Harmful Marine Algal Blooms - P. Lassus, G. Arzul, E. Erard, P. Gentien, C. Marcaillou


# Identification of Harmful Algal Species Using Molecular Probes: An Emerging Perspective

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## 1. INTRODUCTION

A common problem in research and monitoring programs focused on harmful algal bloom (HAB) species occurs when the species of interest is only a minor component of the planktonic assemblage. Many potentially useful measurements are not feasible because of the co-occurrence of numerous organisms and detritus. Autoecological studies must thus rely on tedious microscope counts to enumerate the target species.

Another constraint arises from difficulties in identifying and distinguishing between morphologically similar species or strains. This is a problem not only for those with limited taxonomic training, but also for skilled taxonomists, since considerable time and effort are required to identify a species when its distinguishing characteristics are difficult to discern under the light microscope. Such fine levels of discrimination are not feasible in monitoring programs or studies which generate large numbers of samples for cell enumeration.

This situation is encountered frequently in studies of harmful algae. For example, the diatom *Pseudonitzschia pungens* occurs in two varieties, one toxic and the other non-toxic, but these cannot be distinguished from each other using the light microscope (Smith et al. 1990). Likewise, toxic and non-toxic varieties of the dinoflagellate *Alexandrium tamarense* co-occur (Yentsch et. al. 1979), as do morphologically-similar *A. tamarense* and *A. catenella* (Cembella et at. 1987).

Whether the problem is distinguishing between closely-related strains or enumerating a single species in large numbers of samples, the need for species- or strain-specific "probes" is clear -- probes which can be used to label only the cells of interest so they can then be detected visually, electronically, or chemically. Many different approaches have been developed for pathogenic bacteria and other microorganisms (Macario and Macario 1990), but little effort has been directed to harmful algal species until recently. Progress has been rapid however, and probes of several different types should soon be available for many of the harmful algae, along with techniques for their application in the rapid and accurate identification, enumeration, and isolation of individual species. This paper will review progress on two technologies targeted at identification of particular species -- antibody probes, and nucleic acid probes. The objectives are to describe the methods used to develop these probes and to discuss results of their application and areas where further work is needed.

## 2. ANTIBODIES

**2.1 General Concepts.** Shapiro et al. (1989) and Campbell et al. (1989) provide useful reviews of the application of immunological techniques to marine picoplankton identification. The approach involves the use of antibodies that

bind specifically to proteins in the cell walls of the algal species of interest. Antibodies are produced by inoculating cells of target species into animals, which then produce antibodies in response to the presence of the intact foreign organism or compounds derived from it. The target molecule against which the antibody is directed (termed an antigen) is typically, but not necessarily, a cell wall protein. Fortunately, it is not necessary to purify specific proteins in order to produce antibodies. Whole cells or cell fragments have been used effectively as antigens for small species such as *Synechococcus* or *Aureococcus* (e.g. Campbell and Carpenter, 1987; Anderson et al., 1989) as well as for large species such as *Alexandrium* (Sako et al., 1993) or *Pseudonitzschia* (Bates et al., 1993). Whole cells stabilized by fixation in glutaraldehyde or paraformaldehyde are injected into vertebrates such as rabbits, goats, or mice on a schedule of repeated inoculations and booster shots lasting several months. Serum from the animal then contains polyclonal antibodies or PAbs (i.e. multiple antibodies, each recognizing a different protein site (epitope) on the cell wall).

This process can be taken further to produce a monoclonal antibody or MAB -- a single antibody which recognizes only one epitope on the antigen. The inoculation process is the same as for polyclonals, but instead of drawing blood to obtain serum, spleen cells from the inoculated animal are fused to immortal myeloma cells to produce an antibody-producing cell line called a hybridoma. The hybridoma is used to produce large quantities of the desired antibody.

PAbs are sometimes more useful than MAbs in labeling cells, as the multiple antibodies in one antiserum can bind to several different epitopes and thus are easier to detect. MAb antisera contain a single antibody type that binds to only one site. However, cross-reactions (labeling of non-targeted cells) are more likely with PAbs than with MAbs, for which the hybridoma cell lines to be used can be restricted to the organism of interest by careful screening. In addition, MAb-producing cell lines produce antibody as long as the hybridoma is in culture (i.e. they are immortal), whereas the supply of a PAb is limited to that available from the serum of a single, short-lived animal. Lyophilized or refrigerated PAb antisera are stable for years, however.

