IMMUNOCHEMICAL APPROACHES TO THE IDENTIFICATION OF THE ULTRAPLANKTON: ASSETS AND LIMITATIONS

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INTRODUCTION TO THE ULTRAPLANKTON

During the past decade, it has become obvious that most photosynthetic cells are exceedingly small. We now realize that 50- 80% of the primary production in the open ocean takes place in cells capable of passing through a 3 μm polycarbonate filter (Platt *et al.*, 1983; Li *et al.*, 1983; Glover *et al.*, 1986a). This size fraction is termed the ultraplankton.

Historically, the term "ultraplankton" has been used to describe very small plankton. Various authorities have set the upper limit of "small" as <3 μm to <10 μm. Here, we define the term functionally as cells small enough to pass through a 3 μm pore Nuclepore filter, thus the upper limit of the smallest diameter becomes 4-5 μm. So defined, the ultraplankton contains both the picoplankton (0.2-2.0 μm) and the smaller nanoplankton (2.0-20 μm).

The development of epifluorescence microscopy and the application of the technique to analysis of marine phytoplankton samples has provided the means for visualizing the minute cells of the ultraplankton (Wood, 1956). Cells not previously differentiated from debris particles can now be clearly distinguished. It quickly became obvious that most ultraplanktonic phototrophs were phycoerythrin-dominant cyanobacteria (Johnson and Sieburth, 1979; Waterbury *et al.*, 1979), but that at certain times, and under certain conditions, the size fraction was dominated by minute, chlorophyll-dominant

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eukaryotes (Johnson and Sieburth, 1982; Murphy and Haugen, 1985). Thus far, it has not been possible to differentiate the separate contributions to primary production of these two moieties, but laboratory studies (Wood, 1985; Glover *et al.*, 1986b and 1987) indicate that deep in the photic zone the eukaryotic moiety should be far more efficient at photosynthesis than the prokaryotic moiety.

TABLE 1.

COMPONENTS OF THE ULTRAPLANKTON

	CLASS	CHLOROPHYLLS
*	Bacillariophyceae	a + c
*	Chrysophyceae	a + c
	Dinophyceae	a + c
*	Eustigmatophyceae	a
	Prymnesiophyceae	a + c
	Cryptophyceae	a + c
*	Chlorophyceae	a + b
	Euglenophyceae	a + b
*	Micromonadophyceae	a + b (+"c-like")

* Contain picoplankton-sized species

The eukaryotic component is diverse and contains representatives of most phytoplankton classes (Shapiro and Guillard, 1986; Stockner and Antia, 1986). Electron micrographs of natural assemblages show many different types of cells and representatives of nine classes have been cultured (Table 1). However, many ultraplankters explode or dissolve in preservative solution, and others are relatively impervious to preservatives so that internal details do not preserve well enough to permit resolution of taxonomic features. Electron microscopy thus gives neither reliable quantitative counts of the various ultraplankton taxa, nor relative abundances. Culture techniques also may not yield estimates of relative abundances since culturable types are not necessarily a random sample of the natural assemblage. Thus, although we know the assemblage is diverse, we have no knowledge of relative abundances of the different components.

Knowledge of taxonomic composition is essential since the various algal components do not behave similarly. Alternate species or genotypes can respond differently to environmental changes and provide different food potential to higher trophic levels. To explore these different responses, we must be able to identify and trace the various components. It seemed to us that the best initial approach would be an immunological assay using a specific tag. We sought to develop antibodies directed against specific ultraplankton taxa, and then to trace the relative abundances of the different taxa in natural assemblages using an immunofluorescence assay.

