNA	31
Cyanophyceae		
Anabaena spp.	DNA	32
Microcystis spp.	Ab, DNA, lectin	33, 34
Eubacteria		
Pseudomonas sp. (from *A. tamarense*)	DNA	35

or chemiluminescent reactions [3, 36]. One has numerous choices as to how a label is attached to a probe, the number of labels that can be incorporated per probe, etc. Therefore, the researcher must not only choose a particular type of probe (lectin, antibody, nucleic acid) but also the type of probe label, the latter often being dictated by the format in which the probe will be applied: whole cell or cell homogenate.

Application of probes to whole cells versus cell homogenates (crude lysates or purified subcellular preparations) defines in large measure the range of possible sample processing techniques, regardless of the type of probe chosen. In the whole cell format, cells must remain intact throughout the labeling process. Detection strategies that utilize this approach include application of antibodies and lectins to label cell surface markers [e.g., 5, 7], the use of antibodies to localize toxins within various compartments of a cell [chloroplast, nucleus; 37, 38], and the use of DNA probes to label nucleic acids also within different compartments of a cell [cytoplasm, chloroplasts, nucleolus; e.g., 6, 11]. Many of these strategies rely on microscopy or flow cytometry to reveal fluorescently labeled probes that in turn allow one to view labeled cells within the sample matrix [culture or natural population; e.g., 12, 38, 48, refs. therein]. Indirect detection of whole cells is also possible, for example by using primary or secondary antibodies coupled to enzymes. In the latter type of assay, a colorimetric reaction develops in proportion to the number of target cells in the sample; color intensity is determined spectrophotometrically, providing an estimate of target species abundance without actually viewing the labeled cells [49].

In contrast to a whole cell approach, a cell-free format demands disruption of the cells such that their contents are liberated. Probes are then applied to a crude (unpurified) homogenate, or to some fraction of that homogenate that has undergone further purification, to "search" that material and report on the presence (and in some cases the abundance) of targeted molecules indicative of a HAB species. Specific examples of detecting HAB species using a cell-free method include the use of antibodies to detect toxins [e.g., 40, 41], sandwich hybridization to detect rRNA in crude cell homogenates directly [2, 42], or some combination of nucleic acid analyses [polymerase chain reaction (PCR), nested PCR, restriction enzyme digestion, etc.] that ultimately yield an indication as to whether or not a nucleic acid "signature" indicative of a particular species is present in the original sample [e.g., 13, 16].

Mixed assays that utilize more than one type of probe are also possible. For example, one might employ an antibody to select cells from a complex population, and then apply DNA probes to captured cells for further analyses [43]. For the purposes of this review the most important points to be made are that there are different types of probes that will label cells specifically, that various compartments within cells are accessible to these probes, and that both whole cell and cell-free probe application strategies are viable means by which one might detect HAB species.

CULTURE STUDIES

Considerable effort has been spent testing the reactivity of probes against organisms held in culture. Typically, a set of probes potentially specific for a given species is screened against a panel of target and non-target organisms to identify a subset of probes that show the greatest promise and warrant further evaluation. One may ultimately reject many, sometimes most, of the candidate probes to find those that excel with respect to specificity, sensitivity and stability.

Critical to the effective use of probes is an appreciation of how probes react towards cells in different physiological conditions. Studies of *Gyrodinium aureolum, Gymnodinium nagasakiense* and *Alexandrium tamarense* are perhaps most advanced in this regard. For the first two species Vrieling et al. [48] have demonstrated consistent labeling with antibodies despite the cells being cultivated under different nutrient/physiological regimes. Similary, Anderson et al. [49] have shown that antibody probes to cell surface antigens are less sensitive to changes in physiological condition than are DNA probes that recognize cellular rRNA (Fig. 1).