Many universities and institutions have animal facilities for PAb production, but it is also easy to mail preserved cells to private companies who do the injections, maintain the animals, and draw blood for a minor cost (US$300-500 for a single animal). MAbs can also be produced in-house or through private companies, but the cost is 10 times higher than for PAbs.

**2.2 Detection.** Most immunological assay methods for cell identification use indirect immunofluorescence for visualization or detection of the label. Cells are first exposed to the primary antibody (i.e. that produced by the inoculated, host animal) and then to a secondary antibody which will bind to all antibodies produced by that particular host animal. The secondary antibody is typically conjugated to a reporter molecule such as the fluorescent compound fluorescein isothiocyanate (FITC). Visual detection of the labeling is possible using an epifluorescence microscope. Alternatively, samples can be processed using a flow cytometer or other instrument that can detect and quantify fluorescence. Assays can also be conducted on filters using fluorescent or colorimetric

detection. Under the microscope, the fluorescent label is often visible as a colored "halo" or ring outlining the periphery of the cell (Fig. 1).

The ability to recognize immunolabeled cells depends in part on the difference in fluorescence between the cells of interest (positives) and the background fluorescence of control or unlabeled cells. Three factors contribute to this background fluorescence - the choice of filter set, the type of fixation, and non-specific binding or cross-reactivity of the antibody. Autofluorescence can be reduced by selecting fluorescent compounds and filter sets with wavelength and bandwidth characteristics that maximize probe fluorescence and minimize autofluorescence. Even with careful filter selection, however, non-labeled cells have significant autofluorescence -- equivalent to 10-30% of the signal from labeled cells. This background level increases further when non-specific binding occurs. This can be assessed by incubating cells without the primary antiserum, using pre-immune or "normal" serum from the host animal (for PAbs) or myeloma protein (for MAbs) instead. The combination of autofluorescence and non-specific binding can often lead to background or control fluorescence that is 30% or more of the intensity of positively labeled cells. The choice of fixative can affect this as well, since autofluorescence varies dramatically among preservatives ( Shapiro et al. 1989; Anderson et al. 1989; Vrieling et al. 1994).

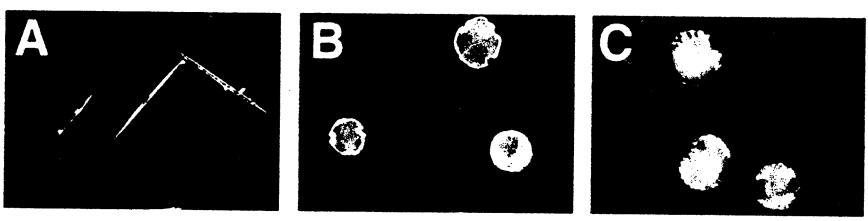


**Figure 1.** Antibody and oligonucleotide probe-labeled cells, visualized using fluorescence. A). Immunofluorescent image of *Pseudonitzschia pungens* var. *multiseries* labeled with PAb antisera developed by Bates et al. (1993). Note the bright fluorescent outline around the frustule, and the autofluorescence of chloroplasts inside the cell; B). Immunofluorescent image of *Alexandrium fundyense* labeled with MAb antisera M8751 produced by Sako et al. (1993); C). Fluorescence image of *A. fundyense* labeled with oligonucleotide probe targeting rRNA. Note the signal throughout the cytoplasm.

**2.3 Specificity.** Table 1 lists the harmful algal species for which high-specificity PAb and MAb antisera have been developed. To the surprise of many, the specificity obtained thus far with PAbs has been remarkable, despite the potential for cross reactions inherent in such preparations. For example, a PAb developed for the brown tide chrysophyte *Aureococcus anophagefferens* is species-specific, showing no cross reactions with 46 phytoplankton cultures representing 5 algal classes, including 20 species from the Chrysophycophyta (Anderson et al. 1989). An even higher level of specificity was demonstrated for the diatom *Pseudonitzschia pungens*, where PAbs were able to distinguish toxic from non-toxic varieties of the same species (Bates et al., 1993).

