IMMUNOFLUORESCENT DETECTION OF THE BROWN TIDE ORGANISM, *AUREOCOCCUS ANOPHAGEFFERENS*

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INTRODUCTION

"Brown tide" is the name given to a new and serious threat to shellfish and submerged aquatic vegetation resources in the northeastern United States. The first known outbreaks occurred in 1985 in Narragansett Bay, Rhode Island, in Peconic Bay and other bays of Long Island, and possibly in Barnegat Bay, New Jersey (Anonymous, 1986). Subsequent blooms have occurred in Long Island waters in 1986-1988 as well. The tiny alga responsible for these outbreaks is a previously undescribed chrysophyte recently given the designation *Aureococcus anophagefferens*, which translates to "golden yellow sphere causing the lack of feeding" (Sieburth *et al.*, 1988). The name derives from one of the major impacts of the brown tide, namely the mortality of high *A. anophagefferens* concentrations (Bricelj and Siddall, 1986).

At the present time, only Long Island and Rhode Island have been adversely impacted by the brown tide. There is an obvious concern in neighboring states which justifiably fear the sudden appearance and dominance of *A. anophagefferens* in their waters, as was the case in Narragansett Bay and Peconic Bay in 1985. Aerial overflights that year showed that the discolored waters of the bloom in Narragansett Bay extended into Rhode Island and Block Island Sounds, stretching around Montauk Point and along the southern Long Island shoreline (Smayda, 1986). Since the species was undoubtedly present

at lower concentrations outside the perimeter of the massive visible bloom, it is reasonable to infer that *A. anophagefferens* was either also present or was introduced into the waters of neighboring states such as New Jersey, Connecticut or Massachusetts.

One of the major constraints to research on the causes, effects, dynamics, and geographic distribution of the brown tide organism is the difficulty investigators have in identifying the cells of *A. anophagefferens* in a mixed natural assemblage. This species (Figure 1A) is very small (*ca.* 2 μm diameter) and lacks morphological features which can be used to differentiate it from similar sized picoplankters using either phase contrast or epifluorescence microscopy (Sieburth *et al.*, 1988). Some workers have developed sufficient familiarity with the species to have reasonable confidence in the accuracy of their light microscope cell counts, but others who do not have constant exposure to *A. anophagefferens* are easily confused. Identification difficulties are especially problematic in efforts to define the geographic distribution of *A. anophagefferens* since discrimination of this species from other picoplankters is very difficult when it is .in low abundance. The situation in Barnegat Bay, NJ is a good example of how water can be discolored from a mixed assemblage of picoplankton within which it has not been possible to positively identify *A. anophagefferens*.

To help resolve this dilemma, we initiated a project with the objective of obtaining antibodies specific to the outer cell wall of *A. anophagefferens* as has been done in the past for the cyanobacterium *Synechococcus* (Fliermans and Schmidt, 1977; Campbell *et al.*, 1983) and some bacteria (e.g. Hill and Gray, 1967; Schmidt, 1974; Taubman and Smith, 1974). This paper describes the procedures used to develop the antibody and the optimal protocol for its use. We also present some preliminary results from an ongoing geographic survey of the distribution of *A. anophagefferens* from Massachusetts to New Jersey.

MATERIALS AND METHODS

Several 2 l Erlenmeyer flasks containing 1 l of K medium (Keller and Guillard, 1985) were inoculated with *A. anophagefferens* (clone BP3B, obtained from E. Cosper) and maintained at 20°C at 250 μEinst m^{-2} s^{-1} on a 14:10 h L:D cycle. Upon reaching stationary phase, the cells were harvested in 250 ml aliquots by centrifugation (1900 X g, 25 min., 20°C). The pelleted cells

were transferred to 50 ml round bottom tubes, and centrifuged again (1500 X g, 10 min., 20°C). All but 5 ml of the supernatant was aspirated from these samples, and 5 ml of 1.2% E.M. grade glutaraldehyde prepared in sea water was added to preserve the cells. After several weeks of storage at 4°C, the supernatant was removed and 5 ml phosphate buffered saline (PBS; 0.02M PO_4^{3-}, 0.15 M NaCl, pH 7.45) containing 0.6% glutaraldehyde was added. The cells were washed three times in the PBS/glutaraldehyde solution and the contents of several tubes mixed together in 15 ml conical centrifuge tubes to obtain a total of approximately 10^9 cells. The cells were shipped by overnight mail to Ventrex Inc., Portland, Maine for antiserum production.