Although the cellular content of rRNA will likely

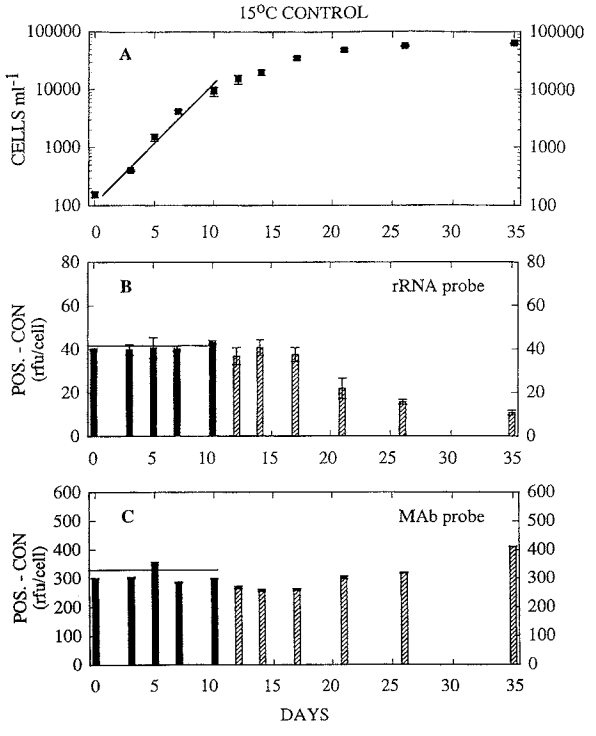

Fig. 1. Changes in cell density (A) and probe labeling intensities (B and C) in a nutrient-replete batch culture of *Alexandrium tamarense* as a function of time [49]. Labeling intensities depict cell fluorescence intensity obtained with species-specific rRNA (B) and monoclonal antibody (C) probes minus background fluorescence (negative rRNA and non-specific primary antibody control treatments, respectively). Solid line indicates data average during exponential growth. rfu = relative fluorescence units. Error bars denote ±1 S.D.

change with alterations in physiological state, the apparent extent of this change may also depend on the chosen analytical method. For example, using rRNA-targeted DNA probes for *A. tamarense/catenella*, Scholin et al. [46] have observed a difference in the apparent loss and gain of signal per cell as the cells experience changes in growth stage; a whole cell-based assay shows far greater changes in signal per cell than one that is cell-free even though the probes target the same sequence of rRNA (Fig. 2). Thus, cells' physiological variability may affect the reactivity of some types of probes to a greater extent than others, and the choice of probe application strategy, even when dealing with the same probe, may also affect the per cell reactivity.

FIELD STUDIES

For many applications, the critical issue is whether a probe is "good enough" to label its intended target reproducibly under the conditions those cells typically encounter in nature. Notable in this regard is the possibility that a putative species-specific probe will cross-react with something found in nature, not in a culture collection. Alternatively, a probe may not react with its intended target because the organism in question exists as series of genetic variants or strains, only a fraction of which are held in pure culture. Thus for a given set of probes, a particular geographic location, and natural populations in those locations, one must empirically determine the range of possible probe responses towards the target species and gain an understanding of any cross-reactions or assay interferences that may occur through observation. Only by applying the probes to a variety of samples, scoring the response, and "truthing" those observations using accepted standards of cell identification and/or toxin analyses can one rigorously assess the utility of a probe for detecting and quantifying specific species in complex natural samples [e.g., 44, 50]. Using a whole cell format, target cells may have variable labeling intensities for any one of a number of reasons (e.g., Figs. 1, 2), but may be nonetheless recognizable as valid "positives" compared to unlabeled cells in the sample. In this case, morphology can confirm the information from the probe label. In contrast, cell-free assays rely on standard curves that relate signal intensity (e.g., color) to cell abundance [e.g., 42], so assay artifacts are difficult to detect. If the signal per cell obtained from

