J. Phycol. **32,** 1–16 (1996)

REVIEW

IMMUNOFLUORESCENCE IN PHYTOPLANKTON RESEARCH: APPLICATIONS AND POTENTIAL

Engel G. Vrieling[1]

Department of Marine Biology and Laboratory for Electron Microscopy, University of Groningen, Biological Centre, P.O. Box 14, 9750 AA Haren, The Netherlands

and

Donald M. Anderson

Woods Hole Oceanographic Institution, Biology Department, Redfield 3-32, Woods Hole, Massachusetts 02543

During the last decade, immunofluorescence has become an important tool in studies of the systematics, biogeography, physiology, and ecology of freshwater and marine phytoplankton. The technology originated in the medical sciences (e.g. Coons et al. 1941, 1955) and was gradually applied to soil bacteria (Fliermans et al. 1974, Josserand and Cley-et-Marel 1979) and bacteria from aquatic environments (Fliermans and Schmidt 1977, Gates and Pham 1979, Ward and Perry 1980, Baker and Mills 1982, Dahl and Laake 1982, Yoshioka et al. 1982, Ward and Carlucci 1985, Currin et al. 1990). Applications of immunology to phytoplankton originated over two decades ago with the innovative studies of Bernhard et al. (1969), who attempted to differentiate phytoplankton species using antibodies. Modern immunological studies of phytoplankton are more diverse in their applications, but the same principles of using antibodies to label and visualize target cells or cellular constituents still apply. Coupled with the power and sensitivity of fluorescence, immunological techniques open the door to physiological and ecological investigations that formerly were not possible. This is especially true in studies that focus on a single species within a complex field assemblage of organisms and detritus. Applications of immunofluorescence include identification and enumeration of phytoplankton species, localization and quantification of cell constituents (enzymes, toxins, structural proteins, and polysaccharides), and labeling of cells for better quantification of grazing rates and mixotrophic or heterotrophic potential. Given the rapid pace of development in this field, it seems appropriate at this time to review the methods, applications, and potential of immunofluorescence as a tool in phytoplankton research, in hopes that such an overview will help to sustain and focus future investigations.

[1] Author for reprint requests.

PRINCIPLES OF IMMUNOFLUORESCENCE

The use of antibodies as "recognition" molecules is essentially an exploitation of a biological phenomenon, the antibody : antigen reaction. Immunofluorescence is the visualization or detection of that reaction. Antibodies are immunoglobulin proteins secreted from activated lymphocytes. They are large molecules, ranging from 150,000 to 950,000 daltons, depending on their class. All are composed of two identical short peptide chains (called light chains) and two identical long peptide chains (called heavy chains) in a Y-shaped molecule. There are five different classes of immunoglobulin molecules, designated IgA, IgG, IgD, IgE, and IgM according to the type of heavy chain they possess. Both heavy and light chains are divided into domains, with the regions of the amino acids that bind to target antigens termed variable domains. The remaining domains are constant or conserved. Adjacent variable domains of the light and heavy chains together constitute the antigen binding site.

Antigens are substances capable of being recognized and bound by antibodies. Antigens may be whole organisms (e.g. bacteria, phytoplankton) or individual molecules. The actual antibody binding site on a whole cell will likely be a protein, but the cell itself can still be considered the antigen. The number and location of amino acid residues of a protein antigen that bind to an antibody are restricted to a maximum of 17 residues (Jeffris and Deverill 1991). A single protein molecule will thus have many structurally different antigenic sites called epitopes. Antibodies can be produced that recognize or bind to any one of a suite of separate or overlapping epitopes on a target protein. An immune response by an animal exposed to an antigen will activate multiple lymphocytes within that animal. Because each lymphocyte is capable of recognizing only one specific epitope, the serum from that animal will contain many different antibodies, termed a polyclonal response. Alternatively, by fusing spleen cells of an immunized animal with myeloma cells, mul-

tiple hybridoma cell lines can be established, each of which produces antibodies against a single epitope of the antigen. These are termed monoclonal antibodies (MAbs). Polyclonal antibodies (PAbs) can be prepared readily at low costs (<$500) and are normally highly sensitive, resulting in high immunofluorescence labeling intensities. They are sometimes more useful than MAbs because multiple antibodies in one PAb antiserum can bind to multiple epitopes on the target molecule and thus make it easier to detect. However, unwanted cross-reactions are more of a problem with PAbs than with MAbs, because the hybridoma cell lines used to produce the latter can be restricted to the antigens of interest by careful screening. In addition, MAb-producing cell lines will continue to produce antibody as long as the hybridoma is maintained in culture (i.e. they are immortal) whereas the supply of a particular PAb is limited to that available from the serum of a single, short-lived animal. Lyophilized or refrigerated polyclonal antisera are stable for years, however, so if produced in sufficient quantity, they can be used for extended periods.

Immunization procedures. In many cases, the most useful antibodies are obtained when the antigens are highly purified prior to immunization. In some cases, however, this is neither possible nor necessary, as in the production of antibodies to cell-surface proteins that are diagnostic for a particular phytoplankton species. For that purpose, whole cells or cell fragments stabilized by fixation in glutaraldehyde, formaldehyde, or paraformaldehyde have been used as antigens for small species such as *Synechococcus* or *Aureococcus* (e.g. Campbell and Carpenter 1987, Anderson et al. 1990) as well as for large forms such as *Alexandrium* (Sako et al. 1993) or *Pseudo-nitzschia* (Bates et al. 1993), obviating the need to identify or purify a particular target protein. Polyclonal antisera have been produced with very high specificity in this manner (e.g. Anderson et al. 1990), but affinity purification techniques can be used to further improve the specificity if the target antigen is available in pure form. A serum with insufficient specificity can also be improved by exposing it to cross-reacting antigens such as cells of species that are not of interest (Mendoza et al. 1995). The nonreacting antibodies remaining in solution then represent a more specific antiserum for the species that are of interest.

When broad specificity in an antiserum is desired, it can be affinity-purified using protein from a species that is evolutionarily distant from that used as the initial antigen. The rationale is that only antibodies that bind to conserved epitopes will be retained and purified and that these will have the desired broad cross-reactivity. This approach was used by Orellana and Perry (1992) to produce an antiserum to the enzyme ribulose-1,5-bisphosphate carboxylase/oxygenase (Rubisco) that can be used to quantify Rubisco concentrations in individual phytoplankton cells in a mixed field assemblage.

Antibodies to toxic antigens such as phytoplankton toxins can be produced by conjugating the antigen to proteins such as bovine serum albumin (Carlson et al. 1984, Baden et al. 1988, Hokama et al. 1988) or by preparing nontoxic derivatives bearing identical antigenic sites (Guire 1988). Immunization procedures can also be adjusted to minimize the amount of antigen actually injected. Direct injection of antigen into lymph nodes, for example, significantly reduces the amount of antigen required. The amount of antigen used in an immunization and the timing of subsequent booster injections vary considerably among laboratories. General guidelines suitable for use with purified proteins are found in immunochemical laboratory manuals (Harlow and Lane 1988, Hudson and Hay 1989). Many of the procedures involving phytoplankton have used modifications of these standard methods, so common sense and extrapolation from related protocols using similar organisms is often the only option when a new species or a new protein is under investigation. For production of cell-surface antigens for species identification, it is often useful to work from a published inoculum size for a given species (e.g. Anderson et al. 1990, Bates et al. 1993), adjusting the cell concentration in proportion to differences in cell-surface area.

Immunofluorescence protocols. Two general staining protocols are used in immunofluorescence studies of phytoplankton. Direct immunofluorescence involves the detection of fluorescence from a reporter molecule coupled directly to the primary antibody. This results in low background fluorescence, because the label is attached directly to the specific antibody. It also involves fewer reagents and fewer washing steps, considerations that are important in quantitative studies. However, the antibody/fluor complex must pass through cell membranes together if the target antigen is inside the cell, and this is not always possible due to charge or size constraints. The fluor also might block or inhibit the antigen binding site of the antibody, reducing its reactivity (Vrieling et al., unpubl. res.).

Indirect immunofluorescence is often used to avoid these problems. In this procedure, the primary antibody reacts with its antigen and is detected using a secondary molecule that binds to the primary or to another molecule coupled to the primary. Sometimes the secondary molecule is an antibody that binds to all antibodies made by a particular animal (e.g. if the primary is a rabbit antibody, a goat–anti-rabbit antiserum can be used as a secondary). The secondary can be an antibody that is covalently linked to a fluorescent compound, an enzyme, or metal particle for detection, or it can be a nonantibody compound that binds to the primary. One of the most common secondaries is streptavidin, a com-

pound with a strong and specific affinity for the vitamin biotin. In use, the primary antibody is labeled with biotin and allowed to react with its antigen. Fluorescently labeled streptavidin is then applied to the sample, where it binds to the biotin and is detected by its fluorescence. Because the biotin/antibody complex often binds more than one streptavidin molecule, fluorescence intensity can be enhanced over procedures using a secondary antibody linked to a single fluorescent molecule (Vrieling et al. 1993a). Another common secondary molecule is protein A, a bacterial protein that binds to the IgGs of many species.

Compared to direct immunofluorescence, indirect methods can decrease permeability problems and increase labeling signal strength. Most importantly perhaps, indirect methods offer great flexibility with respect to the fluors used for detection. There are, however, disadvantages as well. Additional washing and incubation steps are needed with indirect methods, and therefore time and cells may be lost. Background labeling can also increase due to nonspecific binding of the secondary antiserum and should be checked using "negative" control samples.

METHODOLOGICAL CONSIDERATIONS

Autofluorescence. The ability to recognize immunolabeled cells or cell constituents depends in part on the difference in fluorescence between positive labeling and the background fluorescence (autofluorescence) of unlabeled cells or cell constituents. Three factors contribute to this background fluorescence: the choice of filter set, the type of fixation, and nonspecific binding or cross-reactivity of the antibody. Autofluorescence can be reduced by spectral discrimination, which involves selecting fluorescent compounds and filter sets with wavelength and bandwidth characteristics that maximize probe fluorescence and minimize autofluorescence. Chlorophyll fluorescence, for example, occurs across a broad range of excitation wavelengths (350–650 nm) that can interfere with many fluorophores (L. P. Shapiro et al. 1989a, Vrieling et al. 1993a). Even with careful filter selection, nonlabeled cells sometimes have significant autofluorescence, equivalent to 10–30% of the signal from labeled cells (Anderson 1995). This background level increases further when nonspecific binding occurs. This can be assessed by incubating cells without the primary antiserum or by using preimmune or "normal" serum from the host animal (for PAbs) or myeloma protein (for MAbs) instead of the primary. The combination of autofluorescence and nonspecific binding can often lead to background or control fluorescence that is 30% or more of the intensity of positive labels.

Fixation. The choice of fixative can dramatically affect both autofluorescence and the affinity of an antibody for its antigen. Autofluorescence varies significantly with different preservatives (L. P. Shapiro et al. 1989a, Anderson et al. 1990, Vrieling et al. 1994b). Glutaraldehyde causes intense autofluorescence across a broad range of wavelengths, even after borohydride reduction (Anderson 1995). Another disadvantage of glutaraldehyde is the nonspecific binding of IgGs to free reactive sites of the fixative, leading to false positives (L. P. Shapiro et al. 1989a, Anderson et al. 1990, Vrieling et al. 1993a, Campbell et al. 1994). Extensive blocking with glycine, ammonium chloride, normal sera, or bovine serum albumin can sometimes avoid this problem. Paraformaldehyde or formaldehyde fixation, even followed by rapid freezing, often preserves cell morphology and gives the lowest background autofluorescence readings (Vrieling et al. 1993a, 1994b). Formaldehyde is perhaps the most widely used fixative in immunohistochemistry. While the ultrastructural preservation obtained with formaldehyde is not as good as that obtained with glutaraldehyde, autofluorescence is lower and antigenicity is better. Formaldehyde is a monoaldehyde, so the degree of cross-linking is minimal and better antibody kinetics are therefore obtained.

