MODERN APPROACHES TO THE IDENTIFICATION, ENUMERATION, AND SEPARATION OF *AUREOCOCCUS ANOPHAGEFFERENS* IN NATURAL SAMPLES

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INTRODUCTION

Certainly one of the most significant challenges in field investigations of the brown tide alga, *Aureococcus anophagefferens* relates to its small size and non-descript morphology (Sieburth *et al.* 1988). Measuring only 2 µm in diameter, this organism lacks morphological features which can be used to distinguish it from other similar-sized algae, bacteria, and detritus with either phase contrast or epifluorescence microscopy. Identification with the standard microscopes is uncertain unless the species is at such high abundance that it dominates a particular sample.

In recognition of this problem, an immunofluorescent technique was developed to label or "tag" the cell surface of *A. anophageferens* so that it could be visualized using an epifluorescence microscope (Anderson *et al.* 1989). This antibody-based procedure has since been used in a number of field studies investigating grazing impacts on the brown tide organism (e.g., Tracy *et al.* 1989; Caron *et al.* 1989), as well as in a large-scale biogeographic survey of the distribution of this organism within this region (Anderson *et al.* 1993). Thus far, however, the antibody has only been used in identification and enumeration of the brown tide alga, and then only with manual microscope techniques. In this paper, the methods for identification of this algae using the transmission electron microscope and the antibody are briefly reviewed. Examples are then drawn from immunological investigations of other algal species to demonstrate new approaches that could greatly facilitate autecological studies of brown tides.

TRANSMISSION ELECTRON MICROSCOPE (TEM)

Until the advent of the antibody technique developed by Anderson *et al.* (1989), the only means of positive identification of *A. anophagefferens* was through the use of the TEM. This is because the cell has a rather non-descript morphology, and does not fluoresce in any unique manner. For the TEM procedure, cells are concentrated, fixed, dehydrated, embedded in resin, and cut into thin sections that are then viewed under high magnification according to the methods of Johnson and Sieburth (1982). With this instrument, Sieburth *et al.* (1988) demonstrated that the nucleus of this spherical picoplankter is ovoid in shape, and that one, cup-shaped chloroplast was present with an embedded pyrenoid. There is no cell wall, but the cell is often surrounded by a diffuse layer of extracellular organic material. These and other features are sufficient for the positive identification of *A. anophagefferens* among co-occurring picoplankton, but the method is tedious and is of little use in enumeration of the species.

IMMUNOFLUORESCENT IDENTIFICATION

Following procedures developed earlier for other picoplankton (e.g., Campbell and Carpenter 1987), a polyclonal antibody was raised against cell-surface proteins of *A. anophagefferens* by injecting preserved, cultured cells of this species into rabbits (Anderson *et al.* 1989). An indirect immunofluorescent protocol has been used with this antibody to label brown tide cells in culture and in field samples. Briefly, the protocol starts with a concentration step in which cells are collected on a filter, followed by a blocking step, in which a protein solution (normal goat serum) is applied to the sample to eliminate non-specific binding. The primary antibody that is specific for *A. anophagefferens* is then added to a sample. Those antibodies bind to the cell-surface proteins of the brown tide cell, but visualization of this complex is only possible after the addition of a secondary antibody, typically conjugated to a fluorescent compound such as FITC (fluorescein isothiocyanate). Cells that have been treated in this manner are easily visualized on an epifluorescent microscope, since they have a green "halo" around them (Fig. 1). The entire procedure takes several hours, of which less than 1/3 is actual "hands-on" time.

