Detection of the ichthyotoxic dinoflagellate *Gyrodinium* (cf.) *aureolum* and morphologically related *Gymnodinium* species using monoclonal antibodies: a specific immunological tool

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ABSTRACT· Sixteen monoclonal antibodies which recognize different cell surface antigens of the ichthyotoxic marine dinoflagellate *Gyrodinium* cf *aureolum* were prepared and characterized for use in identification by both immunofluorescence microscopy and flow cytometry. Based on the labeling results obtained from indirect immunofluorescence assays the monoclonals could be divided into 3 groups: (I) fluorescence of the overall cell surface, (II) fluorescence of the overall cell surface together with the flagella, and (III) fluorescence of granular-like structures randomly distributed along the cell surface and at the edges of the sulcus and cingulum. Cross-reactivity of 6 selected monoclonals with other phytoplankton species revealed that labeling was restricted to *G. aureolum*/*G.* cf. *aureolum* and to the morphologically closely related species *Gymnodinium nagasakiense* and *G. mikimotoi*. This suggests a taxonomic proximity of these species. Fixation of samples with paraformaldehyde produced the optimal immunochemical response, resulting in the strongest fluorescence intensities. In flow cytometric analyses, labeled cells of *G.* cf. *aureolum* could be distinguished sufficiently from unlabeled controls when monoclonals of Group II and a pooled serum (combined antibodies of Groups I, II, and III) were used. This immunochemical technique will be applied in an early warning system to detect *G. aureolum* cells at dilute concentrations using flow cytometry or, alternatively, image analysis.

KEY WORDS: Flow cytometry · *Gymnodinium* (cf.) *aureolum* · Immunofluorescence · Monoclonal antibodies · Toxic marine phytoplankton

INTRODUCTION

The number of reports on the occurrence of nuisance and potentially toxic bloom-forming phytoplankton species has increased steadily during the last decades (Anderson 1989, Hallegraeff 1993). Although the bloom frequency may be overestimated due to increased observer efforts, other factors (eutrophication and other forms of pollution, general climatological changes) have been implicated (Smayda 1989). In fact, the toxic dinoflagellate genera *Gyrodinium*, *Gymnodinium*, and *Alexandrium* have been observed in samples taken in Dutch coastal waters only since 1989 (Peperzak 1990, R. P. T. Koeman pers. comm.), while they have been reported before in other parts of the North Sea (Reid et al. 1990). *Dinophysis* species have been observed before in Dutch coastal waters (Kat 1985, 1987, Leewis 1985); the presence of other toxic species remains elusive.

The presence of a harmful species is usually not observed until the full bloom stage, after biotoxins or anoxia have already exerted their effects on sea life or humans. A study of the initial stages of such a bloom would have revealed the ecophysiological conditions

leading to mass occurrence. This would have allowed prediction and modeling of the harmful event. In order to detect potentially toxic phytoplankton species at dilute concentrations – the early bloom stage – an early warning system is now being developed for Dutch coastal waters. This method is based on specific immunochemical tagging of specific organisms which are subsequently enumerated by flow cytometry (Vrieling et al. 1993a, b). To differentiate morphologically closely related toxic and non-toxic phytoplankters within genera, several antisera have already been prepared (e.g. Hiroishi et al. 1988, Nagasaki et al. 1989, 1991, Sako et al. 1992, Bates et al. 1993). These antisera recognize cell surface antigens of the red tide organisms *Chattonella*, *Gymnodinium* and *Alexandrium*, and discriminate toxic from non-toxic diatoms of the *Nitzschia* complex at the species level. For our own early warning system, species-specific antisera are essential to achieving unequivocal identification of a target organism in order to avoid cross reactions with other species present in the mixed populations to be analyzed by flow cytometry.

In this paper we describe the preparation and characterization of monoclonal antibodies directed against cell surface antigens of *Gyrodinium* (cf.) *aureolum* (= *G. aureolum* or *G.* cf. *aureolum*). Attention is focused on this species because of its potential threat to young shellfish at oyster and mussel grounds in parts of the Dutch coastal region.

MATERIALS AND METHODS

Preparation of monoclonal antibodies. Unialgal batch cultures of the dinoflagellate *Gyrodinium* cf. *aureolum* Hulburt (strain Iroise; F. Partensky, Roscoff, France) were established in f/2-Si enriched seawater medium (Guillard & Ryther 1962) at a temperature of 16°C under a light/dark regime of 12 h : 12 h (light intensity of approximately 75 μE m^{-2} s^{-1}). Exponentially growing cells were fixed *in situ* for 2 h at room temperature by adding freshly prepared paraformaldehyde at a final concentration of 2% (w/v). Subsequently, the cells were harvested by mild centrifugation (5 min, 1000 rpm), and washed 3 times with sterile phosphate-buffered saline (PBS) containing 0.02% (w/v) azide. Samples were resuspended, stored at –70°C and washed twice with sterile PBS before immunization.