The choice of fixative can also alter the structural integrity of the antigenic sites on the cell surface (Vrieling et al. 1994b). For some antibodies, the reaction between antibody and antigen remains after Lugols fixation, but with reduced binding affinity (Anderson et al. 1990, Bates et al. 1993, Vrieling et al. 1994b). Osmium tetraoxide has not been used because it severely alters antigen/antibody binding sites resulting in a drammatic loss of labeling intensity (Doucette and Anderson 1993).

Choice of the fluorophore. Selective detection of phytoplankton or labeled constituents requires that the emission wavelength of the fluorophore differ from the autofluorescence of pigments (chlorophyll and phycoerythrin), cell walls (Pomeroy and Johannes 1968, Tsuji & Yanagati 1981, L. P. Shapiro et al. 1989b, Vrieling et al. 1993a, 1995b), and flagella (Müller et al. 1987, Coleman 1988, Kawai 1988, Kawai and Inouye 1989). With heterotrophic phytoplankton, for example, it may be necessary to select orange- or red-fluorescing probes (Roberts et al. 1993) to avoid interference with the yellow-green autofluorescence of cell walls (L. P. Shapiro et al. 1989b). Furthermore, detritus particles and certain minerals may autofluoresce in a broad range of excitation wavelengths. Improper selection of a fluor may cause overlap of fluorescent signals that leads to incorrect interpretations of the location of the fluorescent labeling or to over- or underestimation of fluorescence intensities in quantitative measurements. This is demonstrated using confocal laser scanning microscopy (Fig. 1). Chlorophyll autofluorescence was seen to interfere with the proper localization of fluorescein isothiocyanate (FITC) on the cell surface of the dinoflagellate *Prorocentrum*

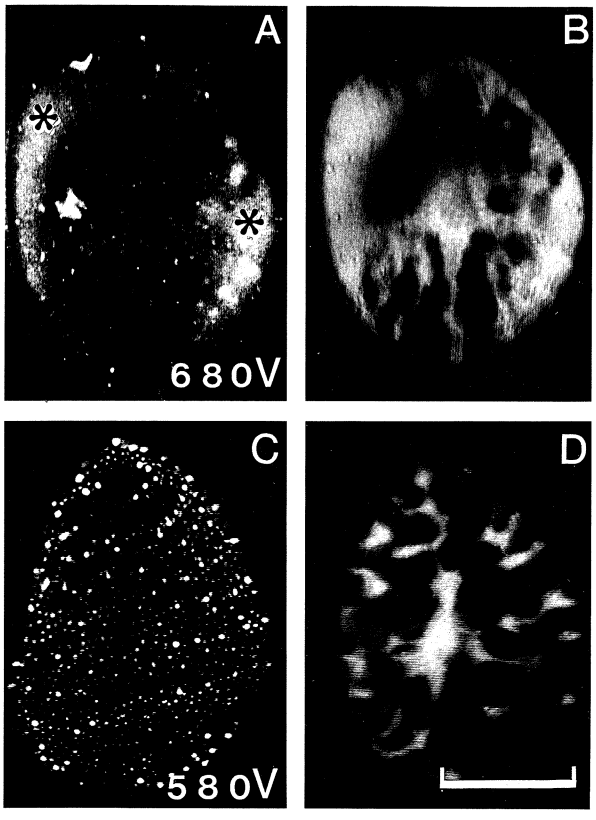

FIG. 1. Micrographs of the dinoflagellate *Prorocentrum micans* labeled with a PAb (PM1; Vrieling et al. 1993a, b) observed by CLSM. A) Using conventional indirect immunolabeling, an optimal image for FITC fluorescence was obtained at a PMT voltage of 680 V; chloroplast images (*) are also visible at this high PMT voltage. B) The same plane as in A but only viewed for chlorophyll fluorescence. C) With enhanced labeling (Vrieling et al. 1993a), an optimal FITC image could be obtained at a PMT voltage of 580 V, without interfering chloroplast fluorescence, which is shown for the same plane in D. Scale bar = 20 μm.

micans Ehrenberg after indirect labeling with a genus-specific polyclonal antibody (Fig. 1A). At a photomultiplier (PMT) voltage of 680 V an optimal image for FITC fluorescence was obtained, but chloroplast features were also visible (asterisks in Fig. 1A). In Figure 1B, however, the FITC fluorescence is solely detectable at a lower PMT voltage due to signal enhancement using the biotin/streptavidin system (Vrieling et al. 1993a). As a result of the decrease of the PMT voltage, no chlorophyll autofluorescence "leaks" into the FITC channel. Alternatively, pigment autofluorescence can be minimized by chemical extraction, though care must be taken to ensure that antigenicity is maintained (Vrieling et al. 1993b, Buma et al. 1995, Orellana and Perry 1995). For example, formalin-fixed phytoplankton cells can be washed free of fixative and then rapidly exposed to ice-cold methanol. Storage at −20° C for days to weeks results in nearly complete elimination of chlorophyll autofluorescence without significant alteration in the affinity of cell-surface antibodies for their antigenic sites (Anderson, unpubl. data). This type of treatment should not be used unless necessary because chlorophyll fluorescence can be an additional, useful parameter that further characterizes a target organism in a mixed assemblage for discrimination using flow cytometry, for example. Careful examination of parameters such as the excitation and emission wavelengths of autofluorescence as well as those of commercially available (immuno) fluororescent probes (Tables 1, 2) can help to maximize signal detection. The probes listed in Table 2 are a partial selection of the numerous fluors that are available (H. M. Shapiro 1985, Haugland 1989, Kasten 1989).

TABLE 1. *Maximal excitation and emission wavelengths of phytoplankton autofluorescent phenomena.*

Autofluorescence	Wavelength (nm)	
	Excitation	Emission
Chlorophyll	440	650–660
Phycoerythrin	460	550–634
Cell wall	460	535
Flagella	440	515–520

Bleaching. Fluorophores excited by the high power levels of epifluorescence microscopes and flow cytometers can rapidly lose their fluorescence, a phenomenon known as bleaching. The temporal loss of fluorescence emission during observation is a well-known irritation for microscopists, but this pro-

TABLE 2. *Commonly used fluorophores in modern marine biology and their excitation and emission wavelengths.*

Fluorophore	Wavelength (nm)	
	Excitation	Emission
Immunofluorophores		
AMCA (7-amino-4-methylocumarine-3-acetic acid)	350	450
FITC	488–495	520–525
RITC (rhodamine B isothiocyanate)	568	595
TRITC (4-*N*-cyclopropylrhodamine isothiocyanate)	540–552	570
CY3 (indocarbocyanine)	550	570
Texas Red (sulforhodamine 101)	590–596	615–620
Nucleic acid probes		
Acridine orange	470–503	523–640
DAPI (4′,6-diamino-2-phenylindole)	340–365	450–488
Ethidium bromide	545	610
Hoechst 33258 (bisbenzimiditrihydrochloride, trihydrate)	365–374	472–480
Hoechst 33342 (bisbenzimidazole derivate)	355	465
Propidiumiodide	530	615
Cell-surface and viability probes		
Calcofluor White	340–360	400–440
CFDA (5(6)-carboxyfluorescein diacetate)	495	520
FDA (fluorescein diacetate)	495	520
Hydroethidine	370,535	420,585
PKH-2	490	504

gressive loss of emitted light is also a potential source of error in quantitative measurements. Observation times in flow cytometers are sufficiently short that bleaching is not a problem, and for photomicrography, high-speed films can be used to reduce exposure times. When it is necessary to retard bleaching, antifade reagents can be used, such as sodium dithionite (Kasten 1989), glycerol (Anderson et al. 1990, 1993), *n*-propylgallate (Giloh and Sedat 1982), *p*-phenylenediamine (Johnson and Nogueira Araujo 1981, Roberts et al. 1993), and Citifluor (Rogers Domozych et al. 1993). Alternatively, the fluorescent intensity can be enhanced using reagents such as 1,4-diazobicyclo-[2,2,2]-octane (or DABCO) (Langanger et al. 1983). Optical microscopes, equipped with variable power supplies permit examination of the specimen at low light intensities, decreasing the rate of bleaching and extending observation times (Vrieling et al. 1993a).

APPLICATIONS OF IMMUNOFLUORESCENCE

Immunofluorescence has been used in a variety of investigations to address ecological, genetic, and physiological questions pertaining to phytoplankton. The unique and powerful technology of using antibodies as recognition molecules that bind to specific targets allows workers to identify whole cells or specific molecules within those cells. Applications of immunofluorescence in phytoplankton studies are diverse and expanding.

Species Identification and Enumeration

It is both expected and suprising that species- or even strain-specific antibodies can be produced to phytoplankton cell wall proteins. This level of selectivity is expected given the diversity of target proteins on a cell, the genetic differences that have evolved in those proteins through time, and the exquisite specificity of the antibody/antigen reaction. It is nevertheless surprising that the process is so easy, that immunizations using fixed, whole cells are often successful, and that isolation and purification of individual proteins is not necessary. The first efforts to produce antibodies in this manner in order to distinguish among phytoplankton species were those of Bernhard et al. (1969), who prepared PAbs to the two diatoms *Leptocylindrus danicus* Cleve and *Pheaodactylum tricornutum* Bohlin. The antibodies cross-reacted with other diatoms as well as with some dinoflagellates, but the results were sufficiently encouraging for the authors to suggest that the approach should be investigated further, and possibly even used for automated cell counting. Nearly a decade later, Fliermans and Schmidt (1977) used PAbs to detect *Synechococcus* and six other morphologically indistinguishable isolates from cultures of other cyanobacteria and bacteria. They were also able to enumerate *Synechococcus* and document the vertical distribution of the species in lakes of north central Minnesota.

Different strains of the marine *Synechococcus* spp. (phycoerythrin- and phycocyanin-containing) and a *Synechocystis* species could be distinguished by Campbell et al. (1983) using PAbs prepared against each species. The antibodies were then used in attempts to enumerate natural populations of these species in Great South Bay, New York. Subsequently, serotypes of oceanic and estuarine *Synechococcus* spp. containing phycoerythrin could be determined using antibodies directed against oceanic, estuarine, and "motile" forms of *Synechococcus* in an immunofluorescent assay (Glover et al. 1986, Campbell and Carpenter 1987). The tropical oceanic serogroup was restricted to a warm-core eddy from the Gulf Stream, whereas the other two groups were abundant in all water masses examined (Glover et al. 1986). Glover et al. (1986) also found that *Synechococcus* cell size increased near the bottom of the thermocline, not as a result of a change in species composition (as indicated by immunofluorescence), but because of elevated nutrient concentrations. In field samples from the Sargasso Sea, phycoerythrin-containing *Synechococcus* spp. were generally not labeled with an antibody directed against one phycoerythrin-bearing species (Campbell and Iturriaga 1988), suggesting that the target species was not abundant in that environment.

L. P. Shapiro et al. (1989a) reported the development of several new PAbs directed against major marine ultraphytoplankton taxa. Antisera were produced that were considered representative of major ultraplankton groups, not including the cyanobacteria. These were then tested for reactivity against numerous clones, and the resulting groupings were shown to agree with high-performance liquid chromatography–based pigment clusters (L. P. Shapiro et al. 1989c). The probes were then used in a study to examine the geographic distribution of Atlantic Ocean ultraplankton that reacted with antisera for *Emiliania huxleyi* (Lohman) Hay et Mohler, *Pycnococcus provasolii* Guillard, *Pelagococcus subviridis* (Norris) Lewin, *Thalassiosira oceanica* Hasle, and an unidentified chlorophyte (Campbell et al. 1994). The first three were present at all stations examined, whereas both *T. oceanica* and the unknown chlorophyte were rarely detected. Overall, only a small portion of the total eukaryotic algae (most of which were ultraplankton) reacted with the probes that were used, suggesting a diverse assemblage containing numerous species belonging to groups not represented by the antisera. The presence of *E. huxleyi* in a variety of regions throughout the Atlantic Ocean demonstrates the cosmopolitan nature of some of these ultraplankton species.