Prior to injection into the marginal ear vein of an SPF New Zealand White rabbit, the cells were washed with PBS and adjusted to 10^9 cells ml^{-1}. This step, and all others mentioned above, was performed aseptically using 0.2 μm sterile-filtered solutions and sterile containers. Our immunization protocol combined an initial large inoculum with monthly booster shots: Day 1, 0.5 ml; day 4, 0.75 ml; days 7, 14, 21, 28, 1.0 ml. Two or three days after the final inoculations, the rabbit was bled from its central artery. The blood was allowed to clot overnight at 4°C and then was centrifuged (1000 x g, 15 min., 4°C) to clear the serum. Each delivery of serum was divided into 1 ml aliquots and stored frozen at -20°C in 1.5 ml micro-centrifuge tubes. Working stock solutions were prepared by diluting 1 part antiserum to 400 parts PBS. These were stored frozen until needed.

Titer and antibody specificity were determined by indirect immunofluorescence (Figure 2). A small quantity of culture or water sample (<200 μl) was added to a 12 x 75 mm test tube along with 1.0 ml of the blocking agent, 3% normal goat serum in PBS. Following a 40 minute incubation, the sample was rinsed with 10 ml PBS into a 25 mm micro-filtration funnel holding a 25mm black polycarbonate filter backed by a 25 mm glass fiber filter, and filtered to a volume less than 2 ml, but not dry. The rinsing process was repeated three times, leaving less than 0.25 ml over the filter at the end. One ml of the A. anophagefferens antiserum was then added (1:800 dilution in PBS for cross reaction testing, and 1:3200 for the geographic survey) and allowed to incubate for 40 min. at room temperature. After a rinse with PBS, the filter was incubated with a 1:800 dilution of FITC conjugated goat anti-rabbit antiserum for 20 min. The sample was rinsed again with PBS and all liquid drawn through the filter. The membrane filter and pad were quickly placed on a microscope slide, and
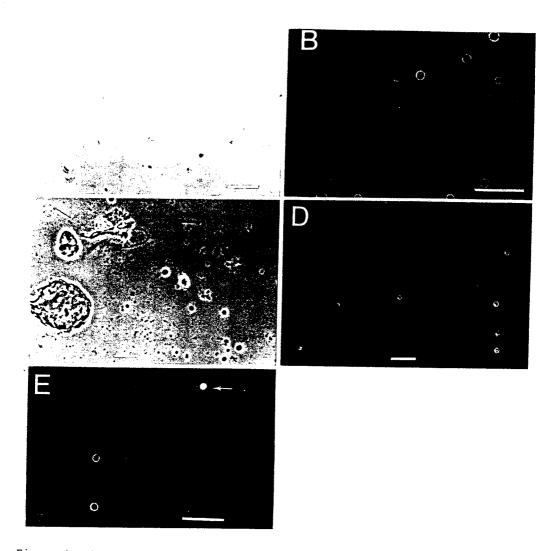
Figure 1. Phase contrast and epifluorescent micrographs of *A. anophagefferens* from laboratory cultures and field samples. Scale bar is 10 μm in all cases. A) Phase contrast image of cultured cells; B) immunofluorescent "halo" around perimeter of cultured *A. anophagefferens*; C) phase contrast image of mixed field assemblage; D) immunofluorescent image of the same field as (C); E) *A. anophagefferens* cells with distinctive perimeter fluorescence and unidentified cross-reacting cell (solid fluorescence, arrow), all in natural sample from Great South Bay, NY.

1 drop of glycerol/PBS (9:1 v/v) was added along with a cover slip. Samples were examined at 400x and 1000x with a Zeiss IM35 inverted epifluorescence microscope (Zeiss filter set 48 77 06 plus red attenuating filter BG 38) with 50 W mercury lamp.

