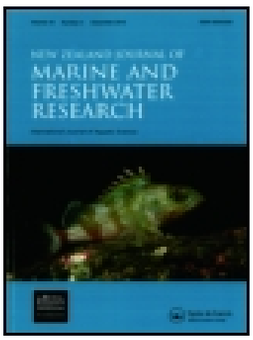

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Immunofluorescent detection of a PSP-producing dinoflagellate, *Alexandrium minutum*, from Bay of Plenty, New Zealand

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Abstract In this study the cross-reactivity of an antibody raised against cell surface antigens of *Alexandrium minutum* Halim, a dinoflagellate isolated from the Bay of Plenty, New Zealand, during the 1993 toxic shellfish outbreaks, was tested on other strains of the same species isolated from a variety of locations, as well as a wide range of unrelated phytoplankton using an indirect immunofluorescence staining technique. The antibody showed positive reactions to all four isolates of *A. minutum* from different localities in New Zealand and one from South Australia. The antibody also showed a positive, but weaker, reaction to *A. ostenfeldii* (two isolates) from New Zealand but no reaction with *A. lusitanicum*, a species which is morphologically similar to *A. minutum*. No cross-reactivity was observed with 51 species or strains from 10 different algal classes tested. The antibody bound to cells of *A. minutum* preserved with either 2.5% glutaraldehyde or 3.5% formaldehyde. Recognition of cells in stationary growth phase, including the encysted form, however, was generally weaker than for cells growing exponentially. The cell surface location of the antigen was confirmed using confocal laser scanning microscopy.

Keywords immunofluorescence staining; antibody; toxic dinoflagellate; *Alexandrium minutum*; Bay of Plenty

INTRODUCTION

Although shellfish poisonings linked to toxic microalgae have affected many shellfish growing countries overseas, the New Zealand shellfish farming industry has been relatively sheltered from such problems until recently. The first shellfish poisonings in New Zealand were reported on the north-east coast in the early summer of 1993 (Chang 1994) when a series of outbreaks resulted in neurological symptoms in more than 180 people lead to a ban of shellfish harvesting throughout New Zealand (Chang et al. 1995).

The 1993 toxic events were notable for the co-occurrence of two major syndromes: neurotoxic shellfish poisoning (NSP) and paralytic shellfish poisoning (PSP) (Chang et al. 1995, 1996). It appears that most of the human shellfish poisonings were attributed to NSP, and the level of PSP detected in shellfish was relatively low (Chang et al. 1995, 1997). Since the 1993 toxic outbreaks, the dinoflagellate *Alexandrium minutum* Halim has been associated with PSP in shellfish in many locations around both North and South Island, New Zealand (Chang 1994; MacKenzie et al. 1994; Chang et al. 1997; MacKenzie & Berkett 1997).

A. minutum was first described in Alexandria, Egypt (Halim 1960) and has now found in several areas in Europe (Montresor et al. 1989), Asia (Chu pers. comm., see Chang et al. 1997), Australia and North America (Hallegraeff et al. 1988, 1991). Before the early 1993 toxic shellfish outbreaks, very little was known about this species in New Zealand. The New Zealand observations add to the known distribution for this species.

A number of *Alexandrium* spp. are morphologically similar to each other, particularly the group of small-celled species which includes *A. minutum*, *A. lusitanicum*, *A. angustitabulatum*, and *A. andersoni* (Balech 1995). Accurate identification of these species relies both on taxonomic skills and access to the time-consuming and inconvenient method of scanning electron microscopy. Since a number of these *Alexandrium* spp. are known to be PSP-producers, it is important to the shellfish industry that a quick and accurate means of identifying these and other species is developed.

In the last decade or so, species-specific immunological and nucleic acid probe techniques (e.g., Anderson et al. 1989; Bates et al. 1993; Sako et al. 1993; Vrieling et al. 1993; Scholin & Anderson 1994; Adachi et al. 1996; Spalter et al. 1997) have been developed to detect targeted phytoplankton species. Among these have been attempts to raise species-specific antibodies against several *Alexandrium* spp. (Adachi et al. 1993; Sako et al. 1993; Aguilera et al. 1996), *Gymnodinium* (Nagasaki et al. 1991; Vrieling et al. 1993, 1994) and other phytoplankton species (Anderson et al. 1989; Nagasaki et al. 1989; Bates et al. 1993; Vrieling et al. 1993) and to use these antibodies as indirect immunofluorescence stains for the identification of these organisms.