In several cases, purified cell wall constituents were used to develop immunofluorescent probes for species identification. Antibodies prepared to coccolith polysaccharides of *Emiliania huxleyi* (Borman et al. 1987) were used to reveal the distribution of two species groups in the northeast Atlantic region (van

Bleijswijk et al. 1991). One type was exclusively present in the Atlantic Ocean, whereas the second dominated in the North Sea. Observations made by immunofluorescence were identical to results based on examination of coccolith bearing *E. huxleyi* cells by scanning electron microscopy. In a similar manner, endosymbiont diatoms were identified in several Foraminifera using antibodies directed against purified cell envelope-membrane fractions (Lee et al. 1988). A variety of algal species were endosymbiotic in the population of Foraminifera studied. In addition to species distinctions, the antibodies were useful in demonstrating how endosymbiont abundance changed seasonally. *Nitzschia frustulum* var. *symbioticum* (Kützing) Grunow, for example, was especially dominant in the winter.

One area where progress in immunofluorescent species identification has been rapid is in studies of harmful or toxic phytoplankton (reviewed by Anderson, 1995). In such work, the species of interest is often only a minor component of the mixed planktonic assemblage, so many potentially useful measurements are not feasible because of the co-occurrence of numerous other organisms and detritus. Another constraint arises from the difficulties in adequately identifying and distinguishing between species or strains that are morphologically similar. This situation is encountered frequently with toxic algae. For example, the diatom *Pseudo-nitzschia pungens* (Grunow) Hasle occurs in two varieties, one toxic and the other nontoxic, but these cannot be distinguished from each other using the light microscope (J. C. Smith et al. 1990). Likewise, toxic and nontoxic varieties of the dinoflagellate *Alexandrium tamarense* (Lebour) Balech can co-occur within a given region (Yentsch et al. 1978), as can morphologically similar *A. tamarense* and *A. catenella* Balech (Cembella et al. 1987) and *A. tamarense* and *A. fundyense* Balech (Cembella et al. 1988).

A variety of different toxic organisms from several classes have been investigated using cell-surface antibodies. MAbs have been used to identify species and strains of the red tide raphidophyte *Chattonella antiqua* (Hada) Ono and *C. marina* (Subrahmanyan) Hara et Chihara (Hiroishi et al. 1988, Uchida et al. 1989). These species cannot easily be distinguished by standard microscopic techniques because of rapid changes of morphology due to the absence of a rigid cell wall. MAbs directed against *C. marina* were recently used to verify the presence of this species in Dutch coastal waters (Vrieling et al. 1995b).

Aureococcus anophagefferens Hargraves et Sieburth (Chrysophyceae) is a tiny, coccoid species that suddenly bloomed in 1985 and devastated shellfish and eelgrass resources in Long Island (Cosper et al. 1989). Its size and nondescript morphology make it difficult, if not impossible, to identify at low concentrations under the light microscope. Anderson et al. (1990) prepared a PAb to this brown tide chrysophyte, and without any purification the antisera proved to be species-specific, showing no cross-reactivity with 46 other phytoplankton species from five classes, including 20 chrysophyte species. Immunofluorescent techniques can detect the species at concentrations as low as 10–20 cells·mL^{-1}. Two field surveys along the northeastern U.S. coast used this approach to document the geographic distribution of *A. anophagefferens* (Anderson et al. 1993). The species distribution centered around Long Island, the area of the most intense and destructive outbreaks, but the species could be detected at low concentrations in many other distant locations with no history of brown tides. Immunofluorescence was thus able to demonstrate that numerous areas have the potential for outbreaks such as those associated with the sudden appearance of the species in high numbers in Long Island in 1985. This would not have been possible with conventional light microscopy.

Another set of highly specific PAbs produced by Bates et al. (1993) can distinguish among diatoms capable of producing domoic acid, the toxin involved in cases of amnesic shellfish poisoning (J. C. Smith et al. 1990). The remarkable aspect of this report is that the PAbs produced by these workers distinguish between two varieties of the same species (toxic *Pseudo-nitzschia pungens* Grunow forma *multiseries* Hasle from the nontoxic *P. pungens* Grunow forma *pungens* Hasle), a distinction that is not otherwise possible without use of electron microscopy (J. C. Smith et al. 1990). It is therefore not always necessary to prepare MAbs for identification of morphologically similar species or strains; PAbs can do an excellent job in this respect.

For toxic dinoflagellates in the *Alexandrium* genus, MAbs developed by Sako et al. (1993) and Adachi et al. (1993) can distinguish species at several levels. A series of MAbs to *A. tamarense* and *A. catenella* were tested against strains of these species from several different countries (Adachi et al. 1993). One antibody was specific for the *tamarense/catenella/fundyense* species complex, and two were highly specific for only *A. tamarense* from Thailand. Groups within this complex could be resolved using four of the other antibodies. For other species in the *Alexandrium* complex, PAbs prepared by Mendoza et al. (1995) can identify *Alexandrium minutum* Halim, *A. lusticanicum* Balech, and *Gymnodinium catenatum* Graham. Those antisera were not species-specific at the outset, but after elimination of cross-reacting antibodies by absorption against nontarget species a set of clone-specific antibodies was obtained. Some reduction in labeling intensity was observed after this purification step, but the increase in specificity was significant. In a similar way, clone-specific antibodies against *Prorocentrum lima* (Ehrenberg) Dodge and *P. triestinum* Schiller were prepared (Costas et al., pers. commun.).

In 1991, a research program was initiated in The Netherlands to develop an early warning system for toxic phytoplankton blooms. This method, based on

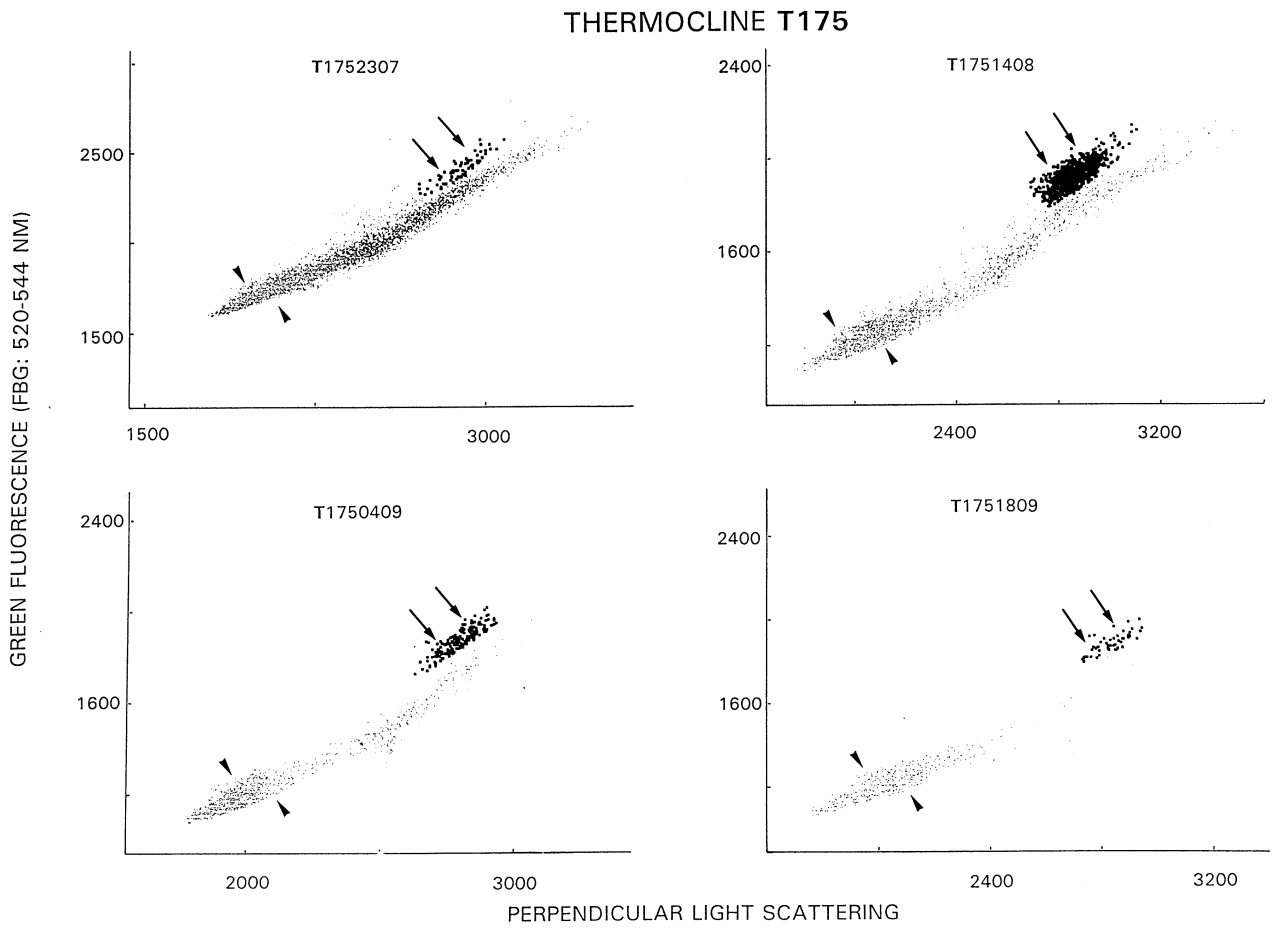

FIG. 2. Flow cytometric scatterplots of four Lugol-fixed field samples from the Dutch part of the central North Sea (175 km northwest of the island of Terschelling). The samples were taken from the thermocline at 2-week intervals in the summer of 1991 and labeled using indirect immunofluorescence for the presence of the ichthyotoxic dinoflagellate *Gyrodinium aureolum* with MAb GA8 (Vrieling et al. 1994b). The arrows indicate the position of labeled cells in the bivariates, which form a distinct cluster. The peak of the bloom occurred on 14 August.

enumeration and recognition of small numbers of potentially toxic species in field samples, requires that the target organisms be quickly and positively identified in mixed populations. Preliminary studies with antibodies raised against the cell wall (Fig. 1) and extruded trichocysts of the dinoflagellate *Prorocentrum micans* showed that FITC-labeled cells of the naked dinoflagellates *Gymnodinium* and *Gyrodinium,* as a result of cross-reaction with the antitrichocyst antibodies, could be identified using flow cytometry (Vrieling et al. 1993b). The labeling intensity, however, had to be enhanced using the biotin–streptavidin approach (Vrieling et al. 1993a). Subsequently, species-specific MAbs were produced, the first of which reacted specifically at the cell surface of *Gyrodinium aureolum* Hulburt and with the closely related species *Gymnodinium nagasakiense* Takayama et Adachi and *G. mikimotoi* Miyake et Kominami ex Oda (Vrieling et al. 1994b). MAbs prepared by Nagasaki et al. (1991) differ from this set because they only identify the ichthyotoxic dinoflagellate *G. nagasakiense.* Preliminary studies using a MAb of Vrieling et al. (1994b) on field samples taken in 1991 in the Dutch central North Sea showed that *G. aureolum* could be followed during the formation and decline of a small bloom (arrows in Fig. 2; Vrieling et al. 1994a, 1995a). In these studies, quantitative enumeration was not accomplished, in part as a consequence of significant cell loss during the multiple blocking and washing steps of the indirect immunofluorescence assay.

To prevent this cell loss, a direct immunofluorescence assay was then developed and is now being evaluated for quantitative analysis of *G. aureolum* in mixed algal populations spiked with this species. At the moment, less than 1500 cells·L^{-1} can be detected in a population of 10^7 cells·L^{-1} with an accuracy of more than 75% (Vrieling et al., unpubl. res.). Total cell losses are still significant, however, but the recovery of *G. aureolum* is sufficient so quantitative enumeration of immunolabeled cells by flow cytometry is feasible.