Tests for cross-reactions were conducted on 33 formalin preserved (5% v/v) cultures of selected phytoplankton. An additional 13 species were screened as well, but these were supplied already preserved following a more elaborate protocol using glutaraldehyde designed by Campbell *et al.* (1989) to keep fragile picoplankton intact.

RESULTS

Preservation Techniques

Several different fixation protocols were tested to determine which was the most effective in maximizing the structural rigidity of *A. anophagefferens* and providing the best antigenic response. Cells preserved in varying concentrations of buffered formalin (0.6-3.5%) rapidly lost their morphological integrity and ruptured easily upon transfer to physiological saline. Glutaraldehyde-fixed cells (0.2-4.0%) retained their shape much better, but many still ruptured when placed in saline. The procedure that was finally adopted used 0.6% glutaraldehyde (final concentration) in seawater and several weeks of cold storage, after which the cells could be safely washed and resuspended in PBS.

Once the antiserum was in hand, 3 different preservatives were evaluated to determine which gave acceptable immunofluorescent results. Formalin, Lugol's iodine, and glutaraldehyde all were equally effective in preserving cells without any appreciable cell loss (Table 1). However, glutaraldehyde-fixation maintained the best morphology and also provided the best and brightest immunofluorescent response. In field studies, we therefore recommend samples be preserved with 0.6-1.0% glutaraldehyde. Five percent buffered formalin or sufficient Lugol's to make a sample "tea colored" are acceptable although slightly less desirable alternatives.
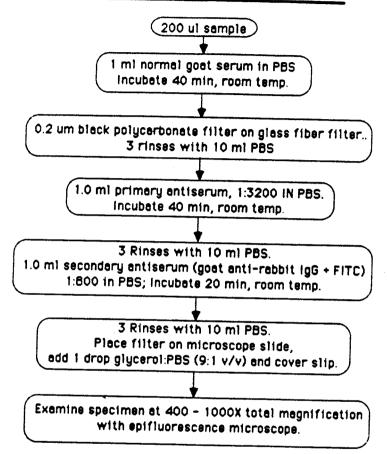
IMMUNOFLUORESCENCE PROTOCOL

Figure 2.   Immunofluorescent protocol.

Antibody Titer and Specificity

When the antiserum was used on cultures or natural samples containing *A. anophagefferens*, the fluorescent labelling was visible around the perimeter of each cell, resembling a green ring or halo (Figure 1B). This cell wall response is to be expected since every effort was made to prepare antibodies to intact cells. The highest dilution of the primary antiserum at which cultured *A. anophagefferens* was visible under epifluorescence was 1:12,800. This would correspond to a remarkably high titer of 12,800. It should be stressed that the addition of glycerol to the microscope slide enhanced fluorescence considerably and consequently the titer as well.

Table 1. Comparison of Counting and Preservation Techniques. Values shown are cell concentration (10^3 cells ml^{-1} (SE)).

Location	Glutaraldehyde[a]		Formalin[b]		Lugol's[c]	
	Phase Contrast	Immunofl.	Phase Contrast	Immunofl.	Phase Contrast	Immunofl.
Blue Point Ave., Great South Bay, Long Island, NY	62 (3.0) n=3	140 (19.6) n=5	68 (4.2) n=3	127 (23.0) n=5	111 (12.4) n=3	124 (5.6) n=8
Islip Marina, Great South Bay, Long Island, NY	19.5 (3.8) n=3	25.9 (4.3) n=5	28.2 (9.3) n=3	46.2 (10.8) n=5	17.7 (2.9) n=3	36 (5.7) n=5
West Neck Bay, Peconic Bay, Long Island, NY	5.7 (3.8) n=3	1.55 (0.13) n=5	5.7 (0.8) n=3	1.32 (0.44) n=3	0.88 (0.22) n=3	1.96 (0.43) n=5

[a] 2.5% glutaraldehyde
[b] 4% formalin
[c] 3.6% Lugol's

TABLE 2. Cross-Reactions with Antiserum to Aureococcus anophagefferens (BP 3B)[1]