In this study an attempt was made to meet the needs of the shellfish industry by exploring a rapid and accurate way of identifying and counting potentially toxic phytoplankton using antibodies raised against cell surface antigens of *A. minutum* (Garthwaite et al. unpubl. data). In this paper we present the results of an extensive examination of the cross-reactivity of one of the antibodies to other phytoplankton species and describe its use in the indirect immunofluorescence labeling of *A. minutum* in a mixed cultures and in field samples collected from Bay of Plenty.

MATERIALS AND METHODS

Cultures

The antibody was raised against cells of a clonal culture of *Alexandrium minutum* Halim (NZBOP006; Chang et al. 1997) isolated from Bay of Plenty, New Zealand. The *A. minutum* cultures used for antibody generation and for the cross-reactivity studies were grown in non-axenic culture in f/2 medium (Guillard & Ryther 1962) at 18°C at a light intensity of 120 $\mu E\ m^{-2}\ s^{-1}$ of Cool-White fluorescent light under a 14:10 h light:dark regime. Cells were harvested and fixed in 1.8% E.M. grade glutaraldehyde, and then concentrated by centrifugation. The concentrated cells were stored refrigerated at 4°C for several weeks before transferring to 5 ml phosphate buffered saline (PBS; 0.02*M* phosphate, 0.15*M* NaCl, pH 7.45) containing 1.8% glutaraldehyde (Anderson et al. 1989).

Other algal cultures used for both specificity and cross-reactivity tests included NIWA's own phytoplankton collection, and those obtained from Cawthron Institute, Nelson, New Zealand, the Bigelow Laboratory of Ocean Sciences, Provasoli-Guillard Center for Culture of Marine Phytoplankton in the United States, and the CSIRO phytoplankton collection in Tasmania, Australia. Most of these cultures were maintained in f/2 medium (Guillard & Ryther 1962), and some in GMP medium (Loeblich 1975).

Polyclonal antibody

Sheep were immunised with preserved *Alexandrium* cells. One of the antibodies obtained for this cross-reactivity study was prepared as described by Anderson et al. (1989) and Garthwaite et al. (unpubl. data). Working stock solutions (Antibody 3730 IgG fraction, 2 mg ml^{-1}) were prepared by diluting 1 part primary antibody to 100 parts PBS (c. 1:100) and tested before being stored at –20°C until needed.

Immunofluorescence assay

A two-step, indirect immunofluorescent staining protocol, essentially as described by Anderson et al.

(1989), was employed to detect the presence of cell surface antigens common to *A. minutum* and other target species. A small volume of *A. minutum* culture (50 ml) either fixed in 2.5% glutaraldehyde or 3.5% formaldehyde were first concentrated by centrifugation (84*g* for 15 min). The cells were resuspended and centrifuged 3 times in 1 ml PBS using an Eppendorf microcentrifuge (Model 5414) (3200*g* for 1 min). 750 µl of 5% Foetal Calf Serum (FCS), the blocking agent, was then added to the pelleted cells. Following a 30 min incubation at room temperature, the cells were centrifuged to remove the FCS, and cells resuspended in 100 µl of the primary antibody (3730) at appropriate dilutions. The cells were incubated with the primary antibody for 30 min at room temperature. After three rinses in 750 µl of PBS, the cells were resuspended in 100 µl of one of the following three secondary antibodies diluted in PBS (1:100): Sigma F5137 anti-sheep IgG-FITC; Sigma F4891 Monoclonal anti-goat IgG-FITC; or Vector FI6000 FITC-Fluorocein anti-sheep for incubation (30 min at room temperature). Negative controls were performed by omitting the primary antibody to estimate non-specific binding of the secondary antibodies. After 2–3 rinses and a final resuspension in 100 µl of PBS, 10 µl of sample was transferred to a 6-well slide with cover slip and examined at 200×, 400×, and 600× magnification using a Nikon Optiphot-2 epifluorescence microscope (Nikon B-2A filter block; excitation filter 450–490 nm; barrier filter 520 nm) with a 100 W mercury lamp. The visual intensity of fluorescent signal in each sample, from weak (+) to moderate (++) to very strong (+++), with a (–) indicating a negative result.