Monoclonal antibodies were prepared with the assistance of MCA Development BV, Groningen, The Netherlands. Aliquots of 100 μl (approximately 10^4 cells) were used to immunize 4 BALB/c mice (3 to 4 wk old) by intraperitoneal injections at Days 0, 30, and 60 respectively. Freund's complete adjuvant was used

(1:1) at Day 0, while at Days 30 and 60, cells were emulsified in incomplete Freund's adjuvant. Serum samples of all immunized mice were screened for antibody reactivity towards target *Gyrodinium* cf. *aureolum* cells using indirect immunofluorescence (see below). One mouse, whose antiserum expressed the highest titer, was selected for fusion. At Day 90 (4 d before fusion) this individual was boosted with an intravenous injection of another 10^4 cells, before it was sacrificed. Suspended spleen cells were used for fusion with mouse myeloma cells of an SP20 HAT-sensitive cell line, using PEG-4000 and HAT selection. Fused cells were spread as oligoclones into 96-well microtiter plates and grown for 2 wk before a screening procedure (see below) was performed. After screening, positive oligoclonal hybridomas were cloned by limiting dilution in culture media to ensure monoclonality and stability of the cells. Specific antibody-producing clones were expanded by sequential transfer to 100 ml culture flasks.

Immunofluorescence. After fusion and cloning, specific antibody-producing hybridoma cells were screened by an indirect immunofluorescence method (Vrieling et al. 1993a) using supernatants of 10 to 15 d old oligo- and monoclonals. This vacuum manifold assay was slightly modified for application of the Multi Screen Assay System (Millipore Intertech). Multiscreen 96-well microtiter plates, containing a permeable bottom of nitrocellulose filter (0.45 μm pore size), were filled with 100 μl cell suspension (about 10^4 cells), which were fixed *in situ* with 2% (w/v) paraformaldehyde (Vrieling et al. 1993a) and blocked with incubation buffer [PBS to which 1% (w/v) bovine serum albumin was added]. Target cells were incubated for 1 h at room temperature after addition of hybridoma supernatants (at a 1:2 dilution). After incubation, supernatants were filtered through the nitrocellulose filter and wells were washed with three 200 μl volumes of incubation buffer. Subsequently, wells were loaded with 100 μl of incubation buffer containing 2% (v/v) of sheep anti-mouse IgG (Amersham) conjugated with fluorescein isothiocyanate (FITC). After incubation for 45 min at room temperature, wells were again vacuum-washed 3 times with 200 μl PBS. Labeled cells were resuspended in 50 μl PBS before fluorescence was examined by both epifluorescence microscopy and real time confocal laser scanning microscopy (Vrieling et al. 1993a).

The titer of the polyclonal antisera of the immunized mice and the resulting 16 monoclonal antibodies was determined by running dilution series using the modified multiscreen immunofluorescence assay as described above. Preservation methods were evaluated for their effect on immunofluorescence intensities according to the procedures of Vaulot et al. (1989) and

Bates et al. (1993). In all immunofluorescence labeling experiments controls were performed by omitting the primary antisera to estimate non-specific labeling of the secondary antiserum.

Cross-reactivity. Six monoclonal antibodies (GA1, GA2, GA7, GA8, GA14 & GA15) were tested in duplicate in the immunofluoresence assay described above to determine the specificity with respect to different strains of *Gyrodinium* (cf.) *aureolum* and other phytoplankton species. Species used in these cross-reactivity experiments are listed in Tables 3 & 4. They were cultured in f/2 medium (Si was omitted for non-diatoms) under the same conditions as *G.* cf. *aureolum* (see above). Additional samples of various strains of the naked dinoflagellates *Gyrodinium* and *Gymnodinium* were obtained elsewhere either fixed (1.0 % glutaraldehyde or 2.0 % paraformaldehyde) or live. Finally, some prefixed (4.0 % Lugol's iodine) and stored (4 °C) field samples, taken in 1991 in the Dutch monitoring program, were used for immunochemical identification of *G.* (cf.) *aureolum*.

Flow cytometry. For flow cytometry, cells were fixed with 2 % (w/v) paraformaldehyde and examined with the Optical Plankton Analyzer (Dubelaar et al. 1989, Peeters et al. 1989), using the filter settings as described previously (Vrieling et al. 1993a). *G.* cf. *aureolum* cells were examined under 4 different labeling conditions: (1) labeled with GA2, (2) labeled with GA8, (3) labeled with GA15, and (4) labeled by a pooled serum consisting of equal amounts of GA2, GA8, and GA15. This last condition was used to examine the fluorescence intensity of the FITC probe by coupling the probe to the antigens recognized by all antibodies. Green FITC fluorescence (FBG; measured between 520 and 544 nm) and red chlorophyll autofluorescence (FBR; measured between 650 and 750 nm) of unlabeled cells and cells at all labeling conditions were compared both in bivariate plots of green vs red fluorescence and in histograms of events vs green fluorescence.

