J Appl Phycol (2007) 19:325–332
DOI 10.1007/s10811-006-9139-2

Development of an immunofluorescence technique for detecting *Prorocentrum donghaiense* Lu

Da-Zhi Wang · Xu-Guang Huang
Leo Lai Chan · Hua-Sheng Hong

Received: 9 May 2006 / Revised and accepted: 18 October 2006 / Published online: 19 January 2007

Abstract *Prorocentrum donghaiense* Lu is a key harmful algal bloom (HAB) species which is widespread along the China Sea coast. In this study, *P. donghaiense*-specific antiserum was developed, and the detection method, based on immunofluorescence (IF), was optimized. The antiserum was raised using 0.5% paraformaldehyde-fixed whole cells (WC) as antigen. The titer and the specificity were examined using whole-cell IF. Results showed that ethanol was an effective decolorization reagent, and 80% ethanol was able to minimize autofluorescence of cells. Samples preserved by freezing at −20°C or −80°C remained above 85% detection efficiency after 1-month storage. The antiserum against WC had a high titer (1:10000), and exhibited high specificity at species level. The antiserum showed a weak cross reaction with the closely related species *P. dentatum* HK, *P. dentatum* CCMP1517 and *P. minimum* HK only at very low dilution (1:5). However, it did not cross-react with the species from the same genus or other phytoplankton species when the dilution reached or exceeded 1:100. Results from different *P. donghaiense* samples collected at different growth phases or grown under different nutrient conditions showed no significant difference in IF intensity. In addition, the antiserum exhibited high specific binding to *P. donghaiense* in both mixed phytoplankton samples and field samples. The results indicate that the technique is a potentially useful tool for the rapid identification of *P. donghaiense* and can facilitate the analysis of various natural samples.

Key words *Prorocentrum donghaiense* Lu · Antiserum · Immunofluorescence

D.-Z. Wang (✉) · X.-G. Huang · H.-S. Hong
State Key Laboratory of Marine Environmental Science/
Environmental Science Research Center,
Xiamen University, Xiamen 361005, China
e-mail: dzwang@xmu.edu.cn

L. L. Chan
Department of Ecology and Biodiversity,
The University of Hong Kong,
Hong Kong, HKSAR, China

Introduction

Prorocentrum donghaiense Lu, a key harmful algal bloom (HAB) species in China Sea coastal waters, was first reported and identified by Lu and Goebel (2001). In the past few years, this species has caused extensive blooms in the East China Sea, and has become of economic and public concern due to its impact on the marine ecosystem, aquaculture and public health (Zhou et al., 2003). Therefore, it has become essential to monitor *P. donghaiense*, to provide important data for assessment and early warning of the hazards of *P. donghaiense* to fisheries and aquaculture.

Generally, the use of light-microscopy is sufficient to classify and enumerate *P. donghaiense* from field samples. However, this becomes difficult when *P. donghaiense* is only a minor component of planktonic

assemblages, or when trying to distinguish between morphologically similar species or strains, such as *P. minimum, P. dentatum* and *P. micans*. Moreover, this method involves training and experience and is time-consuming and labor-intensive, especially when large numbers of samples are to be analyzed. This presents serious problems for routine monitoring programs, and diagnostic tools are needed urgently to identify, enumerate and separate cells of this species from mixed phytoplankton samples. One alternative is the use of molecular or cellular probes to reveal target species.

Antibody probes have shown great potential to advance the pace of harmful algal research and monitoring (Anderson 1995; Vrieling and Anderson 1996), and indirect immunofluorescence assays using polyclonal antibodies have become the most common technique used to identify harmful algae, and to distinguish toxic and non-toxic harmful algal species. Thus, Anderson et al. (1990) used species-specific polyclonal antibodies to successfully detect the brown tide Chrysophyte *Aureococcus anophagefferens;* Bates et al. (1993) developed a specific polyclonal antiserum to distinguish toxic *Pseudonitzschia multiseries* and non-toxic *P. pungens*; and Lopez-Rodas and Costas (1999) investigated morphospecies and strains of *Prorocentrum* using polyclonal antisera. Recently, Lin et al. (2003) developed an immunofluorescence technique to detect *Pfiesteria piscicida*. Monoclonal antibodies have also been applied in distinguishing harmful and toxic algal species (Sako et al. 1993; Adachi et al. 1993; Lopez-Rodas and Costas 1997).