DIVISION Class Genus species Culture ID	ISOLATION Location	Investigator	Immuno- fluorescent Reaction (+ or -)
Cyanophyeace			
Synechococcus sp			
DC-2	33°44.99'N, 67°29.8'W	L. Brand	-
FL-8	Flanders Bay, LI, NY	P. Hargraves	-
CYAN	Narragansett Bay, RI	D. Steele	-
TPB	Narragansett Bay, RI	D. Steele	-
CHLOROPHYCOPHYTA			
Chlorophyceae			
Chlorella stigmatophora			
993	Plymouth, UK	Parke	-
Chlorella sp			
PJ8A	Pt. Judith, RI	P. Hargraves	-
PJ8E	Pt. Judith, RI	P. Hargraves	-
Prasinophyceae			
Unidentified			
X48-23	38°19.5'N, 69°34.5'W	L. Murphy	-
Unidentified			
Nanno	Great South Bay, LI, NY	J. Ryther	-
Bt 3	Great South Bay, LI, NY	D. Caron	-
Bt 8	Great South Bay, LI, NY	D. Caron	-
CHRYSOPHYCOPHYTA			
Chrysophyceae			
Aureococcus anophagefferens			
BP 3B	Great South Bay, LI, NY	E. Cosper	+
Chrysamoeba sp			
IG3	34°N, 65°W	L. Provasoli	-
IG5	34°N, 65°W	L. Provasoli	-
Ochromonas sp			
IC1	34°N, 65°W	L. Provasoli	-
Ochromonas stellaris			
UW329	West San Juan Island, WA	Unknown	-
Pelagococcus subviridis[2]			
Pela C12	North Pacific Central Gyre	R. Norris	-
Pelagococcus sp			
H314	Unknown	L. Provasoli	-
Unidentified			
MCL	North Pacific Central Gyre	R. Levin	-

[1]All samples processed with 1:800 primary antibody dilution, following protocol in text. Positive immunofluorescent reaction required visible FITC fluorescence at 400 or 100 X total magnification.

[2]Cross-reaction not visible at 1:1600 dilution of primary antiserum.

Table 2. (cont.)

DIVISION
  Class
 	
    <u>Genus species</u>
      Culture ID
	ISOLATION
	
	Location
		
		Investigator
			Immuno-
			fluorescent
			Reaction
			(+ or -)

CHRYSOPHYCOPHYTA (Cont.)			
Prymnesiophyceae			
Emiliania huxleyi			
BT6	32°10N, 64°30'W	R. Guillard	-
451B	Oslofjord, Norway	E. Paasche	-
Imantonia rotunda			
IIE6	38°42'ˆN, 72°22'W	L. Provasoli	-
WTRE	Saanich Inlet, Canada	J. Jordan	-
Isochrysis galbana			
Iso	Isle of Man, UK	Clonal-Parke	-
T. Iso	Society Island, Tahiti	Clonal-Haines	-
Pavolova pingus			
IG7	34°N, 65°W	L. Provasoli	-
Pavolova lutheri			
MONO	Finland, rock pool	M. Droop	-
Phaeocystis sp			
1209	29°16'N, 85°54'W	L. Brand	-
Pleurochrysis carterae			
Cocco II	Woods Hole, MA	I. Pintner	-
Xanthophyceae			
Olisthodiscus luteus			
Olistho mx			
Bacillariophyceae			
Minutocellus polymorphus			
Minuto	Great South Bay, LI, NY	D. Caron	-
675-D	6°28'N, 54°59'W	R. Guillard	-
Thalassiosira pseudonana			
3H	Moriches Bay, LI, NY	R. Guillard	-
PYRRHOPHYCOPHYTA			
Dinophyceae			
Amphidinium sp			
Amphi	Great Pond, Falmouth, MA	R. Guillard	-
RHODOPHYCOPHYTA			
Rhodophyceae			
Porphyridium sp			
Porph	Eel Pond, Woods Hole, MA	R. Lewin	-
OTHER UNCLASSIFIED TEST CULTURES			
IS-low1	Gulf Stream	R. Olson	-
PBE10	Narragansett Bay	D. Steele	-
NBEID	Narragansett Bay	D. Steele	-
WNB 722	West Neck Bay, LI, NY	E. Cosper	-
WNB3	West Neck Bay, LI, NY	E. Cosper	-
B. Nuzz 1	Peconic Bay, LI, NY	D. Steele	-
B. Nuzz 4	Peconic Bay, LI, NY	D. Steele	-
B. Nuzz 7	Peconic Bay, LI, NY	D. Steele	-
NBLC	Narragansett Bay, RI	D. Steele	-
ISB	38°23'N, 67°48'W	R. Olson	-