RESULTS

Monoclonal antibodies

Five oligoclonal hybridomas, producing antibodies directed against cell surface antigens of *Gyrodinium* cf. *aureolum*, were identified by the modified vacuum manifold immunofluorescence assay. These hybridomas were cloned and expanded, after which 16 antibody producing clones, designated as GA1 to GA16, were selected and cultured to obtain 100 ml batches of monoclonal antibodies which were used for further characterization.
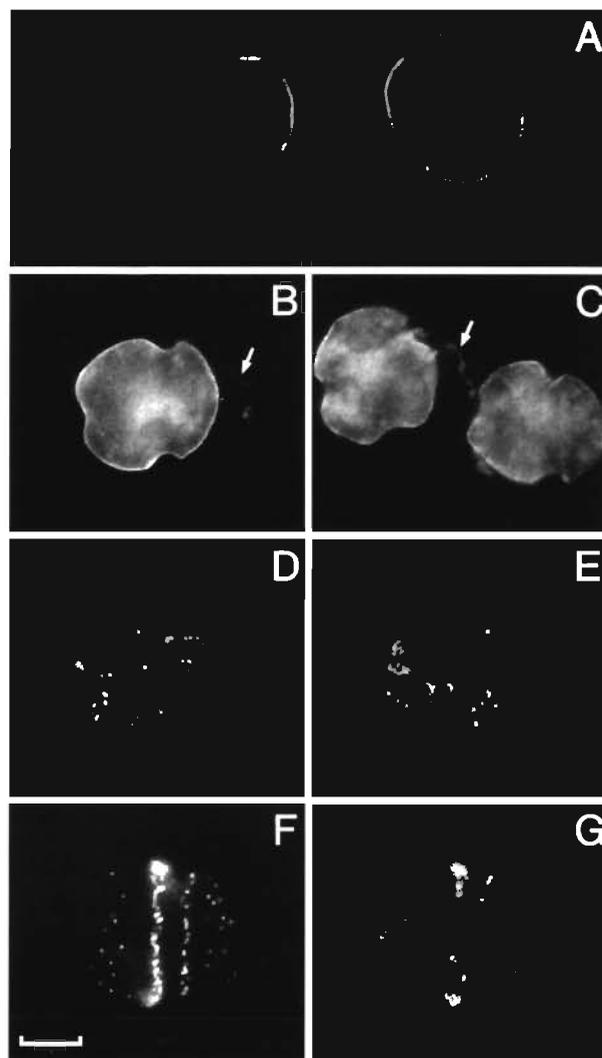

Fig. 1. *Gyrodinium* cf. *aureolum*. Micrographs of dinoflagellates treated with indirect immunofluorescence using the monoclonals GA2, GA8 & GA15. FITC fluorescence is confined to the outer cell wall. (A) Monoclonal GA2 shows bright fluorescence of the outer cell wall. (B & C) GA8 shows bright fluorescence of outer cell wall and flagella (arrows). (D to G) GA15 shows bright fluorescence of granular particles randomly distributed along the cell wall, the sulcus and cingulum. (F & G) Aligned globular-like fluorescence at the edge of the sulcus and especially the cingulum of the same cell at different focal positions. Scale bar = 20 µm

As judged from immunofluorescence microscopy, the labeling patterns of *Gyrodinium* cf. *aureolum* cells varied among the monoclonals, so they could be divided into 3 separate groups. Group I (GA1 & GA2) only showed reactivity with the outer cell surface (Fig. 1A) and differed from Group II (GA3 to GA8) which showed the same fluorescence pattern, though at an enhanced intensity, but the flagella showed a bright fluorescence as well (Fig. 1B, C). Group III (GA9 to GA16) revealed a random spotted fluorescence pattern

on the outer cell surface; in addition the sulcus showed fluorescence (Fig. 1D, E). Fluorescent particles appeared to be concentrated specially at the edges of the cingulum, which was evident after moving through several focal planes (Fig. 1F, G). In control experiments non-specific labeling of the secondary antiserum was absent (not shown).

To select suitable monoclonal antibodies and to define the optimal dilution, the titer of all 16 clones was determined. Two monoclonal antibodies of each of the 3 groups indicated above were selected based on titer, fluorescence intensities, and number of cells labeled (Table 1). The titer is defined as the reciprocal of the highest dilution at which clear FITC fluorescence could be observed (designated as + in Table 1, i.e. over 40 % of the cells showing positive labeling) and distin-

guished from chlorophyll autofluorescence. The titers of the antibodies in Groups I & II varied between 400 and 800, while those of Group III showed a broader variation, ranging from 25 to 200 (not all are listed in Table 1).