Similar specificities have been obtained using MAbs. An MAb produced by Nagasaki et al. (1991) labeled nine strains of *G. nagasakiense* from Japan, but not *G. breve*, *G. catenatum*, or a western European strain of *Gyrodinium aureolum*. In contrast, sixteen MAb antisera produced against*G. aureolum* from western Europe cross reacted with *G. nagasakiense* and *G. mikimotoi* (Vrieling et al., 1994). These results emphasize the need to establish multiple monoclonal cell lines, as MAbs are highly specific for individual epitopes that may not be unique to the target species. A related concern is that the number of epitopes labeled by an antiserum and the number of those epitopes on a cell directly affect the intensity of immunofluorescence and therefore the detectability of the signal.

**2.4 Applications.** Much of the effort in immunological detection of HAB species has been focused on development and characterization of antibodies for individual species, so applications of this probe technology to field populations are limited to date. Experience with a PAb for the brown tide organism *A. anophagefferens* suggests that immunofluorescence has a major role to play in harmful bloom field programs. That antibody has been used in cell enumeration and in grazing studies (Caron et al., 1989), and was recently used to map the geographic distribution of this species over a large region (Anderson et al. 1993). The latter study detected *A. anophagefferens* at extremely low concentrations (10-20 cells $ml^{-1}$), demonstrating that the species is present in many areas with no known history of harmful brown tides. This degree of resolution is of note since *A. anophagefferens* is so small and non-descript that microscopic identification and enumeration are highly uncertain at low cell concentrations.

**Table 1. Antibodies to harmful algal species.**

| Species | Type* | Specificity | P |
|---|---|---|---|
| *Alexandrium catenella*<br>*Alexandrium tamarense* | MAb | strain | Sako et al. (1993);<br>Adachi et al. (1993) |
| *Aurreococcus anophagefferens* | PAb | species | Anderson et al. (1989) |
| *Chatonella marina*<br>*Chatonella antiqua* | MAb | strain or<br>species | Nagasaki et al. (1989) |
| *Gymnodinium nagasakiense* | MAb | species | Nagasaki et al. (1991) |
| *Gyrodinium aureolum* | MAb | species or<br>complex | Vrieling et al. (1993;<br>1994) |
| *Pseudonitzschia pungens*<br>(var. *multiseries*) | PAb | strain | Bates et al. (1993) |

One obvious application of antibody probes would be for automated cell enumeration in field or monitoring programs. This has not been accomplished thus far, but is under active investigation using both flow cytometry and dot blot

or tissue culture plate formats. One obstacle is that the intensity of positive labeling is often not much higher than the background fluorescence of control or unlabeled cells. Even when the mean fluorescence of labeled cells is several times higher than the mean for unlabeled cells, variability in fluorescence around those means can result in unacceptable overlap of the two populations. This problem is depicted in Fig. 2, which shows flow cytometer output of a sample in which cultured *A. tamarense* cells were added to a natural plankton assemblage. With standard indirect labeling using an MAb and an FITC-conjugated secondary antibody, the target cells appear as a cluster with higher fluorescence than the unlabeled cells (Fig. 2A). The difference appears to be sufficient to permit enumeration of the *A. tamarense* population, but the distributions of the labeled and unlabeled cells overlap, even though the means are different (Fig. 2B). It would thus be difficult to specify a minimum fluorescence level that delineates *A. tamarense* cells from all others without setting the threshold so high that many positively labeled (but weakly fluorescent) *A. tamarense* cells would not be counted.