IMMUNOLOGICAL ASSAY

The application of immunological approaches to oceanographic questions has provided a means to label, tag, or identify specific substances or antigens. Such labels have been used to identify predator (Feller, 1984; Theilacker *et al.*, 1986) and prey species (Feller *et al.*, 1979; 1985; Grisley and Boyle, 1985), identify life cycle stages (Gallagher *et al.*, 1988), visualize intracellular location of antigen (Nicolas *et al.*, 1985; Anderson and Cheng, 1988), identify genetically based physiological type (Ward and Carlucci, 1985), recognize phylogenetic relationships (Fawley *et al.*, 1986 and 1987; Friedman and Alberte, 1987), and approach taxonomic problems such as differentiation among sibling species and races (Campbell *et al.*, 1983; Glover *et al.*, 1986a; Campbell and Carpenter, 1987).

An antigen is a substance that is capable of eliciting an immune response. The production of antibodies is the aspect of the immune response that is most useful for labeling. The antibodies produced are generally highly specific in that they bind to specific antigens. Whole cells or components of phytoplankton cells can be used as antigens and can be injected into vertebrates (usually rabbits, goats or mice). These particulate or soluble antigens induce production of antibodies by beta lymphocytes (B cells). A given lymphocyte and all of its progeny (the clone) will produce only one antibody. The antibodies can be collected, labeled, and used for subsequent detection of their specific antigen. Detection of the label indicates presence of antibody, and therefore the presence of the specific antigen (Fig. 1). Choice of label is determined by detection device: radioimmunolabels emit alpha or beta particles which are detected by scintillation counting, enzyme-linked labels usually provide a colored marker detected visually, immunogold or immunoferritin labels can be detected by

electron microscopy, immunofluorescent labels are detected with epifluorescence microscopy or flow cytometry.

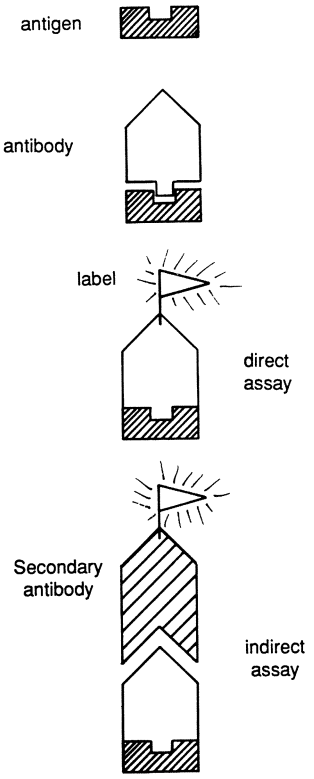

Figure 1. The Anitigen: Antibody Response

We have utilized an indirect immunofluorescence assay. In our system, antigen is located on the surface of the phytoplankton cell. The primary antibody is produced in a rabbit in response to immunization with intact phytoplankton cells. The resulting serum contains antibodies to antigens located on the cell surface. In the assay itself (Fig. 2), cells are first labeled with the primary antibody, then unbound antibody is rinsed away. The sample is then incubated with a secondary antibody which is directed against

the primary antibody and is conjugated with fluorescein isothiocyanate (FITC), a fluorescent dye that emits green light when excited with blue light. The secondary antibody is produced in swine in response to rabbit antibodies; thus, it recognizes the primary antibody as its antigen. Labeled cells, when observed by epifluorescence microscopy, appear brightly outlined by the green FITC fluorescence.