Cell preservation methods and influence on antigenicity

The effects of different preservation methods on antigenicity, expressed as the ability of antigens to bind to the different antibodies, are summarized in Table 2. Paraformaldehyde and formalin fixation displayed high intensities of FITC fluorescence with all monoclonals tested; in addition a bright red fluorescence of chloroplasts was evident. Glutaraldehyde fixation caused partial inhibition of binding (Table 2). In addition to a decrease of fluorescence intensities, an interfering intracellular yellow-greenish background fluorescence appeared (not shown). Also, the antibodies of Group III failed to react with the antigens (Tables 2 & 3) fixed with glutaraldehyde. Freezing at −80°C did not significantly affect antigen binding by Group I & II monoclonals, although the intensity of the fluorescence signal was weaker compared to paraformaldehyde or formalin fixation. Frozen cells were damaged and had probably lost the antigens recognized by the antibodies of Group III (Table 2). Prefixation prior to freezing the cells resulted in quite satisfactory fluorescence intensities, although glutaraldehyde still affected the immunochemical reaction of monoclonal antibodies of Group III (Table 2). Lugol fixation fully destroyed the binding capacity of the antibodies, except for those of Group II (GA7 & GA8).

Table 1 Titers of 6 selected monoclonal antibodies directed against cell surface antigens of *Gyrodinium* cf. *aureolum*. The titers represent the reciprocal of the dilution of the antibodies. Intensity of the immunochemical reaction is expressed as: (+++) 80 to 100 % of the cells stained, (++) 60 to 80 %, (+) 40 to 60 %, and (−) < 40 %

Titer	Immunofluorescence reactivity					
	Group I		Group II		Group III	
	GA1	GA2	GA7	GA8	GA14	GA15
25	+++	+++	+++	+++	+++	+++
50	++	++	+++	+++	++	++
100	++	++	+++	+++	+	++
200	+	+	++	++	+	+
400	+	+	+	+	−	−
800	−	+	+	+	−	−
1600	−	−	+	−	−	−
2400	−	−	−	−	−	−

Cross-reactivity of the antibodies to other species

From specificity and cross-reactivity experiments it became clear that all selected monoclonals reacted with *Gyrodinium* (cf.) *aureolum* and the morphologically closely related species *Gymnodinium nagasakiense* and *Gymnodinium mikimotoi* (Table 3). The fluorescence labeling pattern of all these species was the same as shown for *G*. cf. *aureolum* (Fig. 1). Other representatives of naked dinoflagellates did not react and antibodies of Group III failed to react with glutaraldehyde-fixed cells (Table 3), as expected from the data presented

Table 2. Comparison of effects of various conservation methods on the immunofluorescence reaction among 6 selected monoclonals (2 of each group) and *Gyrodinium* cf *aureolum*. Antibody dilution 1:50. Intensity is expressed as in Table 1

Fixative	Strength	Immunofluorescence reactivity					
		GA1	GA2	GA7	GA8	GA14	GA15
Formalin	2.0 %	+	+	+++	+++	++	++
Frozen, −80°C	−	+	+	++	++	−	−
Frozen (prefixed)[a]	2.0 %	++	++	+++	+++	++	++
Frozen (prefixed)[b]	0.5 %	+	+	++	++	−	−
Glutaraldehyde	0.5 %	+	+	++	++	−	−
Glutarald./paraformald.	2.0 %	+	+	+++	+++	−	−
Lugol's iodine	4.0 %	−	−	++	++	−	−
Paraformaldehyde	2.0 %	++	++	+++	+++	++	++

[a,b] Prefixation was performed with either [a]paraformaldehyde or [b]glutaraldehyde prior to storage at −80°C

Table 3. Reactivity of 6 selected monoclonal antibodies with different strains of *Gyrodinium aureolum*, the closely related species *Gymnodinium nagasakiense* and *G. mikimotoi*, and other members of both dinoflagellate taxa. Antibody dilution 1:50. Intensity is expressed as: (+++) very intensive, (++) intensive, (+) clearly visible, and (−) not detectable. (GA): prefixed in 0.5% glutaraldehyde solution (see 'Materials and methods')