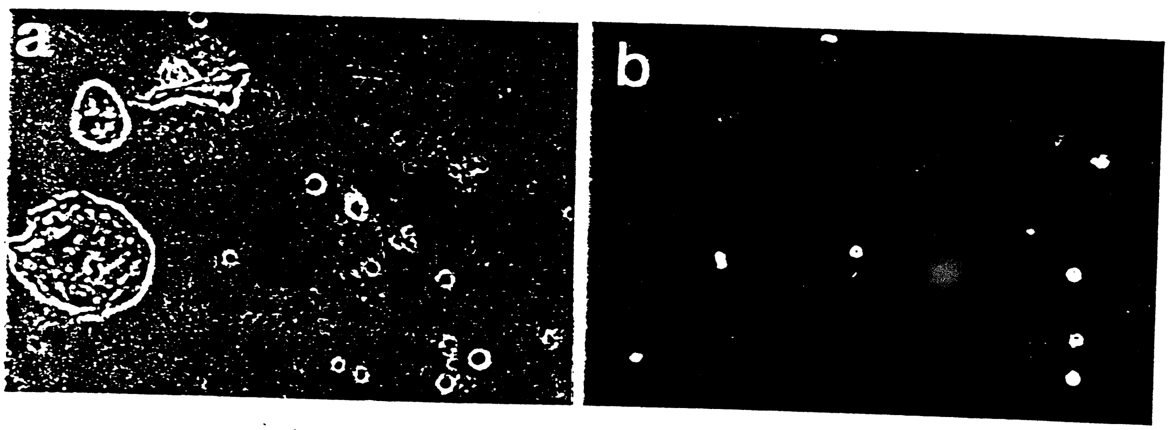

Figure 1. Phase contrast and epifluorescent micrographs of *A. anophagefferens* from a Long Island field sample. a) Phase contrast image of field sample showing detritus and cells of many types and sizes. Cells of the brown tide organism are not easily identified. b) Epifluorescence image of the same field, clearly showing the *A. anophagefferens* cells with a fluorescent halo.

The polyclonal antibody has proven to be highly specific for *A. anophagefferens*. Anderson *et al.* (1989) tested 46 algal species selected on the basis of their phylogenetic or morphological similarity to the brown tide alga. No cross-reactions were observed at antiserum dilutions of 1:3200, which was selected as the working concentration for field samples.

When the immunofluorescent technique is applied to field samples, it is easy to enumerate *A. anophagefferens* cells using a standard epifluorescence microscope. As few as 10-20 cells•ml can be detected with this procedure. This low detection limit allowed Anderson *et al.* (1993) to conduct a biogeographic survey for *A. anophagefferens* cells over a large area between Massachusetts and Delaware Bay.

NEW APPLICATIONS OF ANTIBODY TECHNOLOGY

Thus far, the antibody to *A. anophagefferens* has been used entirely for manual microscope counts. There are, however, several new technologies that could be applied to this organism which would greatly improve enumeration speed and accuracy, and which would also permit a variety of physiological measurements to be made on natural brown tide populations. Application of these methods to the brown tide alga is seen as a high priority activity that will greatly facilitate autecological studies searching for mechanisms underlying the massive blooms.

Flow-cytometry

One obvious application of the fluorescent antibody technique involves the use of a flow-cytometer to enumerate, and possibly to sort *A. anophagefferens* cells. A flow-cytometer is a sophisticated instrument used heavily in hospitals and other medical facilities to characgterize cell types. The instrument uses a laser to probe the optical characteristics of individual cells that are passed single-file in a sample stream at rates of thousands of cells per second. Fluorescence (at several different wavelengths) as well as light scattering properties, (which are sometimes related to cell-size) are recorded for each cell.

The flow-cytometer has been used in studies of phytoplankton which have unique size or autofluorescence characteristics (i.e. the natural pigments emit fluorescence at wavelengths which allow them to be distinguished from co-occurring organisms). For example, the cyanobacterium *Synnechoccus* and the prochlorophyte *Prochlorococcus phave* been investigated throughout the worlds oceans (i.e., Chisholm *et al.* 1988; Olsen *et al.* 1988). Only recently has flow-cytometry been applied to phytoplankton cells which have been labeled with antibodies, however. Vrieling *et al.* (1993, 1994) have used an antibody specific for the toxic dinoflagellate *Gyrodinium aureolum* in flow cytometric analyses of natural samples. Likewise, an antibody to the toxic dinoflagellate *Alexandrium tamarense* is being used in efforts to enumerate and separate that species from co-occurring phytoplankton and detritus in samples from the Gulf of Maine (Anderson, unpublished data).