Cell Structure and Physiology

Antibodies also have great utility in investigations of the location, structure, metabolism, and abundance of individual proteins or other compounds within phytoplankton cells. Production of antibodies for these purposes is more involved than is the case for cell-surface antibodies, because isolation and purification of the specific antigen is usually required, but in some cases commercially available antibodies or those prepared by other investigators for different organisms will suffice. Methodologies differ as well, due to the need to get antibodies and reporter molecules across cell and organelle membranes. Permeability issues are especially important if the study involves whole cells, but problems of antigen accessibility even arise with sectioned and embedded cells (e.g. Doucette and Anderson 1993).

Cell wall proteins and polysaccharides. Considerable effort has been devoted to immunofluorescent investigations of the glycoprotein composition of the cell wall of the chlorophyte *Chlamydomonas.* For *C. eugametos* Moewus, PAbs were prepared to flagella and to a major cell wall protein (Musgrave et al. 1983). The PAb for the latter was only reactive when cell walls were partly digested, so the antibody has been used to investigate the stages of disintegration and/or cell wall digestion during asexual and sexual reproduction. The cross-reactivity of this antibody with cell wall and flagellar glycoproteins suggests similarity in antigenic oligosaccharides. MAbs directed against a cell wall fraction of *C. eugametos,* containing mt^- agglutinin and two cell wall glycoproteins (PAS-1.2, PAS-1.3, and PAS-1.4 respectively, sensu Musgrave et al. 1981), recognized only strain-specific *O*-methyl sugars (Homan et al. 1987). Surprisingly, no reactivity to mt^- agglutinin was found, confirming the immunogenicity of plant carbohydrates in the mammalian immune response (E. Smith et al. 1984, Williams and Woollet 1985). The same was noticed by Eardly et al. (1990); specific MAbs prepared to *Macrocystis* (a macroalga) polysaccharide labeled a sulfated polysaccharide (fucoidan) located on the cell surface of the phaeophyte *M. pyrifera* (Linnaeus) C. Agardh.

E. Smith et al. (1984) also investigated the structure of the *Chlamydomonas* cell wall by preparing MAbs to the major cell wall glycoproteins of *C. reinhardii* Daugeard. These antibodies could be subdivided into six groups, each varying with respect to the location of labeling on the cell wall and the pattern of immunofluorescent staining (e.g. as dots, continuous or in an irregular network). Voigt (1988) compared LiCl-soluble glycoproteins of *C. reinhardii,* which seemed to be immunologically related based on cross-reactivity experiments. For wild-type cells, reactivity varied during the vegetative cell cycle, whereas for a cell wall–deficient mutant, no reaction could be observed.

Enzymes and proteins. Immunofluorescence is also an excellent tool for investigating the location and abundance of proteins and enzymes involved in cellular metabolism. Vladimirova et al. (1982) used a PAb directed against ribulose bisphosphate carboxylase (RuBPCase) for immunofluorescent localization of that enzyme in the pyrenoid of *C. reinhardii* and *Dunaliella salina* (Dunal) Teodoresco. Local luminescence of the fluorophore was investigated in relation to the unique role of the pyrenoid as a "giant carboxysome." In synchronized cultures of *C. reinhardii,* immunofluorescence was used to localize frx B, an iron-sulphur protein related to a subunit of NADH dehydrogenase (Zhang and Wu 1993). During the light period the frx B concentration increased in vesicles near the pyrenoid, whereas in dark periods the concentration declined. In male gametes, the frx B fraction disintegrated as mating progressed, whereas in the female the protein persisted.

In a study of macroalgae, three MAbs were established to Rubisco of *Bryopsis.* These antibodies did not cross-react with spinach Rubisco but were specific to the large subunit of *Bryopsis* Rubisco, which was concentrated in pyrenoids and at the surface of starch grains, revealed by indirect immunofluorescence (Kajikawa et al. 1988). In a related study, Orellana et al. (1988) prepared a PAb to RuBPCase of *Chaetoceros gracilis* Schutt. The antiserum was affinity-purified against spinach RuBPCase and used to detect RuBPCase in nitrate-limited and nitrate-sufficient cultures using immunofluorescence and flow cytometry. Recently, another Rubisco antibody was prepared and used to define two taxonomic groups on the basis of high- and low-binding affinity to the probe (Orellana and Perry 1992). These authors suggest that their affinity-purified serum can be used, after further characterization, as an immunoprobe to quantify Rubisco concentration and photosynthetic rates of individual cells. By optimalization of immunofluorescent measurements of Rubisco (Orellana and Perry 1995), the method reached an applicable stage.

Nitrate reductase, the key enzyme in the reduction of nitrate, was localized at the plasma membrane and in organelle-like spots in phytoplankton and visualized by a diffuse and a bright fluorescence, respectively (Balch et al. 1988). Localization of this enzyme on the plasma membrane is consistent with the role of nitrate reductase in nitrogen uptake. In a similar manner, PAbs raised against the Fe-S protein of the nitrogenase enzyme complex from the bacterium *Rhodospirillum rubrum* (Esmarch) Molisch reacted with nitrogenase from both marine and freshwater N_2-fixing bacteria and cyanobacteria (Currin et al. 1990). Immunofluorescence was then used to reveal the localization of nitrogenase within the heterocysts of cyanobacteria. These techniques make it possible to rapidly determine the presence, relative abundance, and distribution of N_2-fixing microorganisms in natural assemblages.

Microtubules. Immunofluorescence has been a powerful tool in studies of the physiological mechanisms of cell division, typically using antibodies directed against major cytoskeleton proteins such as tubulin and centrin summarized by Melkonian (1992) and Menzel (1992). Here we discuss only a few. For the chlorophyte *Ulothrix palusalsa* Lokhorst, tubulin was localized 1) in nuclear spindles with interzonal fibers during anaphase and early telophase; 2) in a clear fluorescing zone associated with centriolar complexes and the nuclear envelope; and 3) in association with furrows, especially less advanced ones (Segaar and Lokhorst 1987). Throughout mitosis, centriolar complexes stained intensely. An antibody against tubulin was also used to examine fertilization and pathogenesis in the phaeophyte *Laminaria angustata* Kjellman (Motomura 1991). The formation of centrosomes and microtubili was followed successfully during normal mitosis. Pathogenetic development was characterized by occurrence of abnormal mono-, tri-, and multipolar spindles, resulting in irregular nuclear and cytoplasmic division. Antibodies directed against tubulin were also used to reveal the cytoskeletal patterns of different dinoflagellates (e.g. *Amphidinium rhynchocephalum* Anissimowa, *Gymnodinium sanguinium* Hirasaka, and *Gymnodinium* sp.) in order to identify characters of taxonomic importance and to assist in investigations of the flagellar apparatus (Roberts et al. 1988, 1993, Roberts 1991, Roberts and Roberts 1991). Antibodies to a flagellar root protein, centrin, were used to differentiate two types of fibrous flagellar roots in green algae (Schulze et al. 1987, Melkonian et al. 1988).

A good example of how immunofluorescence can be combined with immunogold electron microscopy to reveal the distribution or localization of a target molecule is the work of Schulze et al. (1987). Centrin, a Ca^{2+}-modulated contractile protein in the flagellar apparatus of green algae, was examined in 28 taxa. This study also demonstrates how one antibody can be used on a broad range of species. A comparative study to localize production of mucilage release by the gliding desmid *Closterium* involved both immunofluorescence and immunogold electron microscopy (Domozych and Rogers Domozych 1993, Rogers Domozych et al. 1993).

Toxins. Immunofluorescence has been used in attempts to localize an algal toxin responsible for a human disease known as paralytic shellfish poisoning. A PAb to saxitoxin (Carlson et al. 1984) was used to examine thin sections of cells of the dinoflagellate *Alexandrium tamarense* (Anderson and Cheng 1988). Labeling was restricted to the nucleus near the condensed chromosomes and on the periphery of small granules (Fig. 3). Attempts to repeat these results using different fixation and embedding methods again showed the toxin to be associated with the chromosomes (Doucette and Anderson 1993), but results differed among fixation and embedding techniques. These studies accentuate one of the problems involved with immunolocalization, namely, that in samples exposed to aqueous-phase chemical fixatives, antigen redistribution is possible (Griffiths et al. 1993). It is therefore difficult to ascertain whether the localization that is observed is real or is, instead, an artifact of the processing. This problem can be minimized through cryofixation and molecular distillation drying techniques, but even then localization results must be interpreted with caution (Doucette and Anderson 1993). In a similar manner, the combination of immunofluorescence and immunogold techniques have been used to localize okadaic acid, the toxin responsible for diarrhetic shellfish poisoning, in chloroplasts of the dinoflagellates *Prorocentrum lima* (Ehrenberg) Dodge and *P. maculosum* Faust (Zhou and Fritz 1994).

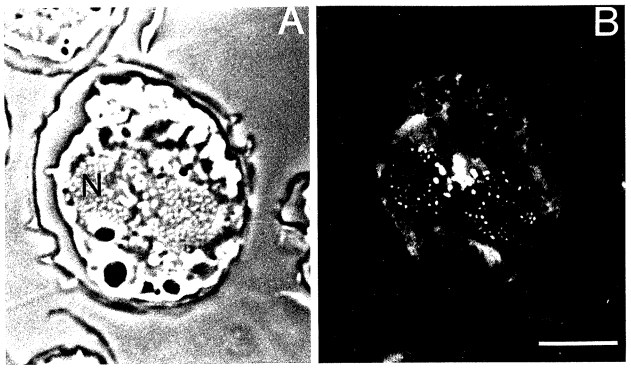

FIG. 3. Phase and fluorescent images of *Alexandrium tamarense* probed with an antibody to saxitoxin. A) Phase-contrast image of toxic cell after fixation, embedding, and sectioning. The prominent horseshoe-shaped nucleus (N) is visible end-on as two lobes in the center of the cell. B) Epifluorescent image of the same field as Figure 1A after treatment with a 1:3200 dilution of anti-saxitoxin antibody. Note the apparent labeling of the chromosomes, appearing as spots or rods depending on their orientation. Some of the brighter labeling appears between the two arms of the nucleus. Scale bar = 10 μm. From Anderson and Cheng (1988).

DNA lesions and cell cycle proteins. In a recent study of the effect of ultraviolet B (UV-B) irradiation on phytoplankton cells, immunofluorescence provided important insights that would be difficult to obtain with other methods. During irradiation with UV-B wavelengths, thymine dimers (T,T-dimers) are formed. A method developed for cancer research was adapted to examine such photoproducts in phytoplankton. Using a MAb directed against T,T-dimers, Buma et al. (1995) determined the effects of UV-B irradiation on the centric diatom *Cyclotella.* Flow cytometry was used to quantify DNA damage in individual cells by double labeling techniques; the DNA was stained directly with propidium iodide and the T,T-dimers using FITC-labeled antibodies. DNA damage increased with increasing UV-B irradiance (Fig. 4A–C), and the extent of DNA repair varied with the length of the dark period following the initial UV-B exposure. The most significant DNA