Species	Strain	Immunofluorescence reactivity					
		GA1	GA2	GA7	GA8	GA14	GA15
***Gymnodinium* spp.**							
G. catenatum	CCMP412	−	−	−	−	−	−
G. catenatum (GA)	D5	−	−	−	−	−	−
G. galatheanum	CCMP415	−	−	−	−	−	−
G. mikimotoi	AUSTRALIA	+	+	+++	+++	+	+
G. nagasakiense	KATSUURA	+	+	++	++	+	+
G. nagasakiense	BUZEN85-2	+	+	++	++	+	+
G. nagasakiense (GA)	G-303-ax2	+	+	++	++	−	−
G. cf. nagasakiense (GA)	TINDUFF	+	+	++	++	−	−
G. simplex	CCMP418	−	−	−	−	−	−
G. varians	CCMP421	−	−	−	−	−	−
Gymnodinium sp.[a]	Y-100	−	−	−	−	−	−
***Gyrodinium* spp.**							
G. aureolum (GA)	CCMP429	+	+	++	++	−	−
G. aureolum (GA)	CCMP430	+	+	++	++	−	−
G. aureolum[b]	PLY497a	++	++	+++	+++	++	++
G. aureolum	SKAGERRAK	++	++	+++	+++	+	+
G. aureolum	TS175GA	++	++	+++	+++	++	++
G. cf. aureolum	IROISE	++	++	+++	+++	++	++
G. instriatum	CCMP431	−	−	−	−	−	−
G. rugosum[a]	GRUGW301	−	−	−	−	−	−
G. uncatenatum	CCMP1310	−	−	−	−	−	−

[a]Species containing endosymbionts
[b]Strains PLY497a and CCMP429 represent the same organism obtained from different laboratories

in Table 2. The specifity of the monoclonals was examined further by testing various phytoplankton species of other taxonomic groups (Table 4). For all the species studied so far, significant labeling was invariably absent. In natural mixed populations, only cells of *G. aureolum* reacted. Occasionally, green autofluorescence of cell walls of small heterotrophic dinoflagellates was observed.

Flow cytometry

The selected monoclonal antibodies were also examined for their use in flow cytometric studies. Labeled cells of *Gyrodinium* cf. *aureolum* were readily distinguished from unlabeled controls in the case of monoclonals of Group II and the pooled serum (red clusters in Fig. 2B, D). For the monoclonals of Groups I & III an insufficient increase of green FITC fluorescence was noticed, resulting in an almost complete overlap of the clusters in bivariate plots of labeled and unlabeled cells (Fig. 2A, C). Histograms of the number of events versus linear green fluorescence revealed that the sep-

aration of the labeled cells from unlabeled controls could readily be observed (Fig. 3). In these histograms labeled cells are easily recognized by their separated peaks for antisera of Group II and the pooled antiserum (arrows in Fig. 3C, D), while the peaks of cells labeled with monoclonals of Groups I & III almost disappeared in the peak of controls (arrows in Fig. 3A, B).

DISCUSSION

For the specific detection of *Gyrodinium* (cf.) *aureolum* 16 monoclonal antibodies directed against cell surface antigens were prepared. By indirect immunofluorescence these monoclonals were divided into 3 different groups based on differences in localization of the fluorescent probe (FITC) after their use (Fig. 1). The major difference between the monoclonals placed in Group I (clones GA1 & GA2) and Group II (clones GA3 to GA8) is that those of Group II were recognized by flagella antigens (Fig. 1C). A granular surface-bound fluorescence pattern was observed using antibodies of Group III (GA9 to GA16); this pattern proba-

Table 4. Cross-reactivity of 6 selected monoclonal antibodies (2 of each group) with various phytoplankton species, as judged from the reaction in an immunofluorescence assay. Antibody dilution 1:50. All strains were fixed with paraformaldehyde. Intensity is expressed as in Table 3. nd: not determined

Species	Strain	Immunofluorescence reactivity					
		GA1	GA2	GA7	GA8	GA14	GA15
Bacillariophyceae							
Asterionella japonica	CCRUGAj	–	–	–	–	–	–
Cyclotella sp.	CCRUGCy	–	–	–	–	–	–
Chaetoceros sp.	CCRUGCh	–	–	–	–	–	–
Ditylum brightwelli	CCRUGDb	–	–	–	–	–	–
Nitzschia closterium	CCRUGNc	–	–	–	–	–	–
Thalassiosira sp.	T135Thsp	–	–	–	–	–	–
T. pseudonana	CCMP1335	–	–	–	–	–	–
T. weissflogii	CCMP1049	–	–	–	–	–	–
Chlorophyceae							
Dunaliella tertiolecta	CCMP1320	–	–	–	–	–	–
Cryptophyceae							
Rhodomonas sp.	CCNIOZ	–	–	–	–	–	–
Cyanophyceae							
Synechococcus	CCMP1334	–	–	–	–	–	–
Dinophyceae							
Alexandrium tamarense	PCC173	–	–	–	–	–	–
A. tamarense	CCMP115	–	–	–	–	–	–
Prorocentrum lima	CCMP685	–	nd	–	–	–	nd
P. micans	T135MC1	–	–	–	–	–	–
P. micans	ELBR.	–	–	–	–	–	–
P. minimum	W40MN1	–	–	–	–	–	–
P. minimum	W40MN2	–	–	–	–	–	–
P. redfeldii	W40RED	–	–	–	–	–	–
Scripsiella trochoidea	OSCHSC1	–	–	–	–	–	–
Haptophyceae							
Chrysochromulina polylepis	CCMP286	–	–	–	–	–	–
Emiliania huxleyi	L	–	–	–	–	–	–
Isochrysis galbana	CCMP1323	–	–	–	–	–	–
Phaeocystis sp. (Antarctica)	Y	–	–	–	–	–	–
Phaeocystis sp. (Antarctica)	W	–	–	–	–	–	–
Phaeocystis sp. (North Sea)	K	–	–	–	–	–	–
Prasinophyceae							
Micromonas pusilla	CCNIOZ	–	–	–	–	–	–
Pyramimonas sp.	FLYNN	–	–	–	–	–	–
P. disomata	E250PD1	–	–	–	–	–	–
Tetraselmis sp.	CCNIOZ	–	–	–	–	–	–
T. suecica	CCNIOZ	–	–	–	–	–	–