Immunofluorescently-labelled cells were also examined using a Leica DMRBE Confocal Laser Scanning Microscope (CLSM), equiped with epifluorescence (filter combination: excitation BP 488 and 568 nm, barrier filter for FITC (BP-FITC), barrier filter for autofluorescence LP590) and a Krypton argon ion laser. Dual channel scanning was used to record red autofluorescence generated by chlorophyll pigments within cells and the green fluorescence generated from FITC on cell walls from 21 focused sections, and the combined images were subsequently processed according to the TCS Leica Operating Instruction Mannual.

Effects of antibody dilution and cell preservation methods

The most appropriate dilution for the primary and secondary antibodies were determined by running a dilution series in the modified immunofluorescence assay as described above and recording the dilution which first gave unambiguous positive labeling of the target cells and the lowest dilution which gave maximum, or near maximum, fluorescence signal. The range of dilution assays was from 1:25 to 1:8000 for primary antibody, and 1:25 to 1:250 for secondary antibody.

Preservation methods using 2.5% glutaraldehyde, 3.5%, 5% formaldehyde, and 1.0%, 3.6% Lugol's iodine solution were evaluated for their effect on fluorescence signal intensity according to the procedures of Anderson et al. (1989).

Specificity and cross-reactivity tests

Tests on species specificity were carried out using five *A. minutum* clones isolated from both New Zealand and Australia. Tests on cross-reactivity with other phytoplankton species were conducted on 24 species (27 isolates) of the algal class of Dinophyceae, and 22 species (26 isolates) of the other 9 algal classes, viz., Chlorophyceae (1), Prasinophyceae (3), Prymnesiophyceae (5), Chrysophyceae (1), Raphidophyceae (3), Cryptophyceae (2), Eustigmatophyceae (1), Bacillariophyceae (9), and Cyanophyceae (1). All cultures were fixed in 2.5% of glutaraldehyde before the tests, although some were fixed with 3.5% formaldehyde also.

Effects of culture age and tests on field and mixed cultures samples

The effects of culture age on the fluorescence signal was determined by harvesting cells from duplicate batch cultures of *A. minutum* in early to late exponential phase (7–28 days), and at stationary phase (beyond 28 days), fixing in 2.5% glutaraldehyde, staining with fluorescent antibodies as detailed above and scoring the intensity of the fluorescence signal. The usefulness of the fluorescence staining technique for counting field samples was assessed by staining and counting stored field samples (collected during the 1993 toxic outbreaks from Tauranga Harbour and Ohope) (see Chang et al. 1997) and a sample from a mixed culture of *A. minutum*, *Prorocentrum micans*, *Scrippsiella trochoidea*, *Gymnodium* sp., *Chroomonas salina*, *Chaetoceros* sp., and *Asterionella japonica*. Both the field samples and the mixed culture were fixed in 1% Lugol's iodine solution and 2.5% glutaraldehyde respectively. *A. minutum* in 20 random field of views across each counting well were enumerated using standard bright field light microscopy, epifluorescence microscopy, and CLSM after antibody staining.

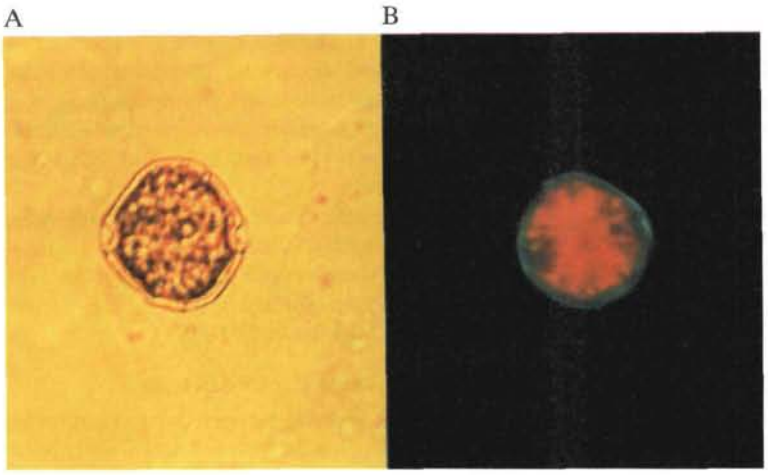

Fig. 1 **A**, Bright field light microscopy of *Alexandrium minutum* cell. **B**, Immunofluorescence label as "halo" ring around *A. minutum* cell using epifluorescence microscopy.