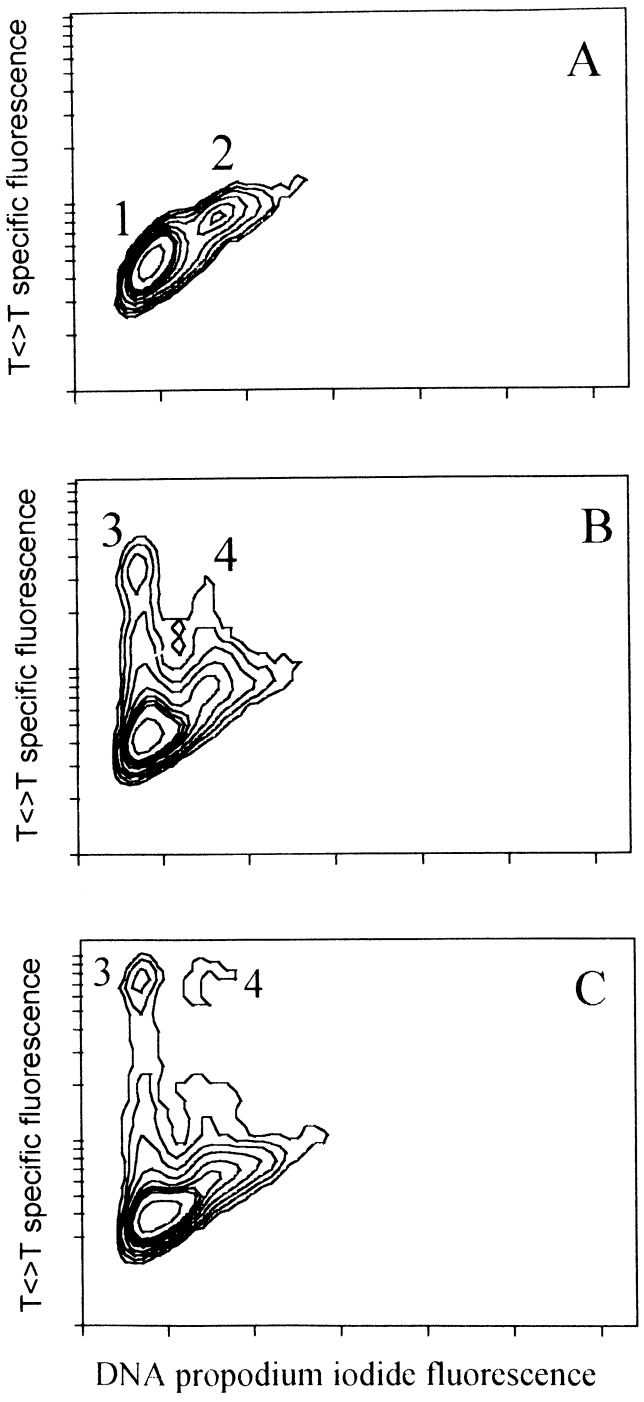

FIG. 4. Flow cytometric contour plots of the centric diatom *Cyclotella* spp. indirectly labeled with a MAb recognizing UV-B–induced T,T-dimers (Buma et al. 1995). DNA damage of G1- and G2-phase cells (marked 3 and 4, respectively) increases with higher weighted UV-B doses. A) Control; no UV-B exposure (G1- and G2-phase cells are marked as 1 and 2). B) Exposure to 1.5 $KJ \cdot m^{-2}$ UV-B. C) Exposure to 3.0 $KJ \cdot m^{-2}$. Courtesy of Drs. A. G. J. Buma and M. Veldhuis (The Netherlands).

damage occurred during the G1 and G2 phases (Fig. 4B, C) of the cell cycle.

Lin et al. (1994, 1995) developed an immunofluorescent method to estimate growth rates of phytoplankton using proliferating cell nuclear antigen (PCNA) as an indicator of division. PCNA in whole cells was labeled with commercially obtained MAbs against rat PCNA and was shown to be located exclusively in the nucleus of the cell by immunofluorescence (Lin et al. 1995). The abundance of PCNA varied in a systematic manner during growth (Lin et al. 1994), with highest abundance during the S phase, none detectable in G_1 or late M, and low levels in G_2 (Lin et al. 1995).

Mucus. Antibodies have been used to investigate the release of mucilage produced during gliding locomotion in placoderm desmids (Domozych and Rogers Domozych 1993, Rogers Domozych et al. 1993). In *Closterium,* mucilage was released at the pole of developing cells in postcytokinetic phases. This also happens to be the region where membrane-associated Ca_{2+} was localized, as determined by staining with the fluorescent probe chlorotetracyclene (Rogers Domozych et al. 1993).

INSTRUMENTATION AND FACILITIES

Fluorescence microscopes (conventional and confocal). Epifluorescence microscopes have traditionally been used for examination of animal and plant cells or tissues labeled with fluorophores. In phytoplankton research, the conventional epifluorescence microscope has been very useful for rapid examination of the location of antigens in cells, but it has its limitations. Rapid bleaching of the fluorophore can make antigen detection and localization difficult, depending on antigen abundance and the quality of the antibodies. Another significant problem is associated with depth-of-focus of conventional microscopes, as antibody/antigen labeling or autofluorescence out of the focal plane can hamper quantitative analysis or resolution of the extent of positive labeling. Video enhancement of the fluorescent signal through the addition of multiple, digitized images using image processing software can help, but this type of electronic enhancement is still constrained by the signal (positive fluorescence)–to–noise (background) ratio.

Confocal microscopes offer an expensive, but effective means to avoid these problems. In these instruments, diffraction-limited laser light is used for excitation. Emitted light emanating from the focal plane and the planes above and below are optically separated so that light from the desired focal plane is observed, while light from other planes is suppressed. Confocal principles (Wilson 1990) allow high-resolution fluorescence imaging of tissue sections as well as thick, whole cells without interference from obscure out-of-focus background (stray-light). New configurations offer laser excitation, auxiliary image intensification, and computer-assisted analysis technology so that even the weakest fluorescence emission can be quantified. Confocal laser scanning microscopes can detect about 10 fluorescent molecules of $FITC \cdot \mu m^{-2}$ (Ploem 1989), where-

as conventional microscope would need 10–100 times more fluor to obtain a clear image. This high degree of detection allows visualization of target antigens that are in low abundance. Furthermore, image analysis software allows fluorescence intensities to be quantified (Buurman et al. 1992, Vrieling et al. 1993a).

Perhaps the most unique feature of the new generation of confocal microscopes is that three-dimensional images can be created of individual cells, especially when they cannot be flattened easily or are quite large (more than 10 μm). Optically confocal sections can be obtained in sequence and in fixed increments. This permits the three-dimensional display of physiological processes or antigen localization in single cells. Surprisingly, this technology has not yet been exploited to any great extent in phytoplankton research. It offers great advantages for chromosome counting in cell cycle studies, for detecting changes in the chloroplast morphology of individual cells, and for localization of cell organelles or constituents such as microtubules, enzymes, or toxins. In an overview of the flagellar apparatus and cytoskeleton of dinoflagellates by Roberts and Roberts (1991), confocal microscopy is advocated as the technique of choice to explore the three-dimensional arrangement of cytoskeletons. Confocal microscopy was used for three-dimensional localization of the cell-surface antigens of the dinoflagellates *Gyrodinium aureolum, Prorocentrum minimum* Pavillard, *P. micans,* and *Gymnodinium nagasakiense* (Vrieling et al. 1993a), demonstrating distinct differences in labeling patterns among species. Labeled antigens in some cells showed intense fluorescence patterns along the edges of the sulcus and cingulum, whereas granular structures on the cell surface were labeled on other cells. Confocal laser scanning microscopy (CLSM) was also used to distinguish FITC fluorescence from chlorophyll autofluorescence in immunochemically labeled cell-surface antigens in dinoflagellates (Vrieling et al. 1993a, b). In those studies, the fluorescent intensity of FITC labeling was estimated and quantified, which could not have been achieved by conventional epifluorescence microscopy. The distinction between FITC (Fig. 1C) and chlorophyll fluorescence (Fig. 1D) can be easily made by CLSM. In other studies, fluorescence lifetime imaging was investigated using another naked dinoflagellate, *Gymnodinium nagasakiense* (Buurman et al. 1992).

Flow cytometer. Flow cytometers are designed for high-speed analysis of cell populations, typically 10,000 events·s^{-1}. Cells are passed single-file through a laser beam, and their optical properties are recorded by an array of photomultipliers. Fluorescence at several different wavelengths can be determined on individual cells, as well as forward-angle and 90° light-scatter, which provide useful information on cell size or surface structure (Visser et al. 1978). Flow cytometry originated as a tool in biomedical investigations of blood cells, either stained by fluorescent DNA probes or fluorophore-conjugated antibodies. It has rapidly become a tool in phytoplankton research, mostly in the analysis of field populations or cultured cells that can be distinguished on the basis of their unique size or natural fluorescence characteristics (e.g. phycoerythrin-containing *Synechococcus* spp. [Olson et al. 1988] or chlorophyll *a*–containing prochlorophytes [Chisholm et al. 1988]). Flow cytometers have even been adapted to operate on-board research vessels (Olson et al. 1985, Cunningham 1990).

Flow cytometers can have a precision of 1–3%, yet they have sufficient sensitivity to detect fewer than 160 fluorescent molecules in about 20 μs, the time required for a cell to traverse the laser beam (Dovichi et al. 1983). Instruments equipped with multiple lasers are able to excite fluors at different wavelengths; special optical filters can provide measurements of as many as eight optical parameters on a single cell. This level of characterization is very useful for species-specific detection using artificial neural networks (Balfoort et al. 1992).

One distinct advantage of flow cytometers over optical microscopes is that large numbers of cells or samples can be processed in a very short time. However, a problem with all commercial flow cytometers is that they are not able to analyze particles larger than 100 μm (Legendre and Yentsch 1989, Phinney and Cucci 1989, Steen 1991), so they are not suitable for large, chain-forming, or colony-forming species (Peeters et al. 1989, Hofstraat et al. 1990, Steen 1991). To overcome this problem a new instrument, the optical plankton analyzer (or OPA), was designed and built in The Netherlands (Dubelaar et al. 1989, Peeters et al. 1989). This flow cytometer, equipped with three lasers, uses a larger cuvette and a larger focus area of the laser beam. It allows analysis of up to eight parameters on particles ranging from 1 to over 1000 μm in length and up to 500 μm in width.

Flow cytometry offers special advantages to immunologists due to its speed, flexibility, and quantitative capabilities. One obvious application of flow cytometry in phytoplankton research and monitoring would be in automated, immunofluorescent cell enumeration. Ward (1990) showed that marine bacteria could be detected when the target species was present in reasonable numbers, and the results of L. P. Shapiro et al. (1989a) and Vrieling et al. (1995a; unpubl. res.) suggest that the method could eventually be useful to detect phytoplankton species using immunofluorescent labeling of cell-surface antigens.

Immunofluorescence coupled with flow cytometry is thus an area of active investigation in several laboratories. One current obstacle to flow cytometric analysis of labeled cells is that the intensity of positive labeling is often not significantly higher than the background fluorescence of control or unla-

beled cells. Even when the mean fluorescence of labeled cells is 3–5 times higher than the mean for unlabeled cells, the variability in fluorescence around those means can result in unacceptable overlap of the two populations. For *Alexandrium tamarense,* for instance, variability in fluorescence intensities and patterns have been observed using a PAb recognizing cell-surface antigens (Taylor and Lewis 1995). It is thus difficult to specify a minimum fluorescence level that delineates cells of a target species from all others without setting the threshold so high that many positively labeled (but weakly fluorescent) target cells would not be counted. Signal enhancement is clearly necessary, and several approaches are promising. Vrieling et al. (1993a) showed that fluorescent intensity can be enhanced using biotinylated secondary antibodies that react with FITC-conjugated streptavidin. More recently, the Dutch group has used pooled antisera to increase the number of labeled epitopes in trying to increase the intensity of the fluorescence signal (Vrieling et al. 1994b). Other approaches to signal enhancement 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 at this writing automated applications of immunofluorescent techniques for harmful algae are still in the developmental stage.

Another problem is that cells can be lost in the many incubation, blocking, and washing steps required to label cells. Preliminary experiments performed to identify *G. aureolum* in field samples of the Dutch monitoring program showed that, due to cell loss, the target organism was only detected properly when at least 10^4 cells$\cdot L^{-1}$ were present (Vrieling et al. 1995a). Accurate cell numbers could not be determined. Despite the cell loss problem, in the summer of 1991 at two stations in the central North Sea, *G. aureolum* could be readily identified during formation and decline of a bloom (arrows in Fig. 2; Vrieling et al. 1994a, 1995a). Preliminary studies using *G. aureolum* cells added to mixed algal populations suggest that a loss of 20% or more is not unusual during processing, despite the use of direct immunofluorescent techniques and careful sample manipulations (Vrieling et al., unpubl. res.). Nevertheless, immunofluorescence combined with flow cytometric countings is still recognized as an important component in a Dutch early warning system to detect toxic marine phytoplankton species at low cell concentrations. Recent results suggest that target species can be detected even when they represent less than 0.01% of the total number of cells in mixed populations (Vrieling et al., unpubl. res.). In theory, a detection limit of less than 1000 cells$\cdot L^{-1}$ is thus possible but largely depends on an optimal signal-to-noise ratio and careful processing of samples.