bly coincides with the granular-like structures which can be observed at the surface of the cell wall of *G. aureolum*, *Gymnodinium nagasakiense* and *Gymnodinium mikimotoi* by scanning electron microscopy (Partensky et al. 1988, Fukuyo et al. 1990, Vrieling unpubl. results). Further biochemical and immuno-(cyto)-chemical electron microscopical studies are needed to reveal the exact nature and precise location of the cell surface antigens recognized by the different monoclonals.

The monoclonal antibodies were very specific in that they recognized only *Gyrodinium* (cf.) *aureolum*

and the 2 morphologically closely related species *Gymnodinium nagasakiense* and *Gymnodinium mikimotoi* (Tables 3 & 4). The autofluorescence of heterotrophic dinoflagellates observed in field samples must not be interpretated as FITC fluorescence, since it was also observed in controls where the primary antibody was omitted. The strong specificity of the monoclonals implies that *G.* (cf.) *aureolum* and the 2 *Gymnodinium* species possess highly similar cell surface antigens. This suggests a taxonomic proximity or strong phylogenetic relationship of these species, although individual cell sizes, cell cycles and DNA con-
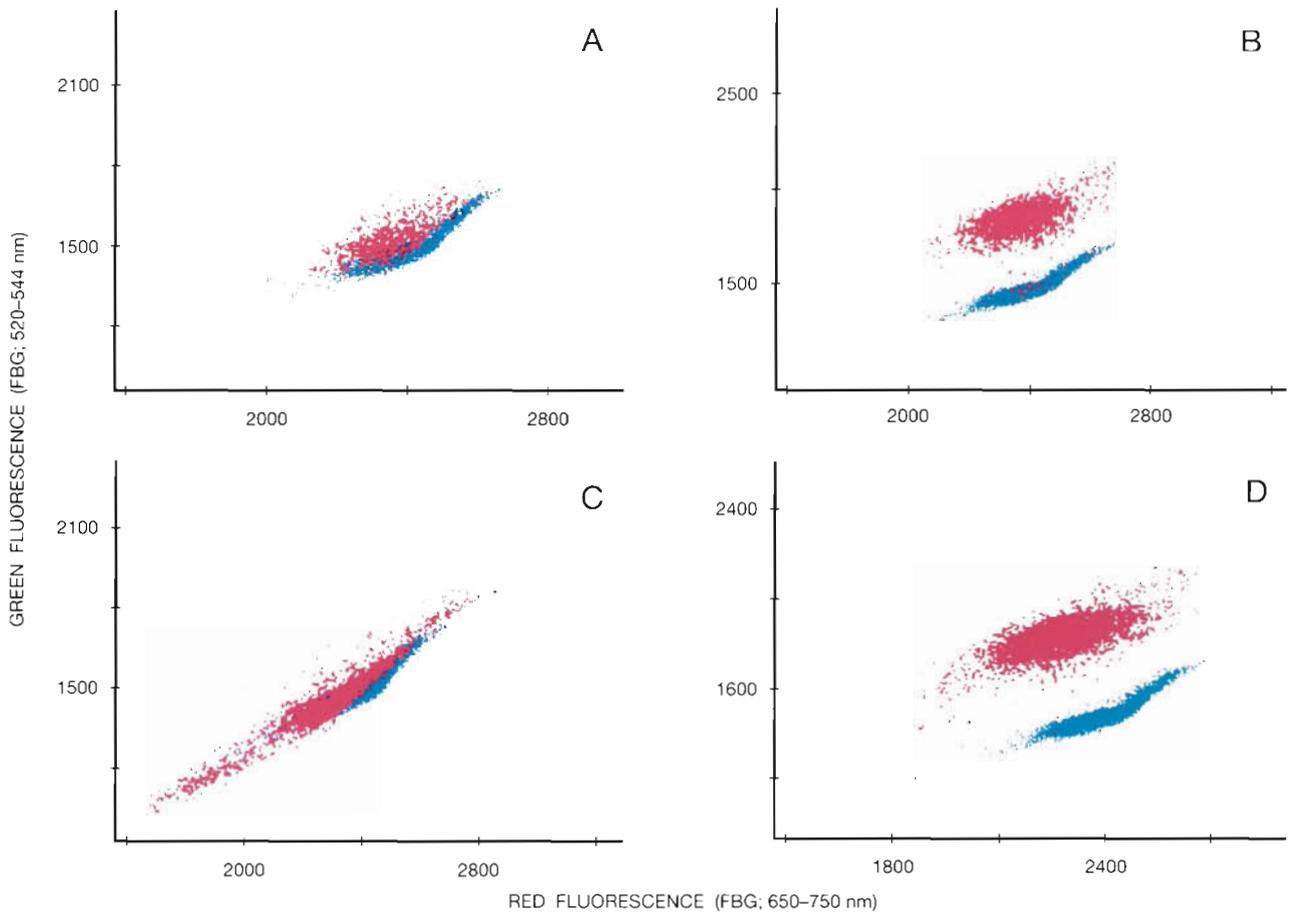
Fig. 2. *Gyrodinium* cf. *aureolum*. Flow cytometric appearance of unlabeled (blue) and labeled (red) cells in bivariate plots of green FITC (FBG; 520 to 544 nm) and red chlorophyll (FBR; 650 to 750 nm) fluorescence. (A) Cells labeled with GA2 (Group I), (B) cells labeled with GA8 (Group II), (C) cells labeled with GA15 (Group III), and (D) cells labeled with a pooled antiserum (combined antibodies of GA2, GA8 & GA15)
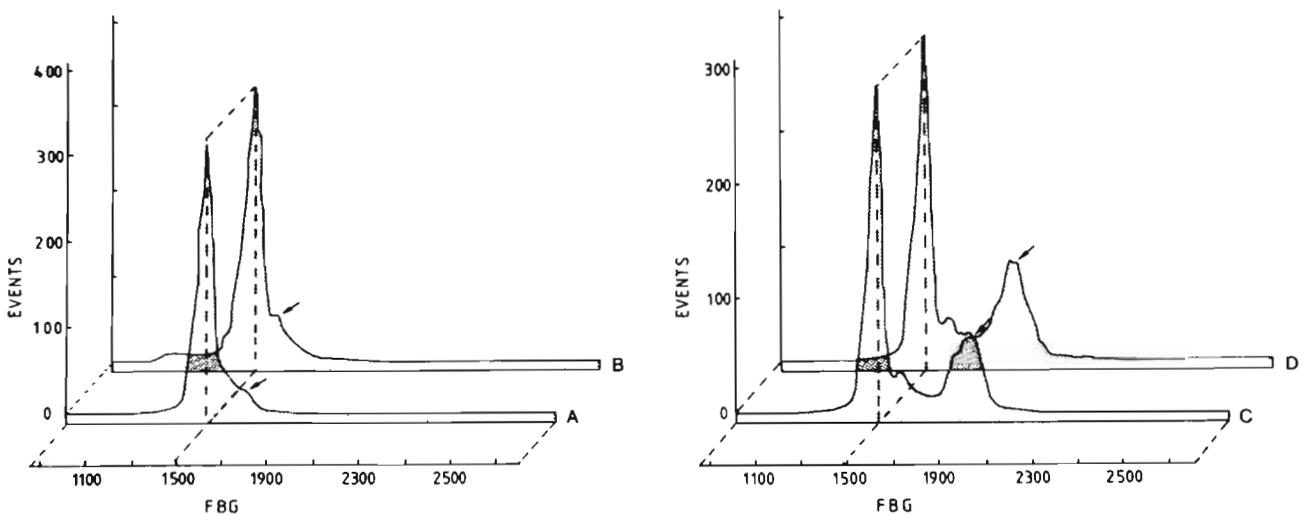

Fig. 3. *Gyrodinium* cf. *aureolum*. Histograms of events versus green FITC fluorescence (FBG) for labeled cells. The first peak of each histogram represents the FBG value of controls (unlabeled cells). The labeling conditions are as follows: (A) cells labeled with antibody GA2 (Group I), (B) cells labeled with GA15 (Group III), (C) cells labeled with GA8 (Group II), and (D) cells labeled with the pooled serum (combined antibodies of GA2, GA8 & GA15). Labeled cells in (A) to (D) are marked with arrows