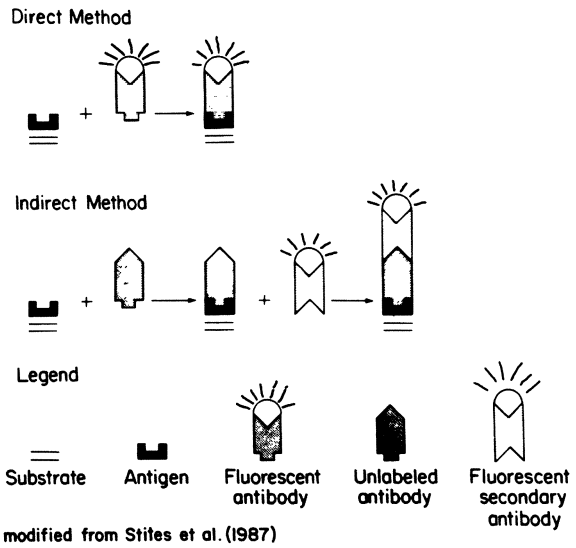

Figure 2. Immunofluorescence Assay

Antibodies can be generated in either a monoclonal or a polyclonal system. The antibodies in a monoclonal system are all produced by a single clone of lymphocytes; those of a polyclonal system are products of multiple lymphocyte clones.

In a polyclonal system, antigen is injected into an appropriate host, and the antibodies generated are harvested from the serum. The procedure is relatively simple and inexpensive, but results in a finite supply of antibodies since it is limited to the antibody production of a single animal.

Additionally, the titer and specificities of antibodies may vary over the life of the animal. The resulting serum may contain several antibodies, each recognizing and binding to different sites (epitopes) on the antigen, since the serum contains antibodies produced by numerous different lymphocytes. This confers greater overall affinity, but can be a disadvantage if the different epitopes are not phylogenetically or physiologically stable.

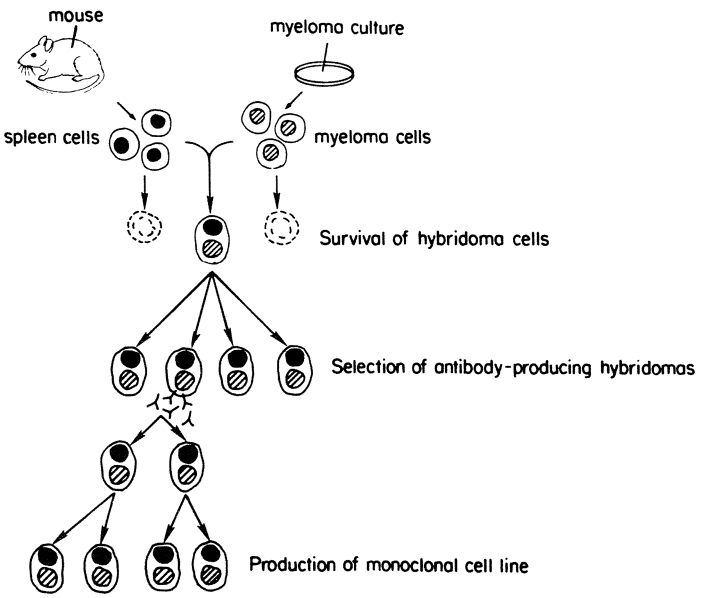

Figure 3. Production of Monoclonal Antibodies

In a monoclonal system (Fig. 3), antigen is injected into an appropriate host (usually a mouse), antibodies manufactured, and antibody-producing cells are harvested from the spleen. These short-lived cells are fused to immortal myeloma cells to produce an immortal, antibody-producing cell line called a hybridoma. The fusing and subsequent screening procedure is relatively complex and expensive, but results in an infinite, unvariable supply of antibody. Since only one lymphocyte clone is involved in any hybridoma line, each hybridoma produces only one antibody which recognizes only one epitope on the antigen. The result is a lessened overall affinity and an unvarying specificity. This may be either an advantage or disadvantage, depending on the question being posed.

We have chosen polyclonal production of antibodies at this step because of the greater sensitivity and ease of production, and the reduced cost.

MATERIALS AND METHODS

Clonal cultures of cells representative of six major algal groups were selected as antigens (Table 2). Two common marine Micromonadophyceae cell types, a coccoid form (Ω48-23) and a flagellated form (DW-8) were used. Cultures were obtained from the Provasoli-Guillard Culture Collection of Marine Phytoplankton (CCMP) at Bigelow Laboratory and maintained in our lab in medium f/2 (Guillard and Ryther, 1962) or "k" (Keller *et al.*, 1987) at 20°C in moderate light on a 14:10 L:D cycle. Aliquots of each cluture were

TABLE 2.