Table 1 Comparison of different preservation techniques on *Alexandrium minutum* using the immunofluorescence assay. (Visual intensity of fluorescent signal in each sample, from weak (+) to moderate (++) to very strong (+++), with a (–) indicating a negative result.)

Preservation method	Strength (%)	Immunofluorescence reaction
Glutaraldehyde	2.5	+++
Formaldehyde	5.0	+
Formaldehyde	3.5	+++
Lugol's iodine	3.6	+
Lugol's iodine	1.0	+

RESULTS

Antibody dilution

Three different secondary antibodies were assessed using a working dilution of 1:50. Sigma F5137 anti-sheep IgG-FITC was found to produce the strongest immunofluorescence signal, intermediate with Sigma F4897 and least with Victor F16000 FITC-Fluorecein anti-sheep antibodies.

The maximum dilution of the primary antibody resulting in visible fluorescence around the outer edge of the cell was 1:1500. Dilution of 1:500 and 1:250 gave signals of moderate intensity but a dilution of 1:100 was required to maximise the fluorescence signal and was subsequently used for the cross-reactivity studies.

Cell preservation

Cells of the thecate dinoflagellate *A. minutum* were preserved equally well in shape and structure in three commonly used fixatives: 2.5% glutaraldehyde, 3.5%, 5% formaldehyde, and 1%, 3.6% Lugol's iodine solution. Under the epifluorescence microscope, the intensity of the fluorescent signal generated by immunofluorescently labeled cells was greatest with 2.5% glutaraldehyde and 3.5% formaldehyde (Table 1). The difference in fluorescence generated by cells fixed in these two fixatives was minimal. Cells fixed in 5.0% formaldehyde gave markedly lower (+ versus +++) fluorescence signals (lower antibody binding) than cells fixed in 3.5% formaldehyde as did cells fixed in Lugol's iodine, whether freshly fixed or fixed for several months. Lugol's iodine darkens cells and this can be overcome by adding 1–2 drops of 10% sodium thiosulphate to the samples.

Specificity and cross-reactivity tests

Under the epifluorescence microscope, a bright green "halo" was clearly visible at the perimeter of positively immunofluorescently-labelled cells (Fig. 1). This contrasts with the background orange-red autofluorescence generated by chlorophyll pigments

