Chinese Journal of Oceanology and Limnology
Vol. 30 No. 3, P. 433-439, 2012
http://dx.doi.org/10.1007/s00343-012-1208-6

Applications of immuno-magnetic bead and immunofluorescent flow cytometric techniques for the quantitative detection of HAB microalgae*

HUANG Jian (黄健)[1], WEN Ruobing (温若冰)[2], BAO Zhenmin (包振民)[1],
SUI Zhenghong (隋正红)[1, **], SUN Ningbo (孙宁波)[3], KANG Kyoungho (康庆浩)[4]

[1] *Key Laboratory of Marine Genetics and Breeding of Ministry of Education*, *Ocean University of China*, *Qingdao 266003*, *China*
[2] *North China Sea Environmental Monitoring Center*, *State Oceanic Administration*, *Qingdao 266033*, *China*
[3] *The Fifth Middle School of Zibo*, *Zibo 255000*, *China*
[4] *Departmentof Aquaculture*, *Chonnam National University*, *Yeosu Jeonnam 550-749*,*Korea*

Received Sep. 30, 2011; accepted in principle Nov. 3, 2011; accepted for publication Dec. 12, 2011

Abstract Over the last several decades, harmful algal blooms (HABs) have become a serious environmental problem in many parts of the world. A rapid and accurate detection process for HAB algae has yet to be developed. *Heterosigma akashiwo* is one of the most important HABs species in China. The objective of this study was to develop an immunologic technique that can rapidly and sensitively count *H. akashiwo* cells. Five HABs species (*Alexandrium catenella*, *Thalassiosira* sp., *Cryptomonas* sp., *Alexandrium tamarense* and *Symbiodinium* sp.,) were used in this study to evaluate the analysis process we developed. A polyclonal antibody with high titers against *H. akashiwo* was obtained by injecting *H. akashiwo* cells into rabbits. Immuno-magnetic beads (IMB) were produced via conjugated polyclonal antibodies with magnetic beads and applied to isolate and count *H. akashiwo* cells from the culture. Results show that 66.7%–91.6% of the cells were captured from unialgal culture by IMBs, and only 5.3%–12.5% of the four other HAB microalgae species were captured, indicating that the constructed IMBs combined specifically with the *H. akashiwo* cells. At the same time, flow cytometry (FCM) sorting was exploited to screen *H. akashiwo* cells after labeling with FITC conjugated polyclonal antibodies. Using the FCM technique, 91.7% of the targeted cells were sorted out from mixed microalgae samples in just a few minutes. These results indicate that both antibody-involved IMB and antibody-based FCM techniques are highly effective at detecting and quantifying HAB species. These techniques, especially immuno-magnetic separation, have low associated cost, and are fast and simple processes compared with other techniques currently in use.

Keyword: *Heterosigma akashiwo*; polyclonal antibody; immuno-magnetic beads; FITC; flow cytometry

1I NTRODUCTION

Over the last several decades, harmful algal blooms (HABs) have been observed more frequently in more places in China than ever before (Long et al., 2008; Zhang et al., 2008). Multidisciplinary studies, ranging from molecular and cell-biology based projects to large-scale field surveys, numerical modeling, and remote sensing have been conducted to study HABs (Anderson, 2009). However, fast and accurately detecting and counting HAB microalgae remain among the key topics in the study of HABs that have yet to be resolved.

Great efforts have been made to develop rapid and sensitive methods for quantitatively detecting microalgae. Use of morphological criteria is still the most widely used technique for detecting and classifying toxic microalgae (Hallegraeff et al., 1995). However, this approach has two major drawbacks: the preparation and counting procedures are tedious and time consuming, and it cannot differentiate non-toxic

* Supported by Specialized Research Fund for the Doctoral Program of Higher Education of China (No. SRFDP 20100132110007), and Shandong Provincial Natural Science Foundation, China (No. ZR2011DZ002)
** Corresponding author: suizhengh@ouc.edu.cn

microalgae from toxic microalgae (Cho et al., 1998). Therefore, new technologies are being developed and implemented to monitor the development of algal blooms (Cho et al., 2001b; Cho and Costas, 2004). These approaches include scanning electron microscopy (SEM), high performance liquid chromatography (HPLC), PCR, immunofluorescence, lectin probes (Cho et al., 2001a), fluorescent in situ hybridization (FISH) (Costas and Rodas, 1994; Costas et al., 1995; Rodas and Costas, 1997; Zhang et al., 2005), peptide nucleic acid (PNA) probes based on DNA sequence analysis (Hou et al., 2005), and two-dimensional gel electrophoresis and proteome markers based on proteomic technology (Chan et al., 2005).

Immunological methods have also been used to detect toxic microalgae (Bates et al., 1993; Nakanishi et al., 1996; Peperzak et al., 2000; Caron et al., 2003). For example, polyclonal antibodies of *Pseudo-nitzchia pungen*s, *P. multiseries*, *P. australis*, *P. frandulenta*, and *P. subcurvata* were raised to differentiate these algal species (Bates et al., 1993). Monoclonal antibodies produced from *Aureucoccus anophagefferens* and *Chatonell marina* were used for qualitative or quantitative detection of these microalgae (Nakanishi et al., 1996; Caron et al., 2003). However, the cross-reactivity of these antibodies with other closely related species limits their application.