An important different benefit to be derived from the combination of immunofluorescence and flow cytometry is that cells of a target species can be sorted from natural samples and used for physiological or toxicological analysis. Here again, signal–noise problems are encountered that can make it difficult to specify which cells are to be selected, but with appropriate signal enhancement strategies species-specific cell sorting based on immunofluorescence will soon be as commonplace as sorting on the basis of pigment fluorescence.

Apart from these efforts to detect species in mixed populations, cellular constituents can also be studied using flow cytometry. For example, Buma et al. (1995) used flow cytometry to demonstrate the relationship between the fluorescence intensity of intracellular T,T-dimers and the amount of DNA damage to phytoplankton cells following UV-B irradiation (Fig. 4). Interference of chlorophyll fluorescence was avoided by extracting cells with ethanol before the flow cytometric analysis. Orellana and Perry (1995) developed a method for flow cytometric quantification of Rubisco in order to estimate photosynthetic rates of marine phytoplankton. By comparing chemical fixatives and extracting almost all pigments (±99%), they were able to identify phytoplankton using the remaining red fluorescence as a triggering parameter to measure the green FITC fluorescence of labeled Rubisco.

CONCLUSIONS AND FUTURE PROSPECTS

The future of immunofluorescence in phytoplankton research is indeed promising. One area where these tools will soon be commonplace is in species identification. Cell-surface antibodies already exist for many species (Table 3), and more are in preparation at this time. Specificity is excellent, as species and even strains of species can be readily distinguished. Sensitivity is excellent, allowing the detection of target organisms at very low concentrations. Considerable effort is still needed, however, to bring antibody probes from the developmental stage to direct applications on natural populations. Techniques must be refined to maximize the fluorescence intensity of labeled cells, and procedures such as enumeration or fluorescence-activated cell sorting of target species using immunofluorescence and flow cytometry must be perfected. In parallel with single cell analysis using expensive instruments such as flow cytometers, efforts must be directed to inexpensive bulk analyses using dot blot or enzyme-linked assays in tissue culture plates.

The natural fluorescence of cell constituents such as chlorophyll or other pigments has often been used in studies of the physiological state, growth, taxonomy, and population dynamics of marine phytoplankton. Immunofluorescence has only recently been used as a tool in this type of study, although it is an ideal method to reveal variations in biomass, cell concentration of individual taxa or even species,

TABLE 3. *Summary off cell-surface antibodies to marine phytoplankton.*

Species	Antibody	Reference
Bacillariophyceae		
Nitzschia frustulum var. *symbioticum*	Polyclonal	Lee et al. 1988
Pseudo-nitzschia pungens forma *multiseries*	Polyclonal	Bates et al. 1993
P. pungens forma *pungens*	Polyclonal	Bates et al. 1993
Thalassiosira oceanica	Polyclonal	L. P. Shapiro et al. 1989a, Campbell et al. 1994
Cyanobacteria		
Synechococcus	Polyclonal	Fliermans and Schmidt 1977, Campbell et al. 1983, Glover et al. 1986, Campbell and Carpenter 1987, Campbell and Iturriaga (1988)
Synechocystis	Polyclonal	Campbell et al. 1983
Chrysophyceae		
Aureococcus anophagefferens	Polyclonal	Anderson et al. 1990, 1993
Pelagococcus subviridis	Polyclonal	L. P. Shapiro et al. 1989a, Campbell et al. 1994
Dinophyceae		
Alexandrium catenella	Monoclonal	Adachi et al. 1993, Sako et al. 1993
A. lusticanicum	Polyclonal	Mendoza et al. 1995
A. minutum	Polyclonal	Mendoza et al. 1995
A. tamarense	Monoclonal	Adachi et al. 1993, Sako et al. 1993
A. tamarense	Polyclonal	Taylor and Lewis 1995
Gyrodinium aureolum[a]	Monoclonal	Vrieling et al. 1994b
G. catenatum	Polyclonal	Mendoza et al. 1995
Gymnodinium nagasakiense	Monoclonal	Nagasaki et al. 1991
Prorocentrum lima	Polyclonal	Costas et al., pers. commun.
P. micans	Polyclonal	Vrieling et al. 1993a, b
P. triestinum	Polyclonal	Costas et al., pers. commun.
Prymnesiophyceae		
Emiliania huxleyi	Polyclonal	L. P. Shapiro et al. 1989a, Campbell et al. 1994
E. huxleyi (types A and B)[b]	Polyclonal	Borman et al. 1987, van Bleijswijk et al. 1991
Raphidophyceae		
Chattonella antiqua	Monoclonal	Hiroishi et al. 1988, Uchida et al. 1989, Vrieling et al. 1995b
C. marina	Monoclonal	Hiroishi et al. 1988, Uchida et al. 1989, Vrieling et al. 1995b

[a] These MAbs also recognize the morphologically related species *Gymnodinium nagasakiense* and *G. mikimotoi* (Vrieling et al. 1994b).
[b] PAbs reacting with polysaccharides of either type A or B.

and location and expression of cell components such as enzymes indicative of metabolism and growth. Modern immunofluorescent techniques clearly represent a powerful but underutilized tool in ecophysiological phytoplankton research. The nearly endless variety of mammalian antibody molecules allows specific detection of antigens related to a very wide range of cell functions, and only a handful of these have been investigated thus far. Various aspects of cellular organization revealed by immunofluorescence can be compared directly with bright-field or phase-contrast images. Rapid visualization of the cell compartments of interest using different fluorophore-conjugated antibodies targeting multiple antigens is also possible. Moreover, the possibility of investigating the localization or abundance of one or several antigens at the whole-cell level (even in three dimensions using CLSM) is perhaps the most valuable aspect of immunofluorescence microscopy. As more workers realize that the species, molecules, or cellular processes of interest to them are accessible to immunofluorescent probes, the applications of this powerful technology should expand considerably in the coming years.

We are grateful to Drs. M. Veenhuis (Laboratory for Electron Microscopy, University of Groningen) and W. W. C. Gieskes (Department of Marine Biology, University of Groningen) for critically reading earlier drafts of the manuscript and for valuable discussions. Drs. A. G. J. Buma (Department of Marine Biology, University of Groningen) and M. Veldhuis (Netherlands Institute of Sea Research) are thanked for contributing Figure 4. This work was supported in part by grants to one of us (D.M.A.) from the National Science Foundation (OCE-9415536), NOAA National Sea Grant College Program Office, Department of Commerce grant NA46RG0470 (WHOI Sea Grant Projects R/B-130), and by the National Marine Fisheries Service through a Saltonstall-Kennedy grant (NA47FD00447). This is contribution 8956 from the Woods Hole Oceanographic Institution.

Adachi, M., Sako, Y. & Ishida, Y. 1993. The identification of conspecific dinoflagellates *Alexandrium tamarense* from Japan and Thailand by monoclonal antibodies. *Nipp. Suis. Gakk.* 59: 327–32.

Anderson, D. M. 1995. Identification of harmful algal species using molecular probes: an emerging technology. *In* Lassus, P., Arzul, G., Erard, E., Gentien, P. & Marcaillou, C. [Eds.] *Harmful Marine Algal Blooms.* Lavoisier, Paris, pp. 3–13.

Anderson, D. M. & P-O Cheng, T. 1988. Intracellular localization of saxitoxins in the dinoflagellate *Gonyaulax tamarensis. J. Phycol.* 24:17–22.

Anderson, D. M., Keafer, B. A., Kulis, D. M., Waters, R. M. & Nuzzi, R. 1993. An immunofluorescent survey of the brown

tide chrysophyte *Aureococcus anophagefferens* along the northeast coast of the United States. *J. Plankton Res.* 15:563–80.

Anderson, D. M., Kulis, D. M. & Cosper, E. M. 1990. Immunofluorescent detection of the brown tide organism *Aureococcus anophagefferens*. *In* Cosper, E. M., Bricelj, V. M. & Carpenter, E. J. [Eds.] *Novel Phytoplankton Blooms: Causes and Impacts of Recurrent Brown Tides and Other Unusual Blooms.* Springer-Verlag, Berlin, pp. 213–28.

Baden, D. G., Mende, T. J. & Szmant, A. M. 1988. Brevetoxins and binding: sodium channels versus antibodies. *In* Yentsch, C. M., Mague, F. C. & Horan, P. K. [Eds.] *Immunochemical Approaches to Coastal, Estuarine and Oceanicographic Questions, Lecture Notes on Coastal and Estuarine Studies,* Vol. 25. Springer-Verlag, New York, pp. 134–44.

Baker, K. H. & Mills, A. L. 1982. Determination of the number of respiring *Thiobacillus ferrooxidans* cells in water samples by using combined fluorescent antibody-2-(p-iodophenyl)-3-(p-nitrophenyl)-5-phenyltertazolium chloride staining. *Appl. Environ. Microbiol.* 43:338–44.

Balch, W. M., Yentsch, C. M., Reguera, B. & Campbell, W. 1988. Examining nitrate reduction by phyto-plankton with an immunoassay. *In* Yentsch, C. M., Mague, F. C. & Horan, P. K. [Eds.] *Immunochemical Approaches to Coastal, Estuarine and Oceanographic Questions, Lecture Notes on Coastal and Estuarine Studies,* Vol. 25. Springer-Verlag, New York, pp. 263–76.

Balfoort, H. W., Snoek, J., Smits, J. R. M., Breedveld, L. W., Hofstraat, J. W. & Ringelberg, J. 1992. Automatic identification of algae: neural network analysis of flow cytometric data. *J. Plankton Res.* 14:575–89.

Bates, S. S., Léger, C., Keafer, B. A. & Anderson, D. M. 1993. Discrimination between domoic-acid–producing and nontoxic forms of the diatom *Pseudonitzschia pungens* using immunofluorescence. *Mar. Ecol. Prog. Ser.* 100:185–95.

Bernhard, M., Lomi, G., Riparbelli, G., Saletti, M. & Zattera, A. 1969. Un metodo immunologico per la caratterizzazzione del fitoplancton. *Estratto Dalle Publ. Staz. Zool. Napoli* 37(2 Suppl.):64–72.

Borman, A. H., de Vrind-de Jonge, E. W., Thierry, R., Westbroek, P. & Bosch, L. 1987. Coccolith associated polysaccharides from cells of *Emiliania huxleyi* (Haptophyceae). *J. Phycol.* 23:118–23.

Buma, A. G. J., van Hannen, E. J., Roza, L., Veldhuis, M. & Gieskes, W. W. C. 1995. Monitoring ultraviolet-B induced DNA damage in individual diatom cells by immunofluorescent thymine dimer detection. *J. Phycol.* 31:314–21.

Buurman, E. P., Sanders, R., Draaijer, A., Gerritsen, H. C., van Veen, J. J. F., Houpt, P. M. & Levine, Y. K. 1992. Fluorescence lifetime imaging using a confocal laser scanning microscope. *Scanning* 14:155–9.

Campbell, L. & Carpenter, E. J. 1987. Characterization of phycoerythrin-containing *Synechococcus* spp. populations by immunofluorescence. *J. Plankton Res.* 9:1167–81.

Campbell, L., Carpenter, E. J. & Iacono, V. I. 1983. Identification and enumeration of marine chroococcoid cyanobacteria by immunofluorescence. *Appl. Environ. Microbiol.* 46: 553–9.

Campbell, L. & Iturriaga, R. 1988. Identification of *Synechococcus* spp. in the Sargasso Sea by immunofluorescence and fluorescence excitation spectroscopy performed on individual cells. *Limnol. Oceanogr.* 33:1196–201.

Campbell, L., Shapiro, L. P. & Haugen, E. 1994. Immunochemical characterization of eukaryotic ultraplankton from the Atlantic and Pacific oceans. *J. Plankton Res.* 16:35–51.

Carlson, R. E., Lever, M. L., Lee, B. W. & Guire, P. E. 1984. Development of immunoassays for paralytic shellfish poisoning: a radioimmunoassay for saxitoxin. *In* Ragelis, E. P. [Ed.] *Seafood Toxins.* American Chemical Society, Washington, D.C., pp. 181–92.