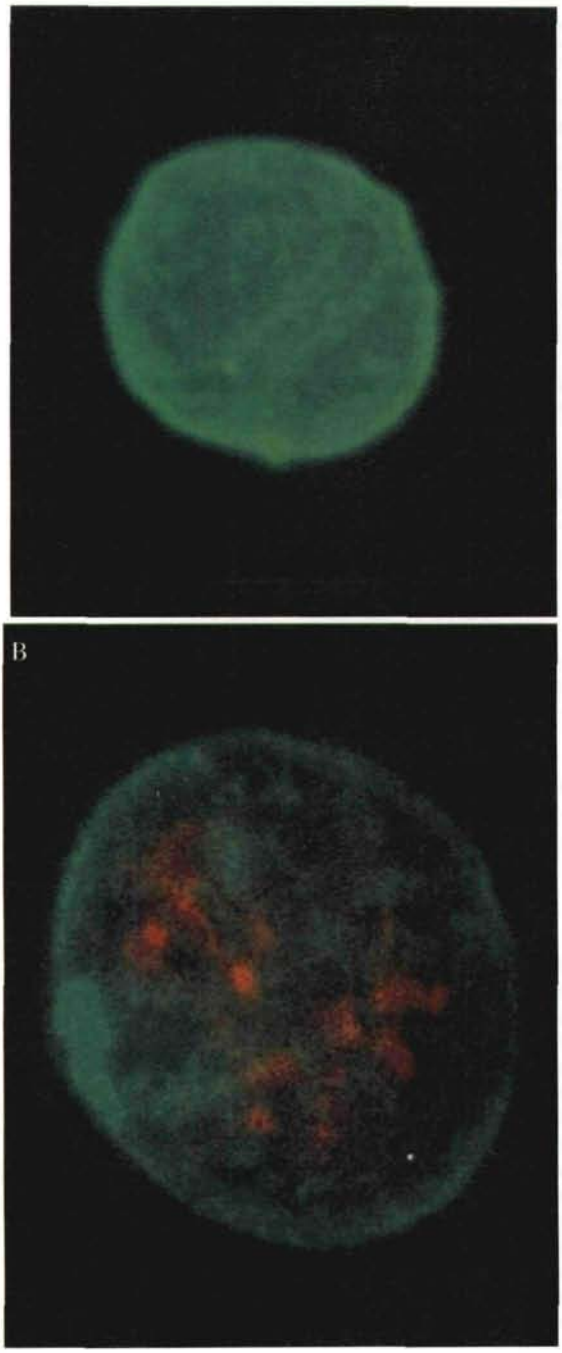

Fig. 2 Processed images of an immunofluorescently labelled *Alexandrium minutum* cell using a confocal laser scanning microscope. **A**, Green fluorescence on the outer cell wall alone (with FITC channel). **B**, Green surface fluorescence, with a suppressed red autofluorescence within the cell (with both channels).

within the cell. The immunofluorescent label on the cell wall was also examined using CLSM. Cells labelled with antibody were focused section by section, at different depths. Green and red fluorescence generated from cells could be discriminated by filters. This made it possible to show either a strong green fluorescence on the outer cell wall alone (Fig. 2A), or the cell with green fluorescence at the edge of the cell, with suppressed autofluorescence from within the cell (Fig. 2B).

The antibody raised against the Bay of Plenty *A. minutum* isolate (clone NZBOP006), reacted with all of the *A. minutum* isolates from New Zealand (clones NZBOP014, Cros 1, Anakoha A, and CS-323) and the isolate from south Australia (Table 2). All but one (Cros 1) of the five clones showed strong, positive immunofluorescence signal demonstrating high antibody binding. Cros 1 was labeled by the antibody but with a lesser signal intensity.

A total of 53 other species/strains representing 10 algal classes were also tested for cross-reactivity with the antibody. Of all the seven *Alexandrium* species/strains tested (other than *A. minutum*), only the two New Zealand *A. ostenfeldii* isolates (Timaru, Cros 2) were recognised by the *A. minutum* antibody, giving a positive but weaker fluorescence signal than the target species (Table 3). It is notable that *A. lusitanicum*, a species not easily distinguishable morphologically from *A. minutum*, was not recognised by the antibody. The antibody showed no cross-reactivity to any of the species from all the other algal classes tested (Table 4).

Effect of culture age on immunofluorescence

Cells from early to late exponential growth (7–21 days) in batch culture showed the strongest immunofluorescence signal indicating greatest antibody binding to these cells (Table 5). Cells in the stationary growth phase (beyond 28 days), including the encysted form, generally showed a weaker immunofluorescence signal (less antibody-bound) than cells growing exponentially.

Tests on field and mixed culture samples

Tests conducted on field and mixed culture samples demonstrated that it was easier to positively identify *A. minutum* cells and discriminate them from non-target species using the indirect immunofluorescence staining technique in combination with either epifluorescence microscopy or CLSM than by using direct bright-field microscopy. This led to higher estimates of *A. minutum* cell numbers in the limited number of samples tested (Table 6).

Table 2 Immunofluorescent labelling of *Alexandrium minutum* isolate from both New Zealand and Australia. (Visual intensity of fluorescent signal in each sample, from weak (+) to moderate (++) to very strong (+++), with a (–) indicating a negative result.)

Species	Isolates	Location isolated	Antiserum 3730
A. minutum	NZBOP006	Bay of Plenty, New Zealand	+++
A. minutum	NZBOP014	Bay of Plenty, New Zealand	+++
A. minutum	Cros1	Croisilles Harbour, New Zealand	++
A. minutum	Anakoha A	Anakoha Bay, New Zealand	+++
A. minutum	CS-323	Port River, South Australia	+++

Table 3 Immunofluorescent reactions of *Alexandrium minutum* congeners. (Visual intensity of fluorescent signal in each sample, from weak (+) to moderate (++) to very strong (+++), with a (–) indicating a negative result.)