CLONES USED IN THIS STUDY

Class	Representative used as Antigen	Cell Origin	Cell concentration of vaccine (ml^{-1})	Titer*
Bacillariophyceae	*Thalassiosira oceanica* (13-1)	oceanic	7.2×10^6	6,400
Chlorophyceae	*Dunaliella tertiolecta* (DUN)	coastal	2.5×10^6	6,400
Chrysophyceae	*Pelagococcus subviridis* (Pela)	oceanic	1.4×10^7	3,200
Cryptophyceae	*Chroomonas salina* (3C)	coastal	5.9×10^6	12,800
Micromonadophyceae	unidentified coccoid (Ω48-23)	oceanic	4.5×10^7	25,600
	Micromonas pusilla (DW8)	coastal	3.7×10^7	6,400-12,800
Prymnesiophyceae	*Emiliania huxley* (BT-6)	oceanic	8.1×10^6	12,800

* highest dilution still producing positive reaction

centrifuged and washed free of media salts at 8° and 4° C before preservation with paraformaldehyde, pH 7.4 to a final concentration of 0.6%, and were stored at 4° C until used for titer or cross reactivity tests.

Whole cell vaccines were prepared as previously described (Campbell *et al.*, 1983) with modifications developed to prevent lysis of the more delicate eukaryotic cells (BT-6, DW-8, 3C, PELA and DUN). These clones had to be centrifuged at lower speeds (4000 vs 6000 rpm) using a Sorvall RC-5B refrigerated centrifuge with either GSA or SA600 rotors, and were washed and resuspended in chilled (4° C) 0.2 μm filter-sterlized seawater (FSW) of 28-32 $^{o}/_{oo}$ instead of sterile saline or 0.02 M phosphate buffered (PBS) at pH 7.5. Cooling these cultures in 2° C stepdowns (*i.e.* 10° , 8° , 6° C) at each successive wash, and resuspending the pellets in 1:1 PBS:FSW in the final step prior to fixation prevented cell lysis and cell clumping. The cryptomonad, 3C, with water soluble phycobilin pigments, was an excellent monitor of the effectiveness of these precautions. Although electron microscopy grade glutaraldehyde produced the best results for fixation and storage (0.5% and 0.25% final concentrations, respectively) of these cells for whole cell vaccines, paraformaldehyde at pH 7.4 (0.6% and 0.2% final concentrations, respectively), as used in prior studies with cyanobacteria, appeared to be as effective as glutaraldehyde for short term storage if these precautions were taken (although morphologically, cells were more distorted).

Polyclonal antisera directed against the cell surface antigens of the 7 clones were produced in rabbits. The immunization was by intravenous injection following the schedule outlined in Campbell *et al.*, 1983). Immediately prior to injection of rabbits, an aliquot of vaccine was washed with sterile PBS. Cell concentrations of the vaccine ranged from 10^6 ml^{-1} for larger cells (13-1, 3C, DUN) to 10^7 ml^{-1} for the smaller (PELA, 48-23, DW-8 and BT-6) (Table 2).

Test bleeds were analyzed for antibody activity using immuno dot-blot and indirect immunofluorescence (IF) assays (Campbell, 1988). The titers and specificity of each antiserum was determined by IF assay on glass slides (Campbell, 1988) or on polycarbonate filters (Campbell *et al.*, 1983). Slides were used to minimize the amount of antiserum required per test, 0.05 ml, as compared to 0.6 ml needed for the 25mm polycarbonate filters. To slides pre-cleaned with 70% ethanol, a drop of each preserved test strain was attached

TABLE 3.