Cembella, A. D., Sullivan, J. J., Boyer, G. L., Taylor, F. J. R. & Anderson, R. J. 1987. Variation in paralytic shellfish toxin composition within the *Protogonyaulax tamarensis/catenella* species complex: red tide dinoflagellates. *Biochem. Syst. Ecol.* 15:171–86.

Cembella, A. D., Therriault, J. C. & Beland, P. 1988. Toxicity of cultured isolates and natural populations of *Protogonyaulax tamarensis* (Lebour) Taylor from the St. Lawrence estuary. *J. Shellfish Res.* 7:611–22.

Chisholm, S. W., Olson, R. J., Zettler, E. R., Goericke, R., Waterbury, J. B. & Welsch-meyer, N. A. 1988. A novel free-living prochlorophyte abundant in the oceanic euphotic zone. *Nature (Lond.)* 334:340–3.

Coleman, A. W. 1988. The autofluorescent flagellum: A new phylogenetic enigma. *J. Phycol.* 24:118–20.

Coons, A. H., Creech, J. H. & Jones, N. R. 1941. Immunological properties of an antibody containing a fluorescent group. *Proc. Soc. Exp. Biol. Med.* 47:200.

Coons, A. H., Leduc, E. H. & Connolly, J. M. 1955. Studies of antibody production. I: A method for the histochemical demonstration of specific antibody and its application to a study of the hyperimmune rabbit. *J. Exp. Med.* 102:49–60.

Cosper, E. M., Bricelj, V. M. & Carpenter, E. J., Eds. 1989. *Novel Phytoplankton Blooms: Causes and Effects of Recurrent Brown Tides and Other Unusual Blooms.* Springer-Verlag, Berlin, 799 pp.

Cunningham, A. 1990. A low-cost, portable flow cytometer specifically designed for phytoplankton analysis. *J. Plankton Res.* 12:149–60.

Currin, C. A., Paerl, H. W., Suba, G. K. & Alberte, R. S. 1990. Immunofluorescence detection and characterization of N_2-fixing microorganisms from aquatic environments. *Limnol. Oceanogr.* 35:59–71.

Dahl, A. B. & Laake, M. 1982. Diversity dynamics of marine bacteria studied by immunofluorescent staining on membrane filters. *Appl. Environ. Microbiol.* 43:169–76.

Domozych, D. S. & Rogers Domozych, C. 1993. Mucilage processing and secretion in the green alga *Closterium.* II. Ultrastructure and immunocytochemistry. *J. Phycol.* 29:659–67.

Doucette, G. J. & Anderson, D. M. 1993. Intracellular distribution of saxitoxin in *Alexandrium fundyense. In* Smayda, T. J. & Shimizu, Y. [Eds.] *Toxic Phytoplankton Blooms in the Sea.* Elsevier, Amsterdam, pp. 863–8.

Dovichi, N. J., Martin, J. C., Jett, J. H. & Keller, R. A. 1983. Attogram detection limit for aqueous dye samples by laser-induced fluorescence. *Science (Wash. D.C.)* 219:845–7.

Dubelaar, G. B. J., Groenewegen, A. D., Stokdijk, W., van den Engh, G. J. & Visser, J. W. M. 1989. Optical Plankton Analyzer: a flow cytometer for plankton analysis, II: Specifications. *Cytometry* 10:529–39.

Eardly, D. D., Sutton, C. W., Hempel, W. M., Reed, D. C. & Ebeling, A. W. 1990. Monoclonal antibodies specific for sulfated polysaccharides on the surface of *Macrocystis pyrifera* (Phaeophyta). *J. Phycol.* 26:54–62.

Fliermans, C. B., Bohlool, B. B. & Schmidt, E. L. 1974. Autecological study of the chemoautotroph *Nitrobacter* by immunofluorescence. *Appl. Microbiol.* 27:124–9.

Fliermans, C. B. & Schmidt, E. L. 1977. Immunofluorescence for autecological study of a unicellular bluegreen alga. *J. Phycol.* 13:364–8.

Gates, J. E. & Pham, K. D. 1979. An indirect fluorescent antibody staining technique for determining population levels of *Thiobacillus ferrooxidans* in acid mine drainage waters. *Microbial Ecol.* 5:121–7.

Giloh, H. & Sedat, J. W. 1982. Fluorescence microscopy: reduced photobleaching of rhodamine and fluorescein protein conjugates by *n*-propylgallate. *Science (Wash. D.C.)* 217:1252–4.

Glover, H. E., Campbell, L. & Prézelin, B. B. 1986. Contribution of *Synechococcus* spp. to size-fractioned primary production in three water masses in the Northwest Atlantic Ocean. *Mar. Biol. (Berl.)* 91:193–203.

Griffiths, G., Parton, R. G., Lucocq, J., van Deurs, B., Brown, D, Slot, J. W. & Geuze, H. J. 1993. The immunofluorescent era of membrane traffic. *Trends Cell Biol.* 3:214–9.

Guire, P. E., Duquette, P. H., Amos, R. A., Behrens, M. W., Josephson, M. W. & Chambers, R. P. 1988. Preparation of model haptens to express common epitopes on PSP toxins. *In* Yentsch, C. M., Mague, F. C. & Horan, P. K. [Eds.] *Immunochemical Aprroaches to Coastal, Estuarine and Oceanico-*

graphic Questions, Lecture Notes on Coastal and Estuarine Studies, Vol. 25. Springer-Verlag, New York, pp. 145–54.

Harlow, E. & Lane, D., Eds. 1988. *Antibodies: A Laboratory Manual.* CSH Laboratory, New York, 726 pp.

Haugland, R. P., Ed. 1989. *Handbook of Fluorescent Probes and Research Chemicals.* Catalogue of Molecular Probes, Eugene, Oregon, 421 pp.

Hiroishi, S., Uchida, A., Nagasaki, K. & Ishida, Y. 1988. A new method for identification of inter- and intra-species of the red tide algae *Chattonella antiqua* and *Chattonella marina* (Raphidophyceae) by means of monoclonal antibodies. *J. Phycol.* 24:442–4.

Hofstraat, J. W., van Zeijl, W. J. M., Peeters, J. C. H., Peperzak, L. & Dubelaar, G. B. J. 1990. Flow cytometry and other optical methods for characterization and quantification of phytoplankton in seawater. *In* Nielsen, H. O. [Ed.] *Environment and Pollution Measurement Sensors and Systems, SPIE Proceedings,* Vol. 1269. Bellingham, Washington, pp. 116–33.

Hokama, Y., Honda, S. A. A., Kobayashi, M. N., Nakagawa, L. K., Shiria, L. K. & Miyahara, J. T. 1988. Monoclonal antibodies (Monoabs) to ciguatoxin and related polyethers. *In* Yentsch, C. M., Mague, F. C. & Horan, P. K. [Eds.] *Immunochemical Approaches to Coastal, Estuarine and Oceanicographic Questions, Lecture Notes on Coastal and Estuarine Studies,* Vol. 25. Springer-Verlag, New York, pp. 155–66.

Homan, W. L., van Kalshoven, H., Kolk, A. H. J., Musgrave, A., Schuring, F. & van den Ende, H. 1987. Monoclonal antibodies to surface glycoconjugates in *Chlamydomonas eugametos* recognize strain-specific O-methyl sugars. *Planta (Berl.)* 170: 328–35.

Hudson, L. & Hay, F. C., Eds. 1989. *Practical Immunolgy* (3rd ed.). Blackwell, London, 507 pp.

Jeffris, R. & Deverill, I. 1991. The antigen antibody reaction. *In* Price, C. P. & Newman, D. J. [Eds.] *Principles and Practice of Immunochemistry.* Stocton Press, New York, pp. 1–18.

Johnson, G. D. & Nogueira Araujo, G. M. de C. 1981. A simple method of reducing the fading of immunofluorescence during microscopy. *J. Immunol. Methods* 43:349–50.

Josserand, A. & Cleyet-Marel, J. C. 1979. Isolation from soils of *Nitrobacter* and evidence for novel serotypes using immunofluorescence. *Microbial Ecol.* 5:197–205.

Kajikawa, H., Okada, M., Ishikawa, F., Okada, Y. & Nakayama, K. 1988. Immunochemical studies on ribulose 1,5-bisphosphate carboxylase/oxygenase in the chloroplasts of the marine alga *Bryopsis maxima. Plant Cell Physiol.* 29:549–56.

Kasten, F. H. 1989. The origins of modern fluorescence microscopy and fluorescent probes. *In* Kohen, E. & Hirschberg, J. G. [Eds.] *Cell Structure and Function by Microspectrofluorometry.* Academic Press, New York, pp. 3–50.

Kawai, H. 1988. A flavin-like autofluorescent substance in the posterior flagellum of golden brown algae. *J. Phycol.* 24:114–7.

Kawai, H. & Inouye, I. 1989. Flagellar autofluorescence in forty-four chlorophyll *c*–containing algae. *Phycologia* 28:222–7.

Langanger, G., de Mey, J. & Adam, M. 1983. 1,4-Diazobizyklo-[2,2,2]-oktan (DABCO) verzögert das Ausbleichen von immunofluoreszenz Präparaten. *Mikroskopie (Wien)* 40:237–41.

Lee, J. J., Chan, Y. & Lagziel, A. 1988. An immunofluorescence approach toward the identification of endosymbiotic diatoms in several spieces of larger Foraminifera. *In* Yentsch, C. M., Mague, F. C. & Horan, P. K. [Eds.] *Immunochemical Approaches in Coastal, Estuarine and Oceanographic Questions, Lecture Notes on Coastal and Estuarine Studies,* Vol. 25. Springer-Verlag, New York, pp. 230–41.

Legendre, L. & Yentsch, C. M. 1989. Overview of flow cytometry image analysis in biological oceanography and limnology. *Cytometry* 10:501–10.

Lin, S., Chang, J. & Carpenter, E. J. 1994. Detection of proliferating cell nuclear antigen analog in four species of marine phytoplankton. *J. Phycol.* 30:449–56.

——— 1995. Growth characteristics of phytoplankton determined by cell cycle proteins: PCNA immunostaining of *Dunaliella tertiolecta* (Chlorophyceae). *J. Phycol.* 31:388–95.

Melkonian, M., Ed. 1992. *Algal Cell Motility, Current Phycology 3.* Chapman & Hall, London, 236 pp.

Melkonian, M., Schulze, D., McFadden, G. I. & Robenek, H. 1988. A polyclonal antibody (anticentrin) distinguishes between two types of fibrous flagellar roots in green algae. *Protoplasma* 144:56–61.

Mendoza, H., López-Rodas, V., González-Gil, S., Aguilera, A. & Costas, E. 1995. Use of polyclonal antisera and blocking of the antibodies in the identification of marine dinoflagellates: species-specific and clone-specific antisera against *Gymnodinium* and *Alexandrium. J. Exp. Mar. Biol. Ecol.* 186:103–15.

Menzel, D., Ed. 1992. *The Cytoskeleton of the Algae.* CRC Press, London, 453 pp.

Motomura, T. 1991. Immunofluorescence microscopy of fertilization and parthenogenesis in *Laminaria angustata* (Phaeophyta). *J. Phycol.* 27:248–57.

Müller, D. G., Maier, I. & Müller, H. 1987. Flagellum autofluorescence and photoaccumulation in heterokont algae. *Photochem. Photobiol.* 46:1003–8.

Musgrave, A., de Wildt, P., Broekman, R. & van den Ende, H. 1983. The cell wall of *Chlamydomonas eugametos.* Immunological aspects. *Planta (Berl.)* 158:82–9.

Musgrave, A., van Eijk, F., te Welcher, R., Broekman, R., Lens, P. F., Homan, W. L. & van den Ende, H. 1981. Sexual agglutination factor from *Chlamydomonas eugametos. Planta (Berl.)* 153:362–9.