Heterosigma akashiwo (Hada), a golden-brown marine alga, known to produce brevetoxins (Khan et al., 1997), is the causal species of dozens of red tides and massive fish deaths leading to losses to aquaculture all over the world (MacKenzie, 1991; O'Halloran et al., 2006). *Heterosigma akashiwo* is also one of the most important HAB species in China (Jiao et al., 2010). The objective of this study was to develop an immunologic technique that can rapidly and sensitively count *H. akashiwo* cells. A polyclonal antibody with high titers against *H. akashiwo* was raised. An immuno-magnetic bead (IMB) separation process then was applied for selective detection and counting of *H. akashiwo* cells. Flow cytometry (FCM) also was used to isolate and count *H. akashiwo* cells labeled by the fluorescent antibody. Quantities of *H. akashiwo* cells in mixed microalgae samples also were processed using the FCM technique. The ultimate goal is to use these techniques for detecting and quantifying *H. akashiwo* from field samples. Compared with other sorting techniques currently in use, especially immuno-magnetic separation, these methods offer great advantages with respect to cost, volume, speed, and simplicity.

2 MATERIAL AND METHOD

2.1 Strains and cultivation condition

Six HAB species (*Heterosigma akashiwo*, *Symbiodinium* sp., *Alexandrium catenella*, *Thalassiosira* sp., *Cryptomonas* sp., and *Alexandrium tamarense*) were used in this study. Four algae (*Symbiodinium* sp., *Alexandrium catenella*, *Thalassiosira* sp., *Cryptomonas* sp.), were isolated from Zhanshan Bay, Qingdao, China, using the limited dilution method (Zhang et al., 2010) normally used in monoclonal antibody techniques. *Heterosigma akashiwo* and *Alexandrium tamarense* were obtained from the strain collection center of Ocean University of China. All microalgae were cultured in f/2 medium under a 12 h:12 h light-dark cycle, a light intensity of 72 μmol/(m^2·s) and a temperature of 20±1°C.

2.2 Preparation of *H. akashiwo* polyclonal antibodies and magnetic beads covalently conjugated with the antibodies

Polyclonal antibodies of *H. akashiwo* were prepared according to the procedure described previously with small modifications (Xiang et al., 2005; Costas and Lopez-Rodas, 1996). Two New Zealand rabbits were immunized to produce polyclonal antibody against cell surface antigens of *H. akashiwo*. First, 1×10^7 *H. akashiwo* cells were collected by centrifugation at 10 000 r/min for 10 min. The cell pellet was resuspended in sterile phosphate buffered saline (PBS) buffer solution and subcutaneously injected into the rabbits with complete Freund's adjuvant. Eight booster injections were given at 10-d intervals over a period of 80 d. When an acceptable titer was achieved, blood was obtained from the anaesthetized rabbits. The blood was allowed to clot at 4°C and then was centrifuged at 2 000 r/min for 5 min to remove red blood cells. The serum was tested and stored at -20°C (Sun et al., 2008).

Immobilization of purified polyclonal antibodies to EDC/NHS-cross-linked carboxylated magnetic beads (Seradyn) (Bangs Laboratories, Fishers, IN, USA) was performed by Jingmei Co. (Beijing, China). One milligram of magnetic beads was mixed with 27 μg of antibody. Next, 10 mg of the IMBs were suspended in 1 mL of stock solution (10 mmol/L PBS, 0.1% BSA, 0.09% NaN_3, 2 mmol/L EDTA, pH 7.4) and stored at 4°C.

2.3 Algal collection and treatment

During the culture period, densities of the microalgae were counted by microscopy. Certain volumes of algal cells at the late exponential growth phase were harvested by centrifugation (800 r/min for 10 min). After washing with 1 mL of cold PBS and being fixed with 2 mL 2% formaldehyde solution for 40 min at room temperature, the suspended solution was centrifuged to precipitate the cells. The cells were washed three times with 2 mL of PBS and then were resuspended in 900 μL of PBE solution (PBS+0.5% BSA+0.08% EDTA) for the IMB separation procedure. For the indirect immuno-FCM experiment, after washing, the cells were resuspended in 1 mL of buffer (PBS+0.2% (v/v) Triton-X100+5% (v/v) BSA).

2.4 IMB separation procedure

The saturation concentration of IMB for *H. akashiwo* was determined by incubating different quantities of IMB with 10^7 *H. akashiwo* cells. Cells were collected, concentrated, and suspended in 900 μL PBE solution as described above. Next, 100 μL of 350, 140, or 70 μg of IMBs were added to the microalgae suspension, which then was incubated for 30 min at 4°C. Mixtures were set on a magnetic rack (TOYOBO, Japan), which allowed capture of microalgae cells attached to IMBs. Cells in the supernatant (not attached to the IMBs) were then counted by microscopy.