tents do vary (Partensky et al. 1988, Partensky & Vaulot 1989, Partensky et al. 1991). Similar results have been obtained previously for polyclonal antisera recognizing toxic and non-toxic diatoms of the *Nitzschia* complex (Bates et al. 1993). Monoclonal antibodies prepared against cell surface antigens of *G. nagasakiense* by Nagasaki et al. (1991) showed an exclusive recognition of this species with respect to the species tested in this study, while our antibodies could not distinguish *G.* cf. *aureolum* from *G. aureolum* or the 2 morphologically similar *Gymnodinium* species. However, because of the morphological similarities and the potential toxicity of *G.* (cf.) *aureolum* and both *Gymnodinium* species, the cross-reaction will not have consequences for our monitoring program because these species are considered to be synonyms. The suggestion of a phylogenetic relationship could be tested by examining the homology of evolutionarily highly conserved ribosomal DNA or RNA sequences of *G.* (cf.) *aureolum, G. nagasakiense,* and *G. mikimotoi.* This method has already been used to study the biogeography of macroalgae (Bakker et al. 1992, Kooistra et al. 1992) and more recently to obtain markers for fine-scale analysis of populations and taxonomy of *Alexandrium* (Scholin et al. 1993).

For long-term storage of samples, we confirmed paraformaldehyde to be the best preservative both for indirect immunofluorescence microscopy as found by Campbell et al. (1988) and Bates et al. (1993) and for flow cytometry. Glutaraldehyde fixation not only reduces the binding capacity, resulting in a decrease of fluorescence intensity, but also induces an interfering yellow-greenish background fluorescence (Campbell et al. 1988). Although this background fluorescence does not necessarily hamper the microscopical identification of *Gyrodinium aureolum* (and the phylogenetically related species), it should be avoided when distinction of labeled cells by flow cytometry is pursued. The use of glutaraldehyde may also have other disadvantages, namely: (1) the occurrence of non-specific, non-immunochemical labeling of cells (Shapiro et al. 1989a, Bates et al. 1993) and (2) a decrease in pigment autofluorescence (Hall 1991, Vrieling et al. 1993a), thus resulting in reduction of fluorescence intensities which may fall below the detection limit of a flow cytometer (Landry et al. 1993). As mentioned before (Vrieling et al. 1993a), we use chlorophyll autofluorescence as the triggering parameter in our flow cytometric early warning system.

Traditionally, simple conservation of phytoplankton samples, especially for delayed examination of population composition, is achieved with Lugol's solution. We found that antigenicity was heavily affected for monoclonal antibodies of Groups I & III (Table 2). Only monoclonals of Group II reacted with Lugol-fixed cells,

although the fluorescence intensities decreased (Table 2) as has been shown previously (Anderson et al. 1990, Bates et al. 1993). In this case, microscopical identification and enumeration can be performed easily, while for flow cytometry forward or perpendicular light scattering has to be used as triggering parameter. The latter results in a significant distinction of labeled cells from unlabeled ones (unpubl. results). However, interference of the FITC label with green autofluorescence of cell walls of heterotrophic dinoflagellates (Shapiro et al. 1989b) and particulate matter influence flow cytometric detection, as has been observed in field samples. Storing intact cells by freezing only is not enough to maintain sufficient antigenic sites recognized by monoclonal antisera of Group III. Furthermore, due to the washing steps during the immunochemical procedure, the overall cell morphology is often affected severely and cells may even lose their chloroplasts. Our results indicated that a prefixation step (we prefer paraformaldehyde) is essential prior to freezing of cells in order to create conditions that allow a subsequent convenient flow cytometric analysis; these results are in line with earlier observations by Vaulot et al. (1989).

Finally, previous studies revealed that at the highest fluorescence intensities of the applied probe, identification of species by flow cytometry is unequivocal (Vrieling et al. 1993a). Therefore, we suggest that paraformaldehyde fixation of samples be combined with immunochemical labeling of *Gyrodinium* (cf.) *aureolum* and the 2 phylogenetically related species *Gymnodinium nagasakiense* and *Gymnodinium mikimotoi* followed by immunochemical labeling using monoclonal antibodies of Group II or the pooled antiserum. Routine (immuno)flow cytometric identification and enumeration of these species in mixed populations can be readily done. With regard to efficiency, the monoclonal antibodies of Groups I & III are less convenient for flow cytometric identification, although fluorescence intensities can be enhanced by applying the biotin-streptavidin system (Vrieling et al. 1993a). Nevertheless, both groups of monoclonals are useful for microscopical observations and/or biochemical investigations. The monoclonals of Group III may be especially useful for a study of the nature and significance of the granular-like structures.

In conclusion, the use of immunochemistry in an early warning system for the presence of *Gyrodinium aureolum* in coastal waters appears to be feasible. Even in mixed populations (field samples) *G. aureolum* could be detected properly. Because of the specificity of the monoclonal antibodies which have been prepared, we should be able to use the method to quantify recovery of *G. aureolum* after immunochemical labeling by using spiked populations.

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