In this study, antiserum against cell-surface antigens was produced using 0.5% paraformaldehyde-fixed whole cells of *P. donghaiense*, and the titer and specificity were examined. An IF protocol based on antiserum was optimized for samples taken from cultures at various growth phases and under various culture conditions, as well as mixed and field samples. The goal of this study is to establish a species-specific detection method for research into, and monitoring of, *P. donghaiense*.

Material and methods

Algal cultures

P. donghaiense Lu was isolated from the East China Sea in April, 2001 and the strain (*A. donghaiense* PR0201-01) was routinely maintained in natural seawater supplemented with f/2-Si nutrients at 20°C, 60 μE m^{-2} s^{-1} light intensity and a 12 h:12 h light: dark cycle.

For antigen preparation and series experiments, *P. donghaiense* was grown in 5000-ml conical flasks containing 4000 ml of f/2-Si medium under the same regime as above, except that the light intensity was adjusted to 100 μE$m^{-2}s^{-1}$. Cell growth was monitored by counting cells daily under a microscope. When the cultures reached their highest density (the early stationary phase), cells were harvested using centrifugation (8000 rpm for 10 min).

Production of polyclonal antiserum

Approximately 10^7 cells were collected using centrifugation for each antigen preparation, and antigens were prepared as the whole cell antigen (WCA), by resuspending the pelleted cells in 0.5% paraformaldehyde (PFA) prepared with phosphor buffered saline (PBS), and then storing them in 15-ml microcentrifuge tubes at 4°C prior to immunization.

Antigen materials preserved in 0.5% PFA were washed five times with PBS and then resuspended in PBS. Four New Zealand white rabbits were immunized for each antigen preparation. For initial inoculation, 10^7 cells, in 1-ml PBS, were mixed with 1 ml of Complete Freund's Adjuvant (from Sigma) and injected at multiple sites: subcutaneously in the inguinal and axillary regions, and intramuscularly in the hind limbs. Subsequent boosts were made intravenously on days 14, 21, 35 and 49, using the same amount of antigen as for the initial inoculation. Test bleeds were collected on day 40, and a final bleed was obtained 10 days after the final boost (Lin et al. 2003).

Immunofluorescence protocol

About 5×10^3 cells were collected from each sample using centrifugation. The pellets were washed with 80% ethanol to extract chlorophyll, so as to avoid auto-fluorescence. Then the pellets were rinsed five times with PBS to remove the ethanol. Incubation with the primary test antiserum was performed at 37°C for 1 h. The different dilutions were designed to obtain a constant primary antibody/cell ratio. After rinsing five times with PBS, the binding activity of