Nagasaki, K., Uchida, A. & Ishida, Y. 1991. A monoclonal antibody which recognizes the cell surface of red tide alga *Gymnodinium nagasakiense. Nipp. Suis. Gakk.* 57:1211–24.

Olson, R. J., Chisholm, S. W., Zettler, E. R. & Armbrust, E. V. 1988. Analysis of *Synechococcus* pigment types in the sea using single and double beam flow cytometry. *Deep Sea Res.* 35: 425–40.

Olson, R. J., Vaulot, D. & Chisholm, S. W. 1985. Marine phytoplankton distributions measured using a shipboard flow cytometry. *Deep Sea Res.* 32:1273–80.

Orellana, M. V. & Perry, M. J. 1992. An immunoprobe to measure Rubisco concentrations and maximal photosynthetic rates of individual phytoplankton cells. *Limnol. Oceanogr.* 37:478–90.

——— 1995. Optimization of an immunofluorescent assay of the internal enzyme, ribulose-1,5-biphosphate carboxylase (Rubisco), in single phytoplankton cells. *J. Phycol.* 31:785–94.

Orellana, M. V., Perry, M. J. & Watson, B. A. 1988. Probes for assessing single-cell primary production: antibodies against ribulose-1,5-bisphosphate carboxylase (RuBPCASE) and peridinin/chlorophyll *A* protein (PCP). *In* Yentsch, C. M., Mague, F. C. & Horan, P. K. [Eds.] *Immunochemical Approaches to Coastal, Estuarine and Oceanographic Questions, Lecture Notes on Coastal and Estuarine Studies,* Vol 25. Springer-Verlag, New York, pp. 243–62.

Peeters, J. C. H., Dubelaar, G. B. J., Ringelberg, J. & Visser, J. W. M. 1989. Optical Plankton Analyzer: a flow cytometer for plankton analysis, I: Design considerations. *Cytometry* 10: 522–8.

Phinney, D. A. & Cucci, T. L. 1989. Flow cytometry and phytoplankton. *Cytometry* 10:511–21.

Ploem, J. S. 1989. Fluorescence scanning instrumentation. *In* Kohen, E. & Hirschberg, J. G. [Eds.] *Cell Structure and Function by Microspectrofluorometry.* Academic Press, New York, pp. 119–30.

Pomeroy, L. R. & Johannes, R. E. 1968. Occurrence and respiration of ultraplankton in the upper 500 meters of the ocean. *Deep Sea Res.* 15:381–91.

Roberts, K. R. 1991. The flagellar apparatus and cytoskeleton of dinoflagellates: organization and use in systematics. *In* Patterson, D. J. & Larsen, J. [Eds.] *The Biology of Free-Living Flagellates.* Systematics Association Special Volume No. 45. Clarendon Press, Oxford, pp. 285–302.

Roberts, K. R., Lemoine, J. E., Schneider, R. M. & Farmer, M. A. 1988. The microtubular cytoskeleton of three dinoflagellates: an immunofluorescence study. *Protoplasma* 144:68–71.

Roberts, K. R., Losoff Rusche, M. & Taylor, F. J. R. 1993. The cortical microtubular cytoskeleton of *Oxyrrhis marina* (Dinophyceae) observed with immunofluorescence and electron microscopy. *J. Phycol.* 29:642–9.

Roberts, K. R. & Roberts, J. E. 1991. The flagellar apparatus and cytoskeleton of the dinoflagellates: a comparative overview. *Protoplasma* 164:105–22.

Rogers Domozych, C., Plante, K., Blais, P., Paliulis, L. & Domozych, D. S. 1993. Mucilage processing and secretion in the green alga *Closterium*. I. Cytology and biochemistry. *J. Phycol.* 29:650–9.

Sako, Y., Adachi, M. & Ishida, Y. 1993. Preparation and characterization of monoclonal antibodies to *Alexandrium* species. *In* Smayda, T. J. & Shimizu, Y. [Eds.] *Toxic Phytoplankton Blooms in the Sea.* Elsevier, Amsterdam, pp. 87–93.

Schulze, D., Robenek, H., McFadden, G. I. & Melkonian, M. 1987. Immunolocalization of Ca^{2+}-modulated contractile protein in the flagellar apparatus of green algae: the nucleus-basal connector. *Eur. J. Cell Biol.* 45:51–61.

Segaar, P. J. & Lokhorst, G. M. 1987. Cell division in the green alga *Ulothrix palusalsa* (Ulvophyceae, Chlorophyta): a combined immunofluorescence and transmission electron microscopy study. *Phycologia* 26:100–10.

Shapiro, H. M., Ed. 1985. *Practical Flow Cytometry.* Alan R. Liss, New York, 353 pp.

Shapiro, L. P., Campbell, L. & Haugen, E. M. 1989a. Immunochemical recognition of phytoplankton species. *Mar. Ecol. Prog. Ser.* 57:219–24.

Shapiro, L. P., Haugen, E. M. & Carpenter, E. J. 1989b. Occurrence and abundance of green-fluorescing dinoflagellates in surface waters of the Northwest Atlantic and Northeast Pacific oceans. *J. Phycol.* 25:189–91.

Shapiro, L. P., Haugen, E. M., Keller, M. D., Bidigare, R. R., Campbell, L. & Guillard, R. R. L. 1989c. Taxonomic affinities of marine coccoid ultraphytoplankton: a comparison of immunochemical surface antigen cross-reactions and HPLC chloroplast pigment signatures. *J. Phycol.* 25:794–7.

Smith, E., Roberts, K., Hutchings, A. & Galfre, G. 1984. Monoclonal antibodies to the major structural glycoprotein of the *Chlamydomonas* cell wall. *Planta (Berl.)* 161:330–8.

Smith, J. C., Odense, P., Angus, R., Bates, S. S., Bird, C. J., Cormier, P., de Freitas, A. S. W., Léger, C., O'Neil, D., Pauley, K. & Worms, J. 1990. Variation in domoic acid levels in *Nitzschia* species: implications for monitoring programs. *Bull. Aquacult. Assoc. Can.* 90:27–31.

Steen H. B. 1991. Flow cytometry instrumentation. *In* Demers, S. [Ed.] *Particle Analysis in Oceanography,* NATO/ASI Series, Series G: Ecological Sciences, Vol. 27. Springer-Verlag, Berlin, pp. 3–29.

Taylor, J. A. & Lewis, J. 1995. Immunofluorescence of *Alexandrium* species from the United Kingdom. *In* Lassus, P., Arzul, G., Erard, E., Gentien, P. & Marcaillou, C. [Eds.] *Harmful Marine Algal Blooms.* Lavoisier Intercept, Paris, pp. 89–94.

Tsuji, T. & Yaganati, T. 1981. Improved fluorescent microscopy for measuring the standing stock of phytoplankton including fragile components. *Mar. Biol. (Berl.)* 64:207–11.

Uchida, A., Nagasaki, K., Hiroishi, S. & Ishida, Y. 1989. The application of monoclonal antibodies to an identification of *Chattonella marina* and *Chattonella antiqua. Nipp.* Suis. Gakk. 55:721–5.

van Bleijswijk, J., van der Wal, P., Kempers, R., Veldhuis, M., Young, J. R., Muyzer, G., de Vrind-de Jong, E. & Westbroek, P. 1991. Distribution of two types of *Emiliania huxleyi* (Prymnesiophyceae) in the Northeast Atlantic region as determined by immunofluorescence and coccolith morphology. *J. Phycol.* 27:566–70.

Visser, J., Haaijman, J. & Trask, B. 1978. Quantitative immunofluorescence in flow cytometry. *In* Knapp, W., Holubar, K. & Wick, G. [Eds.] *Immunofluorescence and Related Stainings Techniques.* Elsevier, Amsterdam, pp. 147–60.

Vladimirova, M. G., Markelova, A. G. & Semenenko, V. E. 1982. Use of the cytoimmunofluorescent method to clarify localization of ribulose bisphosphate carboxylase in pyrenoids of unicellular algae. *Fiziol. Rast.* 29:941–50.

Voigt, J. 1988. The lithium-chloride–soluble cell-wall layers of *Chlamydomonas reinhardii* contain several immunologically related glycoproteins. *Planta (Berl.)* 173:373–84.

Vrieling, E. G., Draaijer, A., van Zeijl, W. J. M., Peperzak, L., Gieskes, W. W. C. & Veenhuis, M. 1993a. The effect of labelling intensity, estimated by real time confocal laser scanning microscopy, on flow cytometric appearance and identification of immunochemically labelled marine dinoflagellate species. *J. Phycol.* 29:180–8.

Vrieling, E. G., Gieskes, W. W. C., Colijn, F., Hofstraat, J. W., Peperzak, L. & Veenhuis, M. 1993b. Immunochemical identification of toxic marine algae: first results with *Prorocentrum micans* as model organism. *In* Smayda, T. J. & Shimizu, Y. [Eds.] *Toxic Marine Phytoplankton Blooms in the Sea.* Elsevier, Amsterdam, pp. 925–31.

Vrieling, E. G., Gieskes, W. W. C., Rademaker, T. W. M., Vriezekolk, G., Peperzak, L. & Veenhuis, M. 1995a. Flow cytometric identification of the ichthyotoxic dinoflagellate *Gyrodinium aureolum* in the central North Sea. *In* Lassus, P., Arzul, G., Erard, E., Gentien, P. & Marcaillou, C. [Eds.] *Harmful Marine Algal Blooms.* Lavoisier, Paris, pp. 743–8.

Vrieling, E. G., Gieskes, W. W. C., van Zeijl, W. J. M. & Veenhuis, M. 1994a. Detection of *Gyrodinium aureolum* in Dutch coastal waters by particle analysis based on immuno-flow cytometry. *Neth. J. Aquat. Ecol.* 28:249–54.

Vrieling, E. G., Koeman, R. P. T., Peperzak, L., Nagasaki, K. & Ishida, Y. 1995b. *Chattonella* and *Fibrocapsa* (Raphidophyceae): first observation of potentially harmful, red tide organisms in the Dutch Wadden Sea. *Neth. J. Sea Res.* 33:183–91.

Vrieling, E. G., Peperzak, L., Gieskes, W. W. C. & Veenhuis, M. 1994b. Detection of the ichthyotoxic dinoflagellate *Gyrodinium* cf. *aureolum* and morphologically related *Gymnodinium* species using monoclonal antibodies: a specific immunological tool. *Mar. Ecol. Prog. Ser.* 103:165–74.

Ward, B. B. 1990. Immunology in biological oceanography and marine ecology. *Oceanography* 3:30–5.

Ward, B. B. & Carlucci, F. 1985. Marine ammonia- and nitrite-oxidizing bacteria: serological diversity determined by immunofluorescence in culture and in the environment. *Appl. Env. Microbiol.* 50:194–201.

Ward, B. B. & Perry, M. J. 1980. Immunofluorescent assay for the marine ammonium-oxidizing bacterium *Nitrococcus oceanus. Appl. Env. Microbiol.* 39:913–8.

Williams, A. F. & Woollet, G. R. 1985. What monoclonals see at cell surfaces. *Biochem. Soc. Trans.* 13:1–3.

Wilson, T. 1990, Ed. *Confocal Microscopy.* Academic Press, London, 426 pp.

Yentsch, C. M., Dale, B. & Hurst, J. W. 1978. Coexistence of toxic and non-toxic dinoflagellates resembling *Gonyaulax tamarensis* in New England coastal waters (N. W. Atlantic). *J. Phycol.* 14:330–2.

Yoshioka, T., Terai, H. & Saijo, Y. 1982. Growth kinetic studies of nitrifying bacteria using immunofluorescent counting method. *J. Gen. Appl. Microbiol.* 28:169–80.

Zhang, Y. & Wu, M. 1993. Fluorescence microscopy on dynamics changes of frx B distribution in *Chlamydomonas reinhardii. Protoplasma* 172:57–63.

Zhou, J. & Fritz, L. 1994. Okadaic acid antibody localizes to chloroplasts in the DSP-toxin–producing dinoflagellates *Prorocentrum lima* and *Prorocentrum maculosum. Phycologia* 33:455–61.