CROSS REACTIONS AMONG MAJOR GROUPS*

Clone	Antisera directed against						
	13-1	DUN	PELA	3C	Ω48-23	DW-8	BT6
BACILLARIOPHYCEAE							
Thalassiosira oceanica (13-1)	+	-	-	-	-	-	-
Cylindrotheca closterium (WT5)	-	-	-	-	-	-	-
CHLOROPHYCEAE							
Dunaliella tertiolecta (DUN)	-	+	-	-	-	-	-
CHRYSOPHYCEAE							
Pelagococcus subviridis (PELA)	-	-	+	-	-	-	-
Aureococcus anophagefferens	-	-	-	-	-	-	-
CRYPTOPHYCEAE							
Chroomonas salina (3C)	-	-	-	+	-	-	-
CYANOBACTERIA							
Synechococcus WH7803	-	-	-	-	-	-	-
WH5701	-	-	-	-	-	-	-
DINOPHYCEAE							
Gonyaulax tamarensis (GT429)	-	-	-	-	-	-	-
Gyrodinium sp. (94 GYR)	-	-	-	-	-	-	-
EUSTIGMATOPHYCEAE							
Nannochloropsis salina (GSB Sticho)	-	-	-	-	-	-	-
Nannochloropsis sp. (7-15 Sticho)	-	-	-	-	-	-	-
MICROMONADOPHYCEAE							
unidentified coccoid (Ω48-23)	-	-	-	-	+	-	-
Micromonas pusilla (DW-8)	-	-	-	-	-	+	-
PRYMNESIOPHYCEAE							
Emiliania huxleyi (BT-6)	-	-	-	-	-	-	+

* "+" positive reaction between antiserum and test clone
"-" no reaction

either by heat fixation or with 0.01% poly-l-lysine, MW>150,000 (Sigma) (Farr and Nakane, 1981). Except for the coccolithophores, better cell adhesion and less cell alteration was achieved with the poly-l-lysine method. Slides were rinsed in PBS for 10 minutes, and air dried before covering the adhered cells with one drop of primary rabbit antiserum. After a 30 minute incubation, slides were washed for 20 minutes in PBS and then incubated 30 minutes with the secondary antibody, swine anti-rabbit antibody conjugated with FITC. Slides were then given a final 20 minute wash and air dried. Coverslips were affixed with a drop of glycerol:carbonate buffer (9:1) mounting medium (pH 9). Controls consisted of tests run concurrently substituting a pre-immunization serum from each respective rabbit for the primary test antiserum in the first incubation. The protocol for IF assay on 0.4 μm polycarbonate filters followed, Campbell *et al.* (1983), except that normal swine serum (diluted 1:200 with 0.2 μm filtered PBS containing 0.05% Tween 20) instead of gelatin was used as a blocking buffer to reduce non-specific staining and background noise. With this procedure, it is not necessary to stain the filters with Irgalan black.

Titers were determined by either IF assay, using a series of two-fold dilutions ranging from 1:100 to 1:51200 of each antiserum in the blocking buffer. Each test was rated for the visual quality of staining from excellent (4+) to poor (1+).

To determine the specificity of each antiserum, we tested for cross reactions, *i.e.* reactions of an antiserum against an antigen(s) not present in the immunization preparation. In the initial screening, we tested representatives of major phytoplankton groups which, in addition to the 7 clones used as antigens, included cyanobacteria (WH7803; WH5701); Dinophyceae (GT429, 94Gyr) and Eustigmatophyceae (GSB Sticho; 7-15 Sticho) (see Table 3). Next, within each algal group, representative coastal and oceanic clones of conspecific, congeneric and unrelated genera were tested for cross reactions (see Tables 4-7).

In initial tests with glutaraldehyde-preserved cells, we experienced non-specific staining that increased with cell storage time. The potential for false positives, as indicated by labeling with control sera, was high. In addition, glutaraldehyde often gave the cells (especially micromonadophytes) a low level of yellowish-green fluorescence that

interfered with the IF assay. For these reasons, paraformaldehyde, pH 7.4, was used exclusively in all subsequent tests.