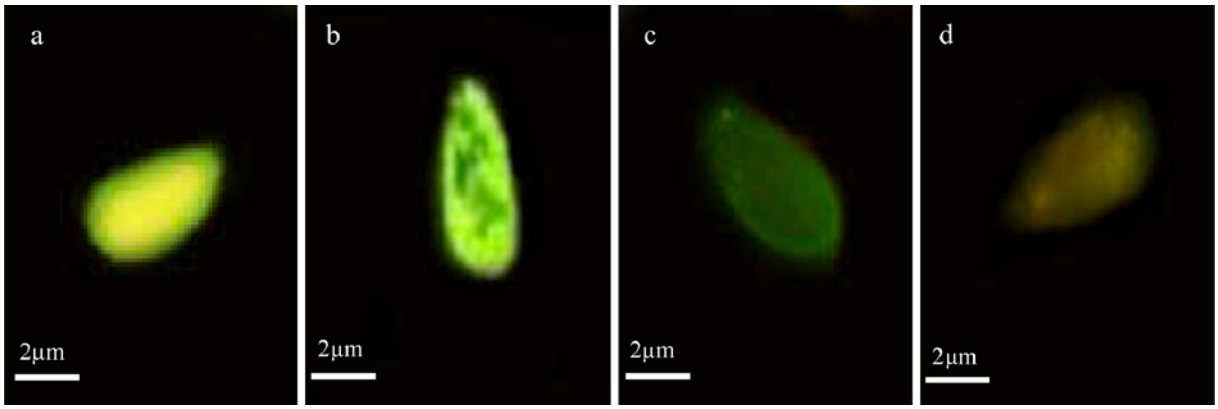

Fig. 1 Labeling intensity of *P. donghaiense* cells at different dilutions of antiserum. **a** <1:500. **b** 1:1000–1:2500. **c** 1:5000–1:10000. **d** >1:10000

the antiserum was gauged using a secondary fluorescein isothiocyanate (FITC)-conjugated goat anti-rabbit antibody (from Sigma) employed at 1.631 mg/ml for 1 h at 37°C. After rinsing five times with PBS, the samples were examined for the quality and quantity of antibody binding, as described previously (Lopez-Rodas and Costas 1997). The quality of stain was estimated using epifluorescence in a Zeiss Axioshop microscope (Carl Zeiss Ltd.) with a filter set (450–495 nm excitation, 520–560 nm emission) using the following scale: (3+) bright stain, 100% of cells stained; (2+) medium stain, 100% of cells stained; (+) low stain but obviously different from controls, and less than 100% of cells stained; (−) non-detectable reaction. All tests were read 'blind', that is, the person reading the test did not know the identity of the tested material.

Decolorization of cultured samples

Three organic solvents were tested for decolorization of the cultured samples fixed with 0.5% paraformaldehyde: (1) 80% ethanol, (2) 80% acetone, and (3) 80% methanol. After incubation with the above solvents for 5, 10, 20, 60 and 120 min at ambient temperature, each sample was analyzed for decolorization efficiency using an IF protocol as outlined below. A sample incubated with water served as the comparison.

Protocol for mixed and field collected samples

In this experiment, *P. donghaiense* cells were mixed with various phytoplankton species, such as *Skeletonema costatum, Alexandrium catanella, Thalassiosira weissflogii, P. dentatum CCMP1517* and *P. micans,* and the detection efficiency was tested using the established IF protocol as the following:

$$Detection\ efficiency = C_{IF}/C_{LM}$$

C_{IF} = Cell number under the fluoroscope microscopy, C_{LM} = Cell number under the light microscopy.

Meanwhile, the detection efficiency of field samples collected from the East China Sea and Xiamen Harbor were also investigated. Briefly, an appropriate volume of seawater was filtered through a 1-μm polycarbonate membrane, and the cells were rinsed and resuspended in 5 ml seawater. Next the cells were fixed in 0.5% buffered paraformaldehyde, then shaken in 5 ml 80% ethanol for 2 h, collected using centrifugation, and rinsed five times with PBS to remove PFA. After decolorization, the cells were incubated with primary and secondary antibodies sequentially, and the detection efficiency was tested.

Results

High titer and specificity antiserum produced with whole-cell antigen

The titer and specificity of the antiserum raised with WC antigen were examined using IF (Fig. 1). When the dilution was below 1:500, there was an intense immunofluorescence reaction (3+) (Fig. 1a). When the dilution increased from 1:1000 to 1:2500, there was a moderately intense immunofluorescence reac-

Table 1 Cross reaction of different phytoplankton species/strains and genera with antiserum against *P. donghaiense*

Species/strains	Dilution	
	1:5	1:100
Prorocentrum dentatum HK	+	–
P. dentatum CCMP1517	+	–
P. triestinum HK1	–	–
P. triestinum HK2	–	–
P. minimum HK	+	–
P. micans DYW	–	–
P. donghaiense	3+	3+
Alexandrium tamarense	–	–
A. catenella	–	–
A. minutum	–	–
Karenia brevis	–	–
Dunaliella salina	–	–
Chlorella sp.	–	–
Dicrateria zhangjianggensis	–	–
Phaeodactylum tricornutum	–	–
Chaetoceros sp.	–	–
Skeletonema costatum	–	–
Thalassiosira weissflogii	–	–
T. pseudonana	–	–

Labeling intensity detected by immunofluorescence, 3+ = intense label; 2+ = moderate label; 1+ = low intensity; – = nondetectable reaction.

tion (2+) (Fig. 1b). From 1:5000 to 1:10000 dilution, the samples showed a weak immunofluorescence reaction (+) (Fig. 1c) and, with further dilution, IF exhibited a non-detectable reaction (−) (Fig. 1d). An antiserum dilution of 1:10000 was the lowest concentration that produced positive labeling.