No significant effort has yet been made to use the flow-cytometer and antibody labeling for brown tide studies, although there's every reason to believe that with some development effort, this could prove to be a useful tool. There are, however, some problems that should be anticipated based on studies of other organisms. The first relates to the natural autofluorescence of planktonic organisms. Even though the human eye distinguishes between antibody-labelled cells and other organisms in a sample, the output from a flow-cytometer when a natural sample is analyzed is often a continuum of fluorescence intensities spanning several orders of magnitude. This sometimes makes it difficult to identify a unique population with optical characteristics that do not overlap with other co-occurring organisms. The first step to circumvent this problem involves making the antibody label brighter, which can be accomplished with several different techniques (Anderson 1995). Even then, it is unlikely that the organism will be separable from others solely on the base of the fluorescence of the antibody, and thus other optical characteristics are needed. In the case of *A. anophagefferens*, its chlorophyll fluorescence and its size can be used as two additional parameters on which to define a population. It remains to be seen whether these characters are sufficient to define a population on the screen of the flow-cytometer that can be enumerated with confidence that other organisms are not included and that the bulk of the brown tide population is represented.

Once it is possible to identify and enumerate brown tide cells among other organisms in a sample using a flow cytometer, the sorting capability of the instrument can be exploited. Despite the tremendous speed with which cells are passed through the laser beam and analyzed, it is possible for the computer to sort or collect cells with particular characteristics (e.g., those with green, antibody fluorescence, red chlorophyll fluorescence, and a certain size as suggested by light scatter). A pure sample of the species of interest can thus be obtained, and used for subsequent analysis. There are constraints to the number of organisms that can be collected in this manner due to the time involved in sorting, but the method would allow physiological or biochemical measurements to be made at the species level without interference from detritus or other organisms. These and other flow cytometric applications of the immunofluorescent assay are clearly an area where developmen effort is needed with respect to the brown tide.

ELISA

Flow-cytometers are sophisticated and expensive instruments, and many investigators and agencies will not have access to them. An alternative technique that can also be used to enumerate cells that are labelled with an antibody probe is called the "enzyme linked immunosorbent assay" or ELISA. With this method, labelled cells are not visualized through the attachment of primary or secondary antibodies, as in the immunofluorescence method described above, but rather by the fluorescence or color produced in solution by an enzyme which has been linked to one of those antibodies. The enzyme portion of the cell/antibody/enzyme complex is able to act on its appropriate substrate, producing color or fluorescence which can be quantified. An ELISA approach to enumeration of phytoplankton species would involve the filtration of samples into individual wells of a special tissue culture plate system fitted with membranes at the bottom of each well, followed by the same blocking and antibody labeling steps described above. An enzyme such as alkline phosphatase is then linked to the cell/antibody complex, typically using biotin-streptavidin linkages, and a substrate is then added. After a suitable incubation time, a vacuum is applied and the liquid surrounding the cells trapped on the membrane is drawn into tissue culture wells lying directly below the membranes, capturing the colored or fluorescent product which is then easily quantified using an automated plate reader.

ELISA procedures have long been used in many areas of medicine and biology, but are only now being applied to the detection of phytoplankton species. In on-going studies of the toxic dinoflagellate *A. tamarense*, we have found that this method holds great promise for the rapid and accurate identification of cells in a sample. Once again, developmental work is required, however, as problems with cross reactions are accentuated by the signal amplification action of the enzymes used for detection. For example, if a sample contains 100 cells of the target species for which the antibody is specific, as well as 10,000 cells of another species for which the antibody has a weak but positive cross reaction, the color or fluorescence produced by enzymes attached to the target cells may well be swamped by that produced by the weak but more numerous cross reactions. Based on our experience, this problem can be circumvented in part by use of the primary antibody at an appropriate dilution, and by careful attention to the blocking steps in the procedure. Thus far, a detection limit of approximately 100 - 200 *Alexandrium* cells seems possible with this method.