All samples were examined with a Zeiss Universal epifluorescent microscope equipped with a 50W mercury lamp and Zeiss filter set #48-77-09 blue excitation (BP450-490) and emission cutoff at 520 mm for FITC fluorescence.

RESULTS

For each of the antigens we obtained an antiserum that was at least genera specific. That is, none cross reacted with strains outside of the genus. Within the Bacillariophyceae, three of four *Thalassiosira* clones cross reacted with anti-13-1 (*T. oceanica*). Of these, one was 13-1 itself, one was a clone of the very closely-related species *T. pseudonana*, and one has not been identified to species. None of the clones from five other diatom genera cross reacted (Table 4).

TABLE 4.

CROSS REACTIONS WITHIN THE BACILLARIOPHYCEAE
ANTISERUM DIRECTED AGAINST

Thalassiosira oceanica (13-1)

CLONE	CLONAL DESIGNATION	CROSS REACTION
Thalassiosira		
oceanica	13-1	3+
pseudonana	3H	3+
sp.	CHB5	3+
weissflogii	4C	-
Chaetoceros		
gracile	SOLCHAET	-
gracile	WTAX1	-
Minutocellus		
polymorphus	SAY7	-
polymorphus	BCN	-
Cylindrotheca		
closterium	WT5	-
Nitzschia sp.	9-9a	-

Within the Prymnesiophyte group, representatives of 7 genera were screened with antiserum directed against naked cells of *E. huxleyi* (anti-BT6). This antiserum was species specific (Table 5). Tests included strains of *E. huxleyi* with and without coccoliths and both were labeled.

TABLE 5.

CROSS REACTIONS WITHIN THE PRYMNESIOPHYCEAE

ANTISERA DIRECTED AGAINST *Emiliania huxleyi*

CLONE	CLONE DESIGNATION	LOCATION	CROSS REACTION
E. huxleyi	BT-6	Sargasso	4+
	MCH1	Sargasso	+
	8613	Gulf of ME	3+
	451B	Oslo fjord	4+
	92A	British coastal waters	3+
	92D	British coastal waters	3+
Chrysochromulina			
herdlansis	NEPCC186	NE Pacific	-
ericina	NEPCC109A	NE Pacific	-
Coccolithus			
carterae	265-04	Salton Sea	-
neohelis	CONE	La Jolla	-
pelagicus	COPEL		-
Imatonia			
rotunda	WTRE	Saanich I.	-
rotunda	IIE6	N. Atlantic	-
Pavlova			
lutheri	MONO	Finland	-
pingus	IG7	Sargasso	-
Phaeocystis			
sp.	1209	Gulf of Mexico	-
Pleurochrysis			
carterae	COCCOII	Woods Hole	-

Similarly, results with antisera directed against the Micromonadophyte *Micromonas pusilla* were species-specific (Table 6). Four conspecific clones reacted, whereas a fifth, con-generic clone, IB4, did not cross react. Results for the coccoid Micromonadophyceae were specific to the morphological

type (Table 6). Because definite species identifications have not yet been assigned to these clones, we cannot ascertain species-specificity; however,

TABLE 6.

CROSS REACTIONS WITHIN THE MICROMONADOPHYCEAE AND RELATED PRASINOPHYTES

CLONE	CLONAL DESIGNATION	CROSS REACTIONS WITH ANTISERA DIRECTED AGAINST:	
		Ω48-23	DW-8
coccoids	Ω48-23	4+	-
	1326-01	4+	-
	1201-2	4+	-
	BT5	4+	-
Micromonas			
pusilla	DW-8	-	4+
pusilla	IIE1	-	3+
pusilla	IVO3	-	+
pusilla	PLY27	-	+
sp.	IB4	-	-
Mantoniella			
squamata	PLY189	-	-
Nephroselmis			
pyriformis	UW460	-	-
Pedinomonas			
minutissima	VA3	-	-
Pseudoscorfielda			
marina	IVP11	-	-
Pyramimonas			
grossi	PLY78	-	-
Tetraselmis			
carteriiformis	UW439	-	-
sp.	REY2	-	-

the lack of cross reactions with other genera is consistent with our observations of species- or genera-specificity. Our immunofluorescence results are consistent with the chemotaxonomic groupings based on pigment types by Hooks *et al.* (1988).