Forty-six species or strains of marine phytoplankton selected on the basis of their phylogenetic or morphological similarity to *A. anophagefferens* were tested for cross-reactivity with the antiserum (Table 2). Of these, 13 showed a slight cross-reaction at a 1:400 dilution. At 1:800, only *Pelagococcous subviridis* was labelled, but the antibody affinity was weak and disappeared at 1:1600. No cross-reactions were observed at antiserum concentrations of 1:3200, which was accordingly selected as the working dilution for field samples.

Several field samples collected during the distributional survey did contain cells that fluoresced when treated with our protocol but that were clearly not *A. anophagefferens*. These cross-reactions were to bacteria (approximately 20% of the total bacterial population, based on DAPI staining) and to a few 2-4 μm spherical cells. The former were rod shaped, always much smaller than *A. anophagefferens* and labelled with a very faint perimeter fluorescence. The latter remain unidentified at this time, but were non-photosynthetic and labelled with a distinct, bright fluorescence of the entire cell which was quite different from the green ring or halo pattern exhibited by *A. anophagefferens* (Figure 1E). These cross reacting cells were rather scarce, and could not be isolated into culture. When these samples were processed through our standard protocol but without the primary antibody, the fluorescence was still visible.

Recommended Protocol

Aureococcus anophagefferens can be quantified easily with our immunofluorescent protocol, shown diagrammatically in Figure 2. Several items should be noted. The most time-consuming steps in this process are the incubations with the blocking agent (normal goat serum), the primary antibody, and the secondary antibody. The incubation times in Figure 2 have not been optimized, and it is possible that times shorter than those indicated will provide the necessary degree of labelling. This can only be determined by varying the duration of each incubation systematically, a process that is underway and that will be completed shortly. Considerable time can also be saved by using multiple filter assemblies (we use six) so that samples can be processed simultaneously. Once a filter is processed, the cells can be counted very rapidly since the fluorescent "halo" is easily visible at 400X total magnification. There is no need to use oil immersion 100X objectives unless an unusual degree of discrimination is required. At

400X, we enumerate *A. anophagefferens* in 50 fields in a cross pattern on each filter, a process that takes 10-15 minutes per sample. The limit of detection for a 200 μl sample is thus approximately 100 cells ml^{-1}. This sample size is small because of clogging problems with a 0.2 μm filter. Use of a 1.0 μm pore size filter is possible, but the vacuum to each filter assembly must be controlled with a valve so that the filter does not dry. Under those conditions, the sample size can be 2 ml or greater and the detection limit < 10 cells ml^{-1}.

A final comment is that the glycerol added to each microscope slide is very important. Without this addition, the fluorescence is so weak that increasing it to an acceptable level would require longer incubations or higher antibody concentrations. We presume that this fluorescence enhancement is a result of the glycerol changing the polarity of the fluid in contact with the cells, which in turn increases the quantum yield of fluorescence.

DISCUSSION

Immunofluorescent detection of the brown tide alga *A. anophagefferens* offers a rapid, accurate alternative to bright field microscope techniques. Samples can now be examined with a high degree of confidence that only *A. anophagefferens* will be enumerated, even when the species is in low abundance relative to other, similar-sized, co-occurring organisms (Figure 1C,D). At dilutions that yield excellent labelling of *A. anophagefferens*, the antiserum did not cross react with any of the 46 phytoplankton cultures we tested, representing 5 algal classes and including 20 species from the class Chrysophycophyta. At higher antiserum concentrations, *Pelagococcous subviridis* showed the greatest affinity for the antibody. This is evidence of some degree of serological similarity in the outer cell wall protein structure that may have important phylogenetic implications. Despite these similarities, it is quite easy to discriminate between *Aureococcus* and *Pelagococcous* using immunofluorescence, a finding consistent with the observations of Sieburth *et al.*, (1988) who compared *A. anophagefferens* ultrastructure to that of *P. subviridis* as described by Lewin *et al.*, (1977) and described differences in size, the number of mitochondria, the extent of vesiculation, and the existence of a well-defined pyrenoid.