As is the case with flow-cytometry, ELISA techniques have not yet been applied to *A. anophagefferens*, but there is every reason to expect that this method can greatly simplify and accelerate enumeration of the species

Magnetic beads

Those studying field populations of *A. anophagefferens* are presently unable to obtain species-specific measurements of important physiological parameters that would yield information about the nutritional status of the cells, their growth-rate, or other physiological parameters. Standard techniques for productivity, nutrient uptake, chlorophyll, and so forth all provide estimates at the community level, and are not readily adapted to species-specific measurements. This problem is especially severe with a species such as *A. anophaefferens* given that its small size precludes microscopic isolation (e.g., Rivkin and Seliger 1981; Rivkin 1985).

We have recently developed a technique which will allow the separation of a phytoplankton species for which an antibody is available from complex planktonic assemblages. This method (Aguilleras *et al.* submitted ms.) involves the use of tiny magnetic beads which can be linked to cells that are labelled with an antibody. The cell/bead complex is then removed from solution using a magnet. Immunomagnetic separation has been a reliable medical tool for the purification and characterization of a wide range of cell types such as tumor and lymphoid cells. This method is also used routinely for the isolation, identification and analysis of DNA or RNA sequences. The viability of certain cell types does not seem to be affected by the attachment process and in some cases, bead detachment is possible without harming the cells. When applied to phytoplankton cells, the process is fundamentally similar to the standard immunological procedures described above. Experiments with *A. tamarense* have yielded samples of better than 90 - 95% purity, both with mixtures of cultured cells, and with natural plankton communities.

Due to loss of some cells during the antibody labelling procedure, this method is not presently suitable for enumeration of a target species, but instead is intended for separation of a species from a sample for subsequent physiological or biochemical measurements. Some measurements would be straight-forward, requiring little additional developmental effort once the actual removal of target cells from solution is accomplished. For example, if this method were applied to the brown tide organism (which seems perfectly feasible since beads are used to collect bacteria), samples of brown tide water could be incubated for a normal ^{14}C protocol. The *A. anophagefferens* cells could then be removed from solution using magnetic bead, and their activity determined directly using a scintillation counter. This would provide estimates of carbon uptake at the species level, something which is presently only possible during the stages of a bloom when *A. anophagefferens* is completely dominant. Other measurements would be more problematic if it proved necessary to remove the beads from the cells to eliminate contamination or interference. For example, given the recent work of Gobler and Cosper (1995) linking the brown tide distribution to levels of iron, it would be of great interest to determine the iron quota of *A. anophagefferens* cells in various waters and at different times during a bloom. Magnetic bead separation offers some promise in this respect, but the obvious problem with iron contamination from the beads must be avoided. In like manner, measurements of nutrient quotas such as C:N:P ratios should be possible, but not before studies have been undertaken to determine the amount of contamination from the beads, or methods are developed for detachment and removal of the beads without damage to the cells.

SUMMARY

The development of an antibody probe for *A. anophagefferens* provided a useful tool which has done much to improve the accuracy and efficiency of cell counts of this organism in natural samples. In this brief overview, new methods have been introduced which rely on this same antibody, and which could be used with great utility in studies of the brown tide. Each method has its own promises and limitations, and in all cases, development work is needed to bring the concepts proposed here to full application. In most cases, the methods are being developed for other organisms, including some that are harmful or toxic such as the dinoflagellate *A. tamarense*, so extension of those procedures to *A. anophagefferens* should be relatively straight-forward. With a suitable investment of time and funds, not only can the identification and enumeration of this species become faster and more accurate, but physiological measurements which at present are impossible at the species level will become feasible.

ACKNOWLEDGEMENTS

This work was supported in part by grants from the NOAA National Sea Grant College Program Office, Department of Commerce grants No. NA46RG0470 (WHOI Sea Grant Projects R/B-121 and R/B-130), and by the National Marine Fisheries Service through Saltonstall- Kennedy grant (No. NA47FD00447). Contribution No.9082 from the Woods Hole Oceanographic Institution.

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Proceedings of the Brown Tide Summit

October 20-21, 1995

New York Sea Grant

Cornell University • State University of New York • NOAA