TABLE 7.

CROSS REACTIONS WITHIN THE CRYPTOPHYCEAE

USING ANTISERA DIRECTED AGAINST

Chroomonas salina (3C)

CLONE	RABBIT NO.	CLONAL DESIGNATION	CROSS REACTION
Chroomonas salina	1	3C	4+
	2		4+
Rhodomonas sp.	1	WT651/60	4+
	2		-
Rhodomonas lens	1	RLENS	2+
	2		-
Cryptomonas sp.	1	ID2	2+
	2		-
Cryptomonas sp.	1	THETA	-
	2		-

Antisera directed against the Cryptophyte, *Chroomonas salina* (anti-3C) and the Chrysophyte, *Pelagococcus subviridis* (anti-Pela) and the Chlorophyte, *Dunaliella tertiolecta* (anti-DUN), are also most likely genus- or species-specific, although tests of these antisera are not yet completed. Results with anti-3C varied between antisera preparations (Table 7). In the first animal, the anti-3C was class-specific, but reacted with at least 3 different genera. The second rabbit produced an antiserum that was genera-specific, and possibly species-specific. The antiserum directed against *Pelagococcus subviridis* has not yet been tested for cross reactions with other *Pelagococcus* clones. It did not react with the brown tide organism, *Aureococcus anophagefferens*. Production of anti-DUN serum is not completed.

ASSETS AND LIMITATIONS

The application of an immunofluorescence assay to the study of marine ultraplankton requires that several precautions be taken. First, one must be able to preserve cells adequately for both vaccine preparation and for enumeration in the IF assay. We have found that paraformaldehyde is the best alternative, at least for short-term sample storage, because cells are fixed with preservation of autofluorescence and without the aldehyde-induced fluorescence associated with glutaraldehyde.

Non-specific staining is a general problem with IF assays. We have found that pre-incubation with normal swine serum as a blocking agent can reduce this problem without affecting the cells.

It is the nature of polyclonal sera that multiple antibodies which recognize different epitopes are produced and that the ratio of these antibodies may change over time. Naturally, different host animals (rabbits, in this case), challenged with the same antigen, are not likely to produce the same combination of antibodies. Thus, changes in specificity over time can occur in a single host animal, and changes must be expected when antisera from different hosts are compared. However, the major and, usually, species-specific antigens are detected with relative consistency.

Each antiserum must be tested rigorously to determine its level of specificity. In most cases we observed species-specific responses. It cannot be assumed, however, that a given antiserum will label every isolate of the species. It is possible that an antiserum may be specific to a subspecies, or race, or even a clone. Because taxonomy of many of the smallest coccoid eukaryotes is not well defined by conventional methods, IF may not be consistent with current identifications. In fact, IF may help in the definition of these species.

In some instances antisera may be too general. Species-specific antisera can be produced by sequential absorption with cross-reacting strains. Highly specific antibody preparations to these strains may then be obtained by eluting with appropriate buffers.

The assets of the IF approach include the ability to identify representatives of extremely small species or clones (such as *Aureococcus*

anophagefferens) and the quantification of specific components in a complex community. Perhaps the major advantage is the identification of a particular cell when no other simple method is possible. Until now we have been limited to studying the ultraplankton community as a qualitatively nonvarying biomass. Development and acquisition of these IF labels allows studies of temporal and spatial changes in dominance and diversity within the ultraplankton community.

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