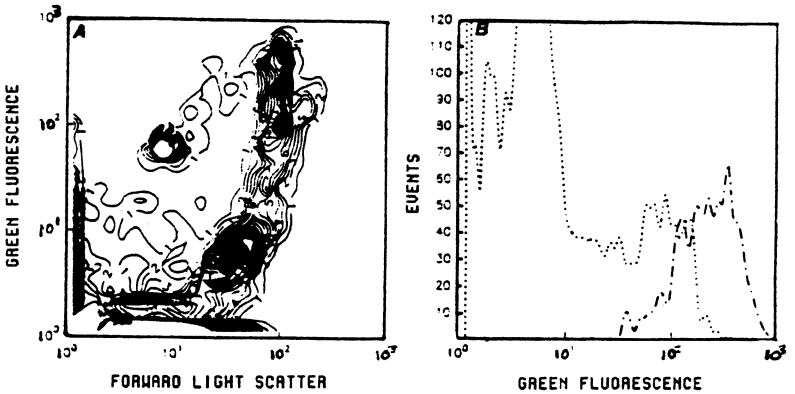


**Figure 2.** Flow cytometric analysis of a 1993 Gulf of Maine field sample containing *Alexandrium fundyense* cells. The sample was concentrated through 20 μm mesh, fixed with 3% formaldehyde, and labeled using indirect immunofluorescence and MAb M-8751 from Sako et al. (1993). The biotinylated secondary antibody was detected with FITC-conjugated avidin. A) Bi-parameter plot of green fluorescence versus forward angle light scatter (an indicator of size). Data are contoured to indicate the number of cells with particular fluorescence and light scatter properties. The target *Alexandrium* cells are found in the top right portion of the distribution. The dark circular region in the left-center of the diagram represents calibration beads. B) Frequency distribution of green fluorescence, showing the *A. fundyense cells* (dashed line) and the background plankton population (dotted line). Note that the mean fluorescence of the two distributions differ significantly, but that the distributions around the means overlap.

Signal enhancement is clearly necessary, and several approaches are under investigation. Vrieling et al. (1993,1994) showed that fluorescent intensity

can be enhanced using biotinylated secondary antibodies which react with FITC-conjugated streptavidin, but even then, the distributions of labeled and unlabeled cells overlapped. Pooled antisera were then used to increase the number of labeled epitopes and thus the intensity of the fluorescence signal (Vrieling et al. 1994). Other approaches are also being investigated such as the use of alternative fluorochromes or additional layers of antibodies (e.g. biotinylated anti-FITC to bind to the FITC-labeled secondary antibody, detected with avidin-FITC). Expectations are high that signal intensity problems can be overcome, but automated applications of immunofluorescent techniques for harmful algae await technique development.

An additional complication is that the nature and abundance of cell surface proteins can vary during different stages of growth, since many are involved in nutrient uptake and other transport processes. It follows that antibodies targeted to those proteins would label cells with differing degrees of intensity at different times or under different conditions. In work that is in progress, we have found that immunofluorescent labeling variability is significant in a single culture during different growth stages, and between cultures grown under different conditions. In nutrient-replete medium, positive immunofluorescence labeling of *A. tamarense* cells was statistically lower during stationary phase than during exponential growth. When nitrogen was limiting, the decrease in labeling intensity between exponential and stationary phase cells was even more dramatic, amounting to a 50% reduction.

For some applications, the objective is simply to label the cells of interest so that they can easily be distinguished from co-occurring species. In some cases, such as in manual microscopic examination of samples, variability in the absolute level of antibody labeling on each cell may not be a critical parameter. Other applications, however, are sensitive to the labeling intensity. Examples would be flow cytometric analysis of a mixed population treated with a species-specific antibody or dot blots or other bulk analyses of samples on filters or in tissue culture plates. In flow cytometry, instrument settings can be adjusted to specify positive identification across a broad range of labeling intensities, but as discussed above, this will result in considerable overlap with the signal from unlabeled cells. Dot blots and similar approaches pool the signal from labeled cells in a sample into a single measurement. Such procedures must be considered semi-quantitative at best, since the fluorescence emitted by some cells could be double that of other cells of the same species due to physiological variability.

**2.5 Future Work.** Additional effort is needed before antibody probes live up to their potential in studies of harmful algae. Antibodies to harmful species not included in Table 1 are needed, and the specificity of existing antibodies must be tested further, especially on mixed field populations. Techniques must be developed to maximize the fluorescence intensity of labeled cells, and other optical parameters such as forward angle light scatter evaluated as additional parameters to help separate a target population from other cells using the flow cytometer. Single cell analysis using flow cytometry shows promise, but a parallel attack must be directed to bulk analyses using dot blots or other solid

support formats. Such assays will be needed by aquaculturists, regulatory officials, and others who must conduct rapid assays for harmful species.