Results of the test for specificity and cross reactivity against 19 phytoplankton species or strains representing 10 genera are shown in Table 1. The antiserum against *P. donghaiense* showed specificity at species level. It did not cross-react with cells from relatively closely related species nor cells from the Bacillariophyceae, Chlorophyceae or Cryptophyceae, although it exhibited a weak cross reaction (1+) with *P. dentatum* HK, *P. dentatum* CCMP1517 and *P. minimum* HK at very low dilution (1:5). When the dilution reached or exceeded 1:100, the cross-reactions disappeared.

Decrease of autofluorescence

The cells of *P. donghaiense* produce intense red autofluorescence due to chlorophyll under epifluorescence microscopy (Fig. 3a), and this masks the green fluorescence produced by FITC. Complete extraction of chlorophyll was thus necessary before analysis under an epifluorescence microscope. In this study, the decolorization efficiencies of three organic solvents—80% ethanol, 80% acetone and 80% methanol—were examined (Fig. 2). Ethanol and acetone were found to decrease the autofluorescence more rapidly than methanol and, in both, about 70–80% of chlorophyll was extracted within 60 min, so that almost no red autofluorescence was observed under epifluorescence microscopy (Fig. 3c and d). However, intense green fluorescence was detected only in the targeted cells extracted with 80% ethanol. The efficiency of cell extraction with methanol was very low; only 60% of chlorophyll was extracted within 60 min, and red fluorescence was still observed in some cells (Fig. 3b).

Preservation method

To examine the stability of preserved samples, four preservation methods were examined. One sample without fixation was stored at 4°C; three other samples fixed with 0.5% paraformaldehyde were stored at 4°C, −20°C and −80°C respectively for 1 month. The detection efficiency decreased significantly in both samples stored at 4°C for 1 month, particularly for the non-fixed sample, which showed an efficiency of only 27%. The fixed samples stored at −20°C and −80°C remained above 85% detection efficiency (Fig. 4). Therefore, preservation by freezing at −20°C or −80°C is an ideal method for long-term sample storage.

Quality of antiserum in various *P. donghaiense* samples

Quality of antiserum was also examined with cells collected at different growth phases and under different nutrient conditions. Antiserum against *P. donghaiense* collected at the exponential phase was active against cells at various growth phases. At a dilution of 1:100, no difference was observed in labeling intensity (it was 3+) among cells collected at the initial, mid-exponential, late exponential, stationary or dissipation phase. Cells from nutrient-deplete and nitrate-limited cultures showed a moderate immunofluorescence reaction (2+), while cells from phosphate-limited and nutrient-replete cultures showed an intense immunifluorescence reaction (3+) (Table 2).

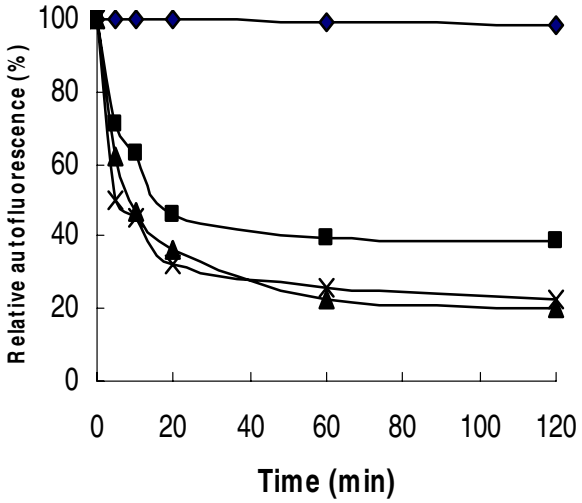

Fig. 2 Decrease of autofluorescence in cell samples after chlorophyll extraction with different solvents. "Decreasing rate of autofluorescence" was determined by comparison with autofluorescence without decolorization treatment (relative autofluorescence at 0 min). ♦ water; ▪80% methanol; × 80% acetone; ▲ 80% ethanol