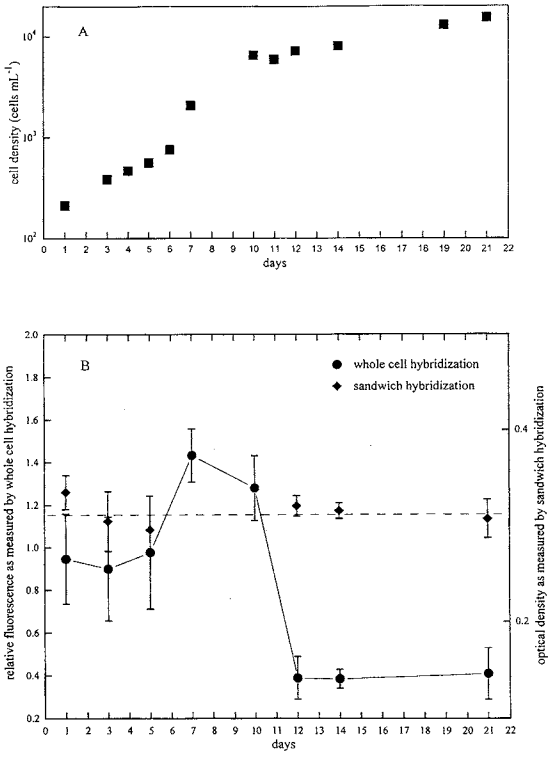

Fig. 2. Changes in cell density (A) and probe labeling intensities (B) in a nutrient-replete batch culture of *Alexandrium* cf. *catenella* as a function of time. A species-specific rRNA probe was applied using whole cell and sandwich hybridization [e.g. 3, 6, 42, 45]. The labeling intensity of whole cells (B, circles; relative fluorescence) was determined by measuring brightness of individual cells (n = 6; pixels per unit area) obtained on a given day and dividing those values by a fluorescence standard to correct for day-to-day variation in bulb intensity, etc. Results of sandwich hybridization (B, diamonds; optical density) for a constant number of cells (~1,000) were determined by measuring color intensity in a microtiter plate-based assay [3, 42 , 45, 46].

natural populations differs from that of the culture model for any reason, then standard curves must be derived empirically. It may not be possible to develop a single curve that rigorously estimates the number of cells in a given sample if the target molecule concentration varies significantly from cell-to-cell or changes in response to variable environmental or physiological conditions. One must apply the assay and evaluate it using independent observations based on different detection strategies (routine microscope counts, whole cell assays, toxin analyses, etc.). In short, intercalibration of whole cell and cell-free detection methods for the same target species requires empirical studies which are time consuming and dependent on analysis of natural samples. Although this is a challenging, protracted and somewhat risky avenue of research, it is nonetheless promising and worth pursing given that cell homogenates are potentially easier and faster to analyze than are their whole cell counterparts [42].

CASE STUDY: DNA PROBES FOR *PSEUDO-NITZSCHIA*

In an effort to assess the utility of species-specific DNA probes for a variety of *Pseudo-nitzschia* spp., Scholin et al. [44] initiated a field-based study in which probes developed and tested on laboratory cultures were applied using whole cell and sandwich hybridization techniques [2, 6, 42]. Compared to culture studies, numerous adjustments of the labeling protocols were required, many of which involved scaling sample and reagent volumes to minimize interference from background material. For example, in order to enumerate target cells using filter-based whole cell hybridization [13 mm filter; see 42] it was found that 10 mL of whole water, or several hundred mL of net tow material, were "optimal" sample volumes; larger volumes can result in too much material (cells and debris) obscuring target species. Large amounts of detritus or non-target species can also clog the filter, forcing a reduction in sample volume in some instances. If the target species is rare (e.g., 100 cells L^{-1}or less), then analysis of replicate filters is required to detect that organism. However, as the number of replicates increases, the reliability of the estimate also increases, along with the lower limit of detection and time required to complete the assay. The application of sandwich hybridization technology to detect target species in cell homogenates can also be problematic for sample types. Because of this, the amount of material collected must be standardized, empirically, so that on average signal-to-noise ratios are maximized for detection of a given species at a particular location. Additional control treatments are also needed to gauge the relative level of background interference as well [46].

FUTURE PROSPECTS

Many different types of probes for a wide range of species are being evaluated under field conditions, and these investigations will likely require several years to complete given the interdisciplinary nature of such trials. In some cases researchers are poised to compare different types of probes for the same species under field conditions, and relate those observations to conclusions derived from culture experiments. We will likely have to "fine-tune" probes for a given region of the world, since genetic variation within a targeted species is possible even though different populations of the organism in question may not appear to vary morphologically [e.g. 47]. Therefore, considerable work remains on a molecular level to characterize target species in regions of the world where application of molecular probes is desired. Nevertheless, the time is fast approaching when we will know if many probes can meet our high expectations and if they are reflective of our predictions from controlled laboratory studies.

Given the data collected so far, it seems likely that some probes will perform better than others, and that there will be no one "best" type of probe or application strategy. Instead, the optimal approach will probably vary depending on the species in question, and the effort expended to develop the capability of any probe to its fullest. The choice of one probe versus another may also be dictated by required lower limits of detection (i.e. established diagnostic standards), if not personal preference. In principle, molecular probes could enhance researchers' ability to detect and quantify a wide range of HAB species significantly. However, many challenges remain before that "promise" can be realized in a meaningful way. One of the greatest challenges is defining the needs of the end-user. In some instances, a plankton net and a microscope may be all that is required.

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