## 3. NUCLEOTIDE PROBES

Another promising probe technology targets particular genes or gene products inside cells using short, synthetic DNA segments (oligonucleotides) which bind selectively to DNA or RNA sequences specific for a particular organism. For marine systems, this technique has been used primarily on procaryotes (e.g. DeLong et al., 1989; Amann et al. 1990; Distel et al. 1991). Work is in its early stages on harmful phytoplankton species, with 2 or 3 species under investigation and most results unpublished at this writing.

**3.1 General Concepts.** In recent years, use of nucleic acid probe technology to detect microorganisms has expanded considerably. This technology is used extensively in the detection of pathogenic bacteria and other microbes (Macario and Macario, 1990), but is only now being explored for HAB species. The procedure involves the detection of target nucleic acid sequences by binding (hybridizing) those sequences to a short strand of DNA containing a homologous complementary sequence. Extraordinary sensitivity and specificity result from the rapid, high-fidelity hydrogen bonding associated with Watson-Crick base pair complementarity (DeLong and Shah, 1990). Many DNA or RNA sequences can be targeted in the organism of interest, including fragments of genes, spacer regions between genes, repeated (non-transcribed) sequences, and transcribed genes in the form of ribosomal RNA (rRNA).

The first step in probe development is the identification of a unique series of bases in the organism's DNA or RNA that are only found in that organism and in others that are to be targeted. Typically, target genes have sequence domains which are highly conserved among all organisms, and other domains which are variable to different degrees. If resolution is sought at the genus, species, or even sub-species levels, highly variable, rapidly evolving domains are targeted. Short, contiguous segments (approximately 20 nucleotides) of sequence are identified that serve as targets for probes. "Oligonucleotide" probes are synthesized inexpensively and in great abundance, and used in a variety of formats to detect the cells of interest.

One problem that arises immediately with HAB species is that little or no sequence information is available. The specificity of a probe will depend on the degree to which a particular sequence is unique, yet that determination requires that sequences of many closely-related or co-occurring algal species be known. Molecular studies of HAB species are in their infancy, so it is often necessary to establish a sequence database at the outset. This is time consuming and expensive, but necessary. For several reasons (large published sequence database, existence of highly variable domains, multiple copies of the target sequences), ribosomal genes (rDNA) and rRNA have become common targets for probe design (e.g. Stahl et al., 1988; DeLong et al., 1989). For HAB species, rDNA has been sequenced only for *Alexandrium* (Scholin and Anderson, 1993) and *Pseudonitzschia* (Scholinet al. in press). In this early stage of probe development for HAB species, it would be ideal if workers focusing on different

organisms could agree on the same target genes for their sequencing efforts and probe design. Large sub-unit rDNA has been the initial choice for *Alexandrium* and *Pseudonitzschia*, but it remains to be seen whether this is the best target.

Another problem is that identification of a unique target sequence does not guarantee that useful probes can be produced. Some regions of the ribosome may not be accessible to rRNA probes due to secondary or tertiary structure.

Probes now exist that hybridize with either the rRNA or the rDNA of *Alexandrium* and *Pseudonitzschia*. These two targets differ in several respects. First, because of their role in protein synthesis, rRNAs are present in high abundance in cells (thousands of copies), and thus provide many targets for probe labeling. This has obvious benefits with respect to detection. However, rRNA abundance varies with physiological condition in procaryotes (DeLong et al., 1989), so labeling intensity varies as well, leading to errors when quantification of the target organism is desired. The extent to which rRNA abundance varies in HAB species is not known. In contrast, rDNA is at least 1-2 orders of magnitude less abundant than rRNA. Labeling intensity and probe detection are thus problematic, especially with whole cell assays, but the number of target molecules per cell will not vary with physiological or environmental conditions (except during a brief period of cell division). Quantification or target species abundance may thus be more accurate using rDNA probes.

**3.2 Detection.** Many assays for bacteria and other procaryotes immobilize extracted DNA on a solid surface such as a nitrocellulose or nylon membrane to which the probe is added and allowed to hybridize and establish a double-stranded molecule (Macario and Macario, 1990). Excess unbound probe is washed off, and the hybrid (target + probe) sequence is detected using radioactivity, fluorescence, chemiluminescence, or colorimetric methods. Modifications of this procedure exist, such as the sandwich hybridization assay in which two probes are used -- one to capture the target DNA and bind it to a solid surface, and the other to permit detection.