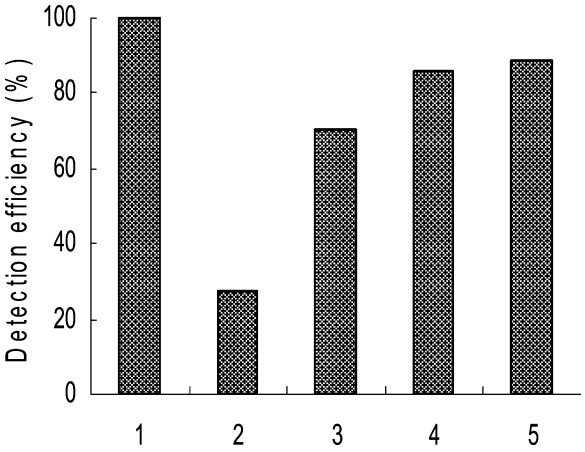

Fig. 4 Effects of different preservation methods on detection efficiency of *P. donghaiense*. "Detection efficiency" was determined in comparison with the fresh sample. *1* = Fresh sample; *2* = non-fixed sample at 4°C; *3* = fixed sample at 4°C; *4* = fixed sample at −20°C; *5* = fixed sample at −80°C

Application of the IF protocol to mixed and field collected samples

The detection efficiency of *P. donghaiense* cells in mixed samples and field collected samples is shown in Fig. 5. *P. donghaiense* cells showed a bright green color, which was clearly distinguishable in the mixed phytoplankton sample, and the efficiency was over 90% (Fig. 5a and b). This protocol was also applied to natural samples collected from the East China Sea and Taiwan Strait in May 2005, and *P. donghaiense* cells were clearly detected in the former samples (5C and 5D), while no positive staining was observed in samples collected from the Taiwan Strait, where no *P. donghaiense* cells have been observed. It was noted that there was no difference in IF between fresh and PFA fixed samples.

Discussion

Generally, the use of light microscopy and settling chambers are sufficient to classify and enumerate HAB species or strains. However, it is difficult to distinguish between morphologically similar species or strains in samples under the light microscope. Another problem is that it is time-consuming to identify and count a particular species using light microscopy, and it is not simple enough for routine

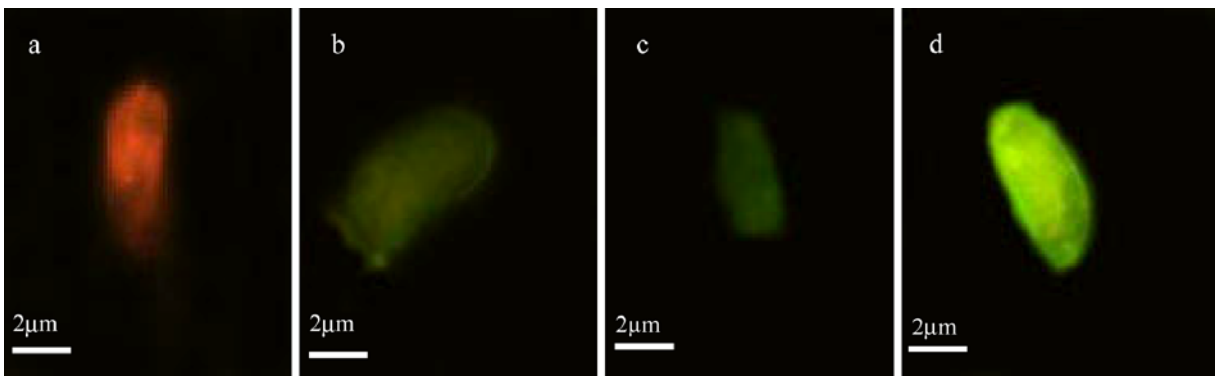

Fig. 3 Labeling intensity of *P. donghaiense* cells decolorized with different solvents. **a** water; **b** 80% methanol; **c** 80% acetone; **d** 80% ethanol