Species	Isolates	Isolator	Location	Antiserum 3730
A. affine	CS-312	S. Blackburn	Tasmania, Australia	–
A. catenella	BOP	L. MacKenzie	Bay of Plenty, New Zealand	–
A. lusitanicum	CS-320	I. Bravo	Ria de Vigo, Spain	–
A. margalefii	Bream	L. MacKenzie	Bream Bay, New Zealand	–
A. ostenfeldii	Timaru	L. MacKenzie	Timaru, New Zealand	++
A. ostenfeldii	Cros2	L. MacKenzie	Croisilles Harbour, New Zealand	++
A. tamarense	CAWD20	–	Plymouth, England	–

Table 4 Representatives of phytoplankton species in major algal classes.

Division, class, species	Isolates	Isolator	Location	Antiserum 3730
Pyrrhophycophyta				
Dinophyceae				
Thecate				
Amphidinium carterae	NIWA101	H. Chang	Big Glory Bay, New Zealand	–
Amphidinium klebsii	CCMP1342	J. Bomber	Flori da, United States	–
Ceratium fusus	CCMP1758	E. Simons	Boothbay, United States	–
Coolia monotis	CCMP304	Steidinger	Ria de Vigo, Spain	–
Coolia monotis	NIWA102	H. Chang	Rarawa Beach, New Zealand	–
Gonyaulax polyedra	CCMP1738	B. Sweeney	La Jolla, United States	–
Gymnodinium catenatum	CS-301	Blackburn	Tasmania, Australia	–
Gymnodinium catenatum	CCMP414	I. Bravo	Ria de Vigo, Spain	–
Heterocapsa triquetra	CCMP448	L. Brand	Falmouth, United States	–
Ostreopsis siamensis	NIWA102	H. Chang	Rarawa Beach, New Zealand	–
Peridinium foliaceum	CCMP626	A. Dodson	Puerto Rico	–
Prorocentrum lima	NIWA112	H. Chang	Rararwa Beach, New Zealand	–
Prorocentrum micans	NIWA021	H. Chang	Westland, New Zealand	–
Protoperidinium sp.	NIWA054	H. Chang	Hauraki Gulf, New Zealand	–
Scrippsiella trochoidea	NIWA056	H. Chang	Hauraki Gulf, New Zealand	–
Scrippsiella trochoidea	CCMP1331	M. Parke	–	–
Non-thecate				
Gymnodinium breve	CCMP718	–	Florida, United States	–
Gymnodinium mikimotoi	–	H. Takayama	Japan	–
Gymnodinium sp. (large)	NIWA033	H. Chang	Hauraki Gulf, New Zealand	–
Gymnodinium sp. (small)	NIWA036	H. Chang	Hauraki Gulf, New Zealand	–
Gyrodinium aureolum	CCMP429	P. Holligan	Plymouth, England	–

Table 4 (*continued*)

Division, class, species	Isolates	Isolator	Location	Antiserum 3730
Chlorophycophyta				
Chlorophyceae				
Dunaliella tertiolecta	NIWA059	–	–	–
Prasinophyceae				
Halosphaera viridis	NIWA025	H. Chang	Westland, New Zealand	–
Tetraselmis chuii	NIWA058	–	–	–
Tetraselmis suecica	NIWA056	–	–	–
Chrysophycophyta				
Prymnesiophyceae				
Chrysochromulina polylepis	CCMP286	L. Edler	Kristineberg, Sweden	–
Isochrysis sp.	NIWA053	–	Tahiti	–
Pavlova luthei	NIWA054	–	–	–
Prymnesium calathiferum	NIWA011	H. Chang	Bream Bay, New Zealand	–
Prymnesium parvum	CCMP708	M. Droop	Gouldsboro Bay	–
Chrysophyceae				
Pelagococcus subviridis	CS-99	J. Stauber	Port Hacking, Australia	–
Raphidophyceae				
Chatonella antiqua	CCMP	T. Okaichi	Harima, Japan	–
Fibrocapsa japonica	NIWA071	H. Chang	Hauraki Gulf, New Zealand	–
Heterosigma akashiwo	NIWA022	H. Chang	Big Glory Bay, New Zealand	–
Cryptophyceae				
Chroomonas salina	NIWA018	H. Chang	Wellington Harbour, New Zealand	–
Chroomonas pauciplastida	CCMP268	L. Provasoli	Woods Hole, United States	–
Eustigmatophyceae				
Nannochloropsis oculata	NIWA051	–	–	–
Bacillariophyceae				
Amphora coffaeformis	CCMP126	T. Galveston	–	–
Asterionella japonica	CS-135	J. ReRoa	Tasmania, Australia	–
Chaetoceros muelleri	NIWA066	–	–	–
Chaetoceros acttois	NIWA065	–	–	–
Corethron hystrix	CCMP307	–	–	–
Cyclotella meneghiniana	CCMP334	Kilham	Yugoslavia	–
Navicula pelliculosa	CCMP543	R. Guillard	Oyster Pond, United States	–
Stephenopyxis turris	CS-100	L. Provosoli	La Jolla, United States	–
Thalassiosira decipiens	CCMP983	A. Dodson	Magdalena Bay, United States	–
Cyanophyceae				
Synechococcus sp.	NIWA077	H. Chang	Big Glory Bay, New Zealand	–