*In situ* hybridization is also possible, using intact cells that are either immobilized on a microscope slide or suspended in solution. In this format, the probe enters the cell and binds to target sequences, excess probe is washed out, and the complex is detected with fluorescence or radioactivity. As with antibodies, the probe can be directly conjugated to a fluorescent reported molecule such as FITC, or it can be biotinylated and detected using fluorescent avidin conjugates. Figure 1C shows an epifluorescent image of an *A. fundyense* cell labeled with an rRNA probe to a fragment of large subunit rRNA.

A well-designed probe will exactly match the homologous sequence in the target organism and have at least 2-3 nucleotides (of approximately 20) which do not match with the equivalent sequence of undesired species. Specificity is controlled by the "stringency" of the hybridization and wash conditions. These can be adjusted empirically to maximize binding to the target sequence and minimize binding to mis-matched sequences.

**3.3 Applications.** Much of the effort in oligonucleotide detection of harmful algal species has been focused on development and characterization of probes for individual species. Applications of this technology to field populations of

harmful algae are limited to a few analyses of field samples, but available data highlight both the promise and the problems of the technique.

Whole cell assays of *Alexandrium* species have been attempted using rRNA probes applied to cultures and to field samples. One rRNA-targeted probe was tested on 15 dinoflagellate cultures representing 5 genera (Anderson, unpub. data). The probe was designed to label toxic *Alexandrium* species from North America, including *A. tamarense*, *A. catenella* , and *A. fundyense*. Preliminary results are encouraging, as all toxic North American *Alexandrium* isolates were labeled strongly and several related but non-toxic species (such as *Alexandrium andersoni*, *A. lusitanicum*, and *Gonyaulax spinifera)* were unlabeled. Minor fluorescence was observed in several *Gyrodinium* species (25% of the intensity observed in target cells) and in one non-toxic Spanish *A. tamarense* , but those levels were low and will likely be eliminated by variations in the stringency of hybridization and wash conditions. However, when this rRNA probe was applied to a field sample spiked with cultured *Alexandrium* cells, background fluorescence of non-target cells was sufficiently high to cause the same problem encountered with the MAb probe -- namely that the *Alexandrium* cells could not be distinguished from other cells on the flow cytometer due to an overlap of their fluorescence distributions. Signal enhancement strategies are thus being pursued.

Scholin et al. (in press ) have found sequence differences in the rRNA of *Pseudonitzschia* species that can be used to distinguish *P. australis* from *P. pungens*. Those workers are developing methods to use rDNA- and rRNA-targeted probes to detect toxic *Pseudonitzschia* species in samples using extracted nucleic acids immobilized on filters and detected using colorimetric or chemiluminescent reactions. It is possible that as few as 50 to 100 cells could be detected using these ribosomally-targeted probes, but additional work is needed before the methods can be directly applied to field samples.

**3.4 Future Work.** Additional research is needed to fully realize the potential that oligonucleotide probes have to change the nature of HAB research and monitoring. The list is similar to that provided earlier for MAbs, and includes studies of the variability of target sequences under different physiological conditions, signal enhancement strategies, optimization of whole cell and cell extract assay formats, and application of the technology to field samples. This work is in progress in several laboratories, so progress should be rapid.

## 4. General Conclusions

Antibody and oligonucleotide probes have great potential to alter HAB research. They can be valuable in quick, qualitative assays that indicate the presence or absence of a target organism, and they should assist in the identification of harmful species when trained taxonomists are not available. Once the limitations of these methods are better understood, probes can be used for the direct (and automated) enumeration of HAB species, and someday, to assist in physically separating those cells from co-occurring organisms for physiological or toxicological analyses. Several different types of probe assays are needed -- some that utilize cell extracts and others that are applied to whole,

intact cells -- as both have applications in monitoring and research. It is also likely that both antibodies and oligonucleotides will have roles to play in these detection systems. There is thus a great deal of work to be done to cover all of these areas and to expand the availability of probes to all HAB species. This area of research will clearly be active and productive for many years

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