Table 2 Labeling intensity of *P. donghaiense* cells at different growth phases and under different nutrient conditions

	Samples	Labeling intensity
Nutrient conditions	Nutrient-replete (f/2 medium)	3+
	Phosphate- limited	3+
	Nitrate- limited	2+
	Nutrient-deplete	2+
Growth phases	Initial phase	3+
	Mid-exponential phase	3+
	Exponential phase	3+
	Stationary phase	3+
	Dissipation phase	3+

monitoring programs. Therefore, recent studies of HAB species, including *Prorocentrum* spp., have focused on the development of new methods of identification, mainly using biochemical, immunological and molecular biological techniques. Immunological techniques using polyclonal and monoclonal antibodies are promising for the identification, separation and enumeration of HAB cells, and have been studied widely (Nagasaki et al. 1991; Adachi et al. 1993; Vrieling et al. 1994; Anderson, 1995).

The present study raised a high titer and high specificity polyclonal antiserum using 0.5% PFA fixed cells as antigens. The lowest effective titer found in this study was 1:10000, which was higher than that reported for other HABs species, i.e., 1:200 for *Pfiesteria piscicida* (Lin et al. 2003), 1:300 for *P. lima*, *P. triestrium*, *P. micans*, *P. rostratum* and *P. minimum* (Lopez-Rodas and Costas, 1999), 1:600–1:1000 for *Gynodinium* and *Alexandrium* (Mendoza et al. 1995), and 1:500–1:1000 for the toxic diatom *Pseudonitzschia pungens* (Bates et al. 1993), but less than that reported in *Aureococcus anophagefferens*, 1:12800 (Anderson et al. 1990). The difference might be caused by the types and abundance of cell surface proteins, which influenced the quality of the antisera.

The antiserum exhibited no cross-reaction with morphologically similar species from the same genus or other phytoplankton species, even at low working dilution (1:100), which indicated that the antiserum against *P. donghaiense* was species-specific. More interestingly, the antiserum showed no cross-reaction with *P. dentatum* CCMP1517, which has been shown