Table 5 Antibody binding to *Alexandrium minutum* at different stages of growth. (Visual intensity of fluorescent signal in each sample, from weak (+) to moderate (++) to very strong (+++), with a (–) indicating a negative result.)

Culture age (days)	Immunofluorescence score
7	+++
21	+++
28	++
35	++

DISCUSSION

This study confirmed that the antibody raised against cell surface antigens of a Bay of Plenty isolate of *A. minutum* is highly species-specific. Four strains isolated from three localities in New Zealand (Bay of Plenty, Anakoha Bay, Crosilles Bay) and one from south Australia all gave strong positive fluorescence scores with the antibody, although in a separate test carried out by Garthwaite et al. (1996) using the

same antibody, an isolate of *A. minutum* from Morlay Bay, France was only weakly bound by the antibody.

The spectrum of toxins produced by the Morlay Bay *A. minutum* isolate, primarily C1, C2, and GTX2,3 (Ledoux pers. comm., see Chang 1997) is quite dissimilar to the toxin spectrum for the five New Zealand *A. minutum* isolates, which is primarily neosaxitoxin, GTX1,4, and GTX2,3, and to the toxin spectrum produced by two isolates from Australia, two from Taiwan, one from Portugal, and three from Spain, which is primarily GTX1,4, and GTX2,3 (Chang et al. 1997). This difference in the toxin spectrum and the weak recognition of the Morlay Bay *A. minutum* isolate by the *A. minutum* antibody raises the questions as to whether different strains of *A. minutum* produce slightly different antigens or whether this Morlay Bay isolate is different from the New Zealand species.

Balech (1995) groups the three other small *Alexandrium* species, notably *A. angustitabulatum*, *A. andersoni*, and *A. lusitanicum* with *A. minutum* in his monograph. Morphologically these species are almost indistinguishable. For example, the presence of a ventral pore in the first apical (1′) plate separates *A. minutum* from *A. angustitabulatum*. The latter species apparently lacks this feature. *A. andersoni* is differentiated from *A. minutum* and other species within the group because of its narrow plate 6″. However, *A. lusitanicum* is very similar to *A. minutum*, and according to Balech (1995) the differences are so small that their independence is doubtful. Accurate identification of these species relies on taxonomic skill and access to expensive tools such as the scanning electron microscope (SEM). There is no quick and easy way of identifying closely related species using light microscopy alone.

Based on the results of this study, it is clear that this group of small-celled species can be separated using indirect immunofluorescence staining. In this study *A. lusitanicum* was not recognised by the antibody raised against *A. minutum*, suggesting that the former species is distinct. Moreover, we have also found that the antibody does not recognise *A. andersoni* (Garthwaite et al. 1996) implying that *A. andersoni* is not con-specific with *A. minutum* either, supporting the finding made by Scholin et al. (1994) using ribosomal RNA sequence analysis. No *A. angustitabulatum* was available for testing in this study. It is thus clear from this work that the antibody raised against *A. minutum* can be used to distinguish at least the two species, *A. lusitanicum* and *A. andersoni*, from *A. minutum*.

The moderate cross-reactivity to *A. ostenfeldii* found in this study and the weak cross-reactivity to one of three strains of *A. catenella*, and one of five strains of *A. tamarense* found by Garthwaite et al. (1996) is not a problem when using the antibody to label *A. minutum* cells for enumeration by epifluorescence microscopy. The weak cross-reaction observed in these species can be eliminated by using a greater dilution of the primary antibody. In addition, cells of *A. ostenfeldii*, *A. catenella*, and *A. tamarense* are larger than those of *A. minutum*, thus it is not likely that cells of these species would be confused with those of *A. minutum*.