Although no cross-reactions were observed in the tests with phytoplankton cultures, some bacteria and small non-photosynthetic cells from field samples did have a fluorescent label. It is now clear that these cross-reactions were due to non-specific binding (*i.e.* labelling was still visible when no primary antibody was used). It should thus be possible to eliminate the problem by changing the concentration and incubation times of the blocking agent or of the secondary antibody. However, since any new protocol will necessarily be more stringent than that presented here (*i.e.* concentrations and incubation times will be decreased) there should not be a need to re-test an array of phytoplankton cultures for cross-reactions.

It should be stressed that these cross-reactions are a minor problem easily recognized by a trained observer. This is because the *A. anophagefferens* labelling pattern around the periphery of the cell (Figure 1B,1D) is both bright and distinct and is difficult to confuse with the very weak fluorescence of bacteria or the solid "spot" fluorescence of the larger cross-reacting cells (Figure 1E). Attempts to count *A. anophagefferens* in an automated fashion, as with a flow cytometer, would require changes in the processing, as discussed above.

Our protocol works best on samples that have been preserved in glutaraldehyde, but formalin or even Lugol's are acceptable alternatives. This statement assumes that each of these preservatives is equally effective in keeping *A. anophagefferens* cells intact. As seen in Table 1, we found good agreement between the immunofluorescent counts of natural samples that had been separated into aliquots, preserved with each of the three fixatives, and counted after approximately one month of storage. The results suggest that these 3 fixatives are equally acceptable in terms of maintaining *A. anophagefferens* cell integrity and in preserving antigenicity for short periods of time, although again we emphasize that glutaraldehyde is preferred due to the brightness of the labelling and the general integrity of the cells.

Although our replicate immunofluorescent counts are reasonably consistent with each other across a range of cell concentrations and different preservatives, they differ considerably from most counts made with light microscope techniques (Table 1). Agreement between the two counting methods is generally the best with Lugol's preserved material, although even then, the phase contrast counts are generally half those made using immunofluorescence at the lower *A. anophagefferens* cell concentrations. It is

of note that the best agreement between counting methods is at the highest cell concentration (Blue Point Ave.). At the lowest concentration (West Neck Bay), the immunofluorescent counts for the three preservatives are reasonably consistent, differing from each other by at most 30%, whereas the phase contrast counts vary by 600%. We believe this difference reflects the low number of *A. anophagefferens* cells in these samples relative to other, similar-sized co-occurring picoplankton which can be 3 orders of magnitude more abundant. Under such conditions, discrimination between the brown tide cells and the other picoplankton is easy with immunofluorescence but difficult (and variable with different preservatives) under phase contrast.

These data alone cannot indicate which counting method is the most accurate, since we have no *a priori* knowledge of the actual *A. anophagefferens* cell concentration. Nevertheless, the internal consistency between the different immunofluorescent counts compared to the large variability between the phase contrast counts argues that the former are the most accurate. In the "blind" comparisons given in Table 1, the phase contrast counts were made by an individual (E.M. Cosper) with extensive exposure to *A. anophagefferens* in natural samples. Comparisons with results from other workers, especially those with little or no familiarity with brown tide cells would likely show even less agreement. In such cases, the immunofluorescent method is the least subjective and most accurate method for enumeration. For experienced workers, immunofluorescence can speed up cell counts and provide an extra degree of confidence in their accuracy, especially when *A. anophagefferens* cells are in low relative abundance.