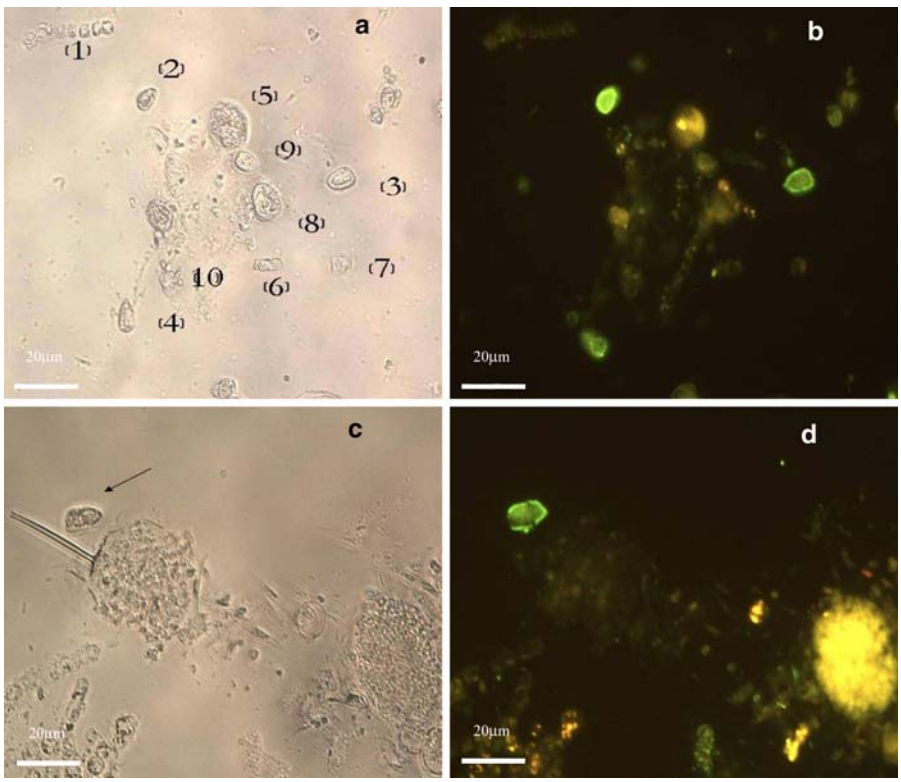

Fig. 5 Detection of target cells in mixed (**a** and **b**) and field (**c** and **d**) samples. **a** and **c**: optical microscopy; **b** and **d**: fluoroscope microscopy. In **a**, *(1) = S.costatum*; *(2)*,*(3)*,*(4)= P. donghainese*; *(5)= A. catanella*; *(6)= T. weissflogii*; *(7)*,*(9)*,*(10)= P. dentatum*; *(8)= P. micans*

to be morphologically and genetically identical to *P. donghaiense* (Lu et al. 2005). Our results demonstrated that *P. donghaiense* and *P. dentatum* might be different geographic phenotypes based on the IF results, which also proved the point made by Lu and Goebel (2001). Bates et al. (1993) reported that polyclonal antiserum was able to successfully distinguish between two forms of *P. pungens f. multiseries, P. australis, P. frandulenta* and *P. subcurvata*. Lopez-Rodas and Costas (1999) reported that polyclonal antisera against *P. lima* and *P. rostratum* were species-specific. However, they found that the antisera from *P. minimum*, *P. micans* and *P. triestinum* exhibited weak-reactions. In one previous study, Mendoza et al. (1995) also found that *Alexandrium minutum* and *A. lustitannicum* could not be distinguished using polyclonal antisera. In some cases, polyclonal antibodies against whole algal cells are species-specific, but if different species have common surface antigens, cross-reaction can occur.

Although indirect immunofluorescence has been used successfully for HAB species detection, several methodology problems have been found, such as the high autofluorescence produced by cell chlorophyll (Vrieling and Anderson 1996). The present study tested three solvents, and demonstrated that 80% ethanol was an effective decolorization reagent to minimize autofluorescence of chlorophyll in *P. donghaiense* cells. Recently, Hosoi-Tanabe and Sako (2005) reported that acetone was an effective decolorization reagent to minimize autofluorescence of chlorophyll in *Alexandrium tamarense* and *A. catenella*, and 90% of the chlorophyll was extracted within 10 min. In the present study, 80% acetone could minimize autofluorescence efficiently within 1 hour. However, the IF was very poor and no green color was observed. Acetone might change the structure or characteristics of cell surface proteins (antigens) during decolorization, so that antibodies could not recognize and bind to the target proteins. Moreover, this difference also reflected the species-specificity of cell-surface proteins in different dinoflagellate species.

Mendoza et al. (1995) indicated that immunofluorescence reactions were not influenced by the physiological stage of the cells. Vrieling and Anderson (1996) also found that immunofluorescence was not affected by growth conditions in one of their studies on immuno-flow cytometric identification of two dinoflagellates, *Gyrodinium aureolum* and *Gymnodinium nagasakiense*. The present results proved this point. Cells collected at different growth phases and under different nutrient conditions showed the same fluorescence intensity (3+), although a slight decline was observed in nutrient-deplete and nitrate-limited cells (2+). In addition, the binding quality was excellent in both fresh and buffered-paraformaldehyde-preserved cells, which was very similar to the results obtained by Mendoza et al. (1995), who obtained excellent labeling in buffered-formaldehyde-preserved cells. This indicated that the immunological protocol developed in this study is constant and stable, and is a useful tool for the rapid identification of *P. donghaiense*.

Conclusion

Our results show that using 0.5% PFA fixed *P. donghaiense* cells as antigens produced specific and effective antibodies for *P. donghaiense,* which did not cross-react with morphologically similar species from the same genus or with other phytoplankton species. Ethanol was an effective decolorization reagent to minimize the autofluorescence of cells. The established IF protocol using this specific antibody shows promise for application in the identification of *P. donghaiense* in mixed culture and in natural samples. Further study of its application to quantify *P. donghaiense* in natural samples is required, as is its comparison with other methods, so as to determine if this new technique can be used in routine monitoring.