The moderate cross-reaction that the antibody exhibited to *A. ostenfeldi* indicates that there may be a closer genetic affinity between *A. minutum* and *A. ostenfeldii* than between *A. minutum* and other *Alexandrium* species. This is consistent with observations made by Spalter et al. (1997) who used ribosomal DNA (rDNA) sequences to demonstrate that *A. ostenfeldii* has a closer relationship to *A. minutum* than to any of the other four New Zealand *Alexandrium* spp. tested (*A. margelefii*, *A. tamarense*, *A. catanella*, *A. affine*). In the Spalter et al. (1997) rDNA sequencing studies, *A. margelefii*

Table 6 Comparison of enumeration of field samples, and mixed culture spiked with *Alexandrium minutum* using standard bright field light microscopy, immunofluorescence epifluorescence microscopy, and confocal laser scanning microscopy (CLSM).

	Concentration of *A. minutum* ($\times 10^3$ cells litre^{-1})		
		Immunofluorescence labelled	
Samples	Bright field	Epifluorescence	CLSM
Field			
Tauranga Harbour	12	14	–
Ohope Beach	10	12	–
Mixed culture	16	18	18

was found to be closer to both *A. minutum* and *A. ostenfeldii* than the other three *Alexandrium* spp. isolated from New Zealand. In our study, no cross-reaction between cells of *A. margalefii* and the antibody raised against *A. minutum* were observed.

Evaluation of preservatives used for fixing the *A. minutum* cells showed that all three preservatives (at different concentrations) were satisfactory in maintaining the general shape and structure of the cell. Strong glutaraldehyde (2.5%) and formaldehyde (3.5%) concentrations were used for preservation with equal success. Although Bates et al. (1993) demonstrated stronger immunofluorescent labelling of *Pseudonitzschia pungens* with paraformaldehyde than with glutaradehyde, they used more dilute glutaraldehyde (0.5%) in their study. In contrast to non-specific labelling as observed by Campbell et al. (1988) and Shapiro et al. (1989) in cells preserved with glutaraldehyde, cells of *A. minutum* fixed in all three preservatives showed specific staining with the antibody produced for this study.

The antibody generally displayed better binding to cells of *A. minutum* in exponential phase than to cells in late stationary phase. This is consistent with the small drop in the immunofluorescence signal reported by Sako et al. (1996) in their study of the labeling of cells of *A. tamarense* in different stages of growth. In another study of *A. tamarense*, significant decreases in immunofluorescent labeling intensity were observed in plateau versus exponential phase cells in batch culture (D. M. Anderson pers. comm.). As nutrient concentrations started to decline in our study, cells of *A. minutum* in late stationary phase went through different stages of the encystment process. The generally weaker reaction observed in cells during the late stationary phase is thus likely to be an indication of the qualitative and quantitative changes of cell surface (antigens) over time, as suggested by Bates et al. (1993). Although this might be a problem when attempting to quantify the number of cells present on the basis of the strength of the immunofluorescence signal in a fluorimeter it is not a problem when using the antibody to stain individual cells for direct counting using epifluoresence microscopy.

It is clear that our replicate immunofluorescent counts made using either epifluorescence microscopy or CLSM are reasonably consistent with each other across a range of cell concentrations from mixed cultures, and were higher than from most counts made with bright field light microscopy. Enumeration of *A. minutum* cells from two Bay of Plenty field samples were also made easy and with greater estimates of *A. minutum* cells using epifluorescent staining technique than with the bright field light microscopy. This is consistent with observations made by Anderson et al. (1989) in their enumeration of *Aureococcus anophagefferens* cells. In their studies a greater accuracy of cell counts was also suggested using immunofluorescent staining technique than the standard bright field light microscopy.

In summary, this study demonstrates that an antibody developed against *A. minutum* can discriminate the target species from closely related and morphologically similar *Alexandrium* spp. as well as all other phytoplankton species. The indirect, whole cell immunofluorescent staining is shown to be a relatively simple detection technique which offers an accurate alternative to bright field microscopy. This immunofluorescence technique provides for more rapid and accurate cell identification and enumeration. The ability to focus at different sections with two channels using CLSM also made it possible to show the immunofluorescent label on the outer cell wall of *A. minutum*.

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