Most of the samples that we examined were collected weeks to months before we processed them. However, we also processed several one and two year old samples, the former preserved in formalin and the latter in Lugol's. One two year old sample from Great South Bay contained approximately 10^4 *A. anophagefferens* cells ml^{-1}. The fluorescent "halo" around the perimeter of the cells was present, but the labelling was not as intense as that seen in fresh samples. Another two year old Lugol's-preserved sample collected from Barnegat Bay, NJ in September, 1986 contained approximately 400 *A. anophagefferens* cells ml^{-1}, but again the fluorescence was dim. Unfortunately, in both of these cases, we do not know what the original concentrations of brown tide cells were prior to the 2 years of storage. This was not the case with the one year old samples preserved in 0.4% formalin which had been counted at the time of collection. For all four of these samples, the

original light microscope counts were higher than our immunofluorescent counts - by a factor of 2 in the two samples with high *A. anophagefferens* cell concentrations (10^5 cells ml^{-1}) and by a factor of 10 in samples with low concentrations (5×10^3 cells ml^{-1}). Although we have not been able to test old samples preserved in glutaraldehyde, the results thus far indicate that some antigenic activity is retained in preserved samples stored in the cold for a year or more but that it would be unwise to attempt to use immunofluorescence quantitatively for samples stored for longer than one or two months. Even though only a variable fraction of the original cells can be positively identified as *A. anophagefferens* in older samples, these results are nevertheless encouraging as they suggest that it may be possible to use the immunofluorescent technique on archived plankton samples to determine whether this species was present in the region prior to the 1985 outbreak.

Just as this method will allow us to look backward in time to learn more about the temporal pattern of *A. anophagefferens* abundance, it also makes it possible for us to screen water samples from areas distant from the population "center" in southern Long Island waters. In work to be reported elsewhere, over 65 samples have been collected from nearshore sites along the coast between Boston MA and western Connecticut during August and September, 1988. In addition, we are examining samples from the entire length of Barnegat Bay collected by personnel from the New Jersey Department of Environmental Protection. Only a few of these samples have been processed at this time, but we can already report that *A. anophagefferens* was identified (and verified with a second analysis) in very low concentrations in one sample from Massachusetts (Pleasant Bay, Chatham; 100 cells ml^{-1}), 9 other samples from Cape Cod being negative. Of the 6 Narragansett Bay samples examined thus far, 2 contained *A. anophagefferens*, again near 100 cells ml^{-1}. Of 4 Connecticut samples, three were positive (Niantic River, Guilford Harbor, and Milford Harbor) with 100-350 cells ml^{-1}. Eight of 10 samples from New Jersey have contained *A. anophagefferens* cells. Three of these samples were from northern Barnegat Bay and ranged from 146 to 784 cells ml^{-1}, whereas two collected further south near Manahawkin and Surf City had higher concentrations of 1.4×10^5 and 3.5×10^4 cells ml^{-1} respectively.

All of these survey samples were collected in 1988, and many more are to be analyzed to complete the study. It is of note that one reason personnel from New Jersey were not able to positively identify *A. anophagefferens* in their samples was the dominance of other 2 μm size

picoplankton. In one sample that we examined, the 146 *A. anophagefferens* cells ml^{-1} that we detected with immunofluorescence represented only 0.008% of the total picoplankton concentration (*i.e.* that one cell in 12,500 was the brown tide alga). Even the highest Barnegat Bay concentration of 1.4 x 10^5 cells ml^{-1} represented only 7% of the total number of 2 μm cells in the mixed assemblage.

These preliminary survey results indicate that *A. anophagefferens* has a much larger geographic distribution than its adverse effects on eelgrass beds or shellfish in Rhode Island (1985 only) and Long Island (1985-1988) would suggest. In light of these findings, many coastal areas now have justifiable cause for concern that this species might someday emerge from low and previously undetectable background concentrations and bloom at the tremendous population densities that cause damage and visible brown tides. A challenging but critically important research priority is thus the need to understand the physical, chemical, and biological factors that would make such a future outbreak a reality and that might have triggered the first blooms of *A. anophagefferens* in 1985. We expect that the antibody and immunofluorescent protocol described here will be valuable tools in these future investigations.

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