Acknowledgements The authors thank Prof. John Hodgkiss for fruitful discussion and for helping to revise the manuscript. The study was partly supported by the Ministry of Science and Technology of the People's Republic of China 2001CB409700 and the Program for New Century Excellent Talents in University to Prof. D.-Z. Wang.

References

Adachi M, Sako Y, Ishida Y (1993) The identification of conspecific dinoflagellates *Alexandrium tamarense* from Japan and Thailand by monoclonal antibodies. Nippon Suisan Gakkaishi 59:327–332

Anderson DM (1995) Identification of harmful algal species using molecular probes: an emerging technology. In: Lassus P., Arzul G., Erard E, et al. (eds) Harmful marine algal blooms. Lavoisier, Paris

Anderson DM, Kulis DM, Cosper EM (1990) Immunofluorescent detection of the brown tide organism *Aureococcus anophagefferens*. In: Cosper EM, Bricelj VM, Carpenter

EJ (eds) Novel phytoplankton blooms: causes and impacts of recurrent brown tides and other unusual blooms. Springer-Verlag, Berlin

Bates M, Léger C, Keafer BA, Anderson DM (1993) Discrimination between domoic-acid-producing and non-toxic forms of the diatom *Pseudonitzschia pungens* using immunofluorescence. Mar Ecol Prog Ser 100:185–195

Hosoi-Tanabe S, Sako Y (2005) Rapid detection of natural cells of *Alexandrium tamarense* and *A. catenella* (Dinophyceae) by fluorescence in situ hybridization. Harmful Algae 4:319–328

Lin SJ, Feinstein TN, Zhang H (2003) Development of an immunofluorescence technique for detecting *Pfiesteria piscicida*. Harmful Algae 2:223–231

Lopez-Rodas V, Costas E (1997) Characterization of morpho-species and strains of *Microcystis* Cyanbacteria from natural populations and laboratory clones using cell probes (lectins and antibodies). J Phycol 33:446–454

Lopez-Rodas V, Costas E (1999) Immunochemical character-ization of morphospecies and strains of *Prorocentrum* (Dinophyceae). J Exp Mar Bio Ecol 238:293–308

Lu DD, Goebel J (2001) Five red tide species in genus Prorocentrum including the description of *Prorocentrum donghaiense* Lu sp. nov. from the East China Sea. Chin J Oceanol Limnol 19:337–344

Lu DD, Goebel J, Qi YZ, Zou JZ, Han XT, Gao YH, Li YG (2005) Morphological and genetic study of *Prorocentrum donghaiense* Lu from the East China Sea, and comparison with some related *Prorocentrum* species. Harmful Algae 4:493–505

Nagasaki K, Uchida U, Ishida Y (1991) A monoclonal antibody which recognizes the cell surface of red tide alga *Gymnodinium nagasakiense*. Nippon Suisan Gakkaishi 57:1211–1214

Mendoza H, Lopez-Rodas V, Gonzalez-Gil F, Costas E (1995) The use of polyclonal antisera and blocking of antibodies in the identification of marine dinoflagellates: species-specific and clone-specific antisera against *Gym-nodinium* and *Alexandrium*. J Exp Mar Biol Ecol 186:103–115

Sako Y, Adachi M, Ishida Y (1993) Preparation and character-ization of monoclonal antibodies to *Alexandrium* species. In: Smayda TJ, Shimizu Y (eds) Toxic phytoplankton blooms in the sea. Elsevier, Amsterdam

Vrieling EG, Anderson DM (1996) Immunofluorescence in phytoplankton research: applications and potential. J Phycol 32:1–16

Vrieling EG, Gieskes W, Van Z, Veenhuis M (1994) Detection of toxic phytoplankton species by immunochemical particle analysis based on flow cytometry. Netherl J Ecol 28:249–254

Zhou MJ, Yan T, Zou JZ (2003) Primary study on the HABcharacteristic in the Changjiang estuary and its adjacent sea area. Chin J Appl Ecol 14:1031–1038