Different quantities of *H. akashiwo* cells (130 000, 10 000, and 1 000) were collected and treated using the aforementioned procedures. Based on the IMB saturation concentration determined above, an overloaded quantity of the beads (7 μg) was added to the *H. akashiwo* solution. After setting the mixture on the magnetic rack, the supernatant was discarded. The IMBs were washed twice with PBS buffer, resuspended in 1 mL of PBE, and counted using the Cell Counter XT-2000i (Sysmex, Japan). Recovery (%) was calculated using the following formula: (cell quantity isolated by the IMBs/total cell quantity counted by microscopy) ×100.

Four HAB species (*Thalassiosira* sp., *A. catenella*, *Symbiodinium* sp., and *Cryptomonas* sp.) were selected to test their cross-reactivity with the IMBs. First, 900 μL of concentrated microalgae suspension (containing 10^4 algal cells) were incubated with 7 μg of IMBs for 30 min at 4°C. After magnetic separation, the number of cells that cross-reacted with IMBs was estimated using the Cell Count XT-2000i. The binding coefficient (%) was calculated as follows: (number of cells binding with IMBs/total cell quantity counted by microscopy)×100. This value was used to evaluate the cross-reactivity of these other HABs with IMBs. This procedure was performed in triplicate for every condition.

2.5 Indirect immuno-FCM identification and enumeration of *H.akas hiwo*

H. akashiwo cells (4×10^6) were collected by centrifugation at 800 r/min for 10 min. After washing with 1 mL of PBS, the cell precipitate was resuspended in 1 mL of buffer (PBS+0.2% (v/v) Triton-X100+5% (v/v) BSA). The obtained *H. akashiwo* suspension was divided into two equal aliquots. 100 μL suspensions of the other five HAB species (*Symbiodinium* sp. (1.3×10^6 cell/mL), *A. catenella* (1.08×10^6 cell/mL), *Thalassiosira* sp. (1.8×10^6 cell/mL), *Cryptomonas* sp. (1.08×10^6 cell/mL), and *A. tamarense* (4.1×10^5 cell/mL)) were added to one aliquot. The other aliquot remained unchanged. Both tubes were placed on ice for 10 min, followed by centrifuging at 800 r/min for 5 min. Next, 1 mL of antibody (1:10 dilution with PBS) was added to each tube, and the tubes were incubated on ice for 40 min. After washing twice with 2 mL of cold PBS, 200 μL of FITC-labeled goat anti-rabbit IgG (1:100 dilution) were added to the mixtures, followed by incubation on ice for 40 min in the dark. After washing twice with 2 mL of cold PBS, the mixtures were resuspended in 1 mL of PBS. Samples were analyzed using a flow cytometer (FACSVantage, BD, USA). FCM counts of *H. akashiwo* from the mixed algal sample were compared with those from the unialgal sample. Three replicates were performed under every condition.

3R ESULT

3.1 Enumeration *of H. akashiwo* by IMB separation

To determine the amount of antibody-conjugated IMB used in each trial, different amounts of IMB ranging from 35 mg to 140 mg were tested for their ability to absorb the *H. akashiwo* cells (Table 1). Table 1 shows the binding efficiency of IMB with *H. akashiwo* cells. The number of *H. akashiwo* cells needed to saturate 1 μg of IMB was $(6.55\pm1.05)\times10^4$. This parameter is necessary for enumeration of *H. akashiwo* by IMB separation.

Different amounts of *H. akashiwo* cells ranging from 10^3 to 10^5 were mixed with IMB for species specific separation and enumeration. As illustrated in Table 2, *H. akashiwo* cells can be detected and quantified using the IMB separation technique. High recovery (86.7%–91.6%) was achieved when the cell quantity was in the range of 1.0×10^4 to 1.3×10^5. However, when the cell quantity decreased to 10^3, only 66.7% ± 1.44% of the cells could be detected and quantified.

Cross-reactivity of IMB with *Thalassiosira* sp., *A. catenella*, *Symbiodinium* sp., and *Cryptomonas* sp. was also tested and was very low (Table 3). The binding coefficients of IMB with these four microalgae ranged from 5.3% to 12.5%, indicating that the raised antibody was specific to *H. akashiwo*.

3.2 Quantitative isolation of *H. akashiwo* by FCM method

H. akashiwo cells were labeled with FITC-conjugated antibodies and then screened with the flow cytometer (as in the method section) either from unialgal sample or from multiply-algal sample. Results showed that *H. akashiwo* cells labeled with FITC could be separated from unlabeled cells using different fluorescence gates of the flow cytometer (Fig.1). The target cells labeled with FITC showed a single peak in the FL1 histogram (Fig.1b), whereas the autofluorescence of unlabeled cells showed a single peak in the FL2 histogram (Fig.1a). When the mixed sample was analyzed, target cells labeled with FITC also showed a single peak in the FL1 histogram (Fig.1c).

The number of *H. akashiwo* cells counted using the FCM technique (2.17×10^6) was consistent with the cell number counted by microscopy (2×10^6) (Table 4). In addition, 91.7% of the targeted cells were sorted out from the mixed samples.

4D ISCUSSION

A polyclonal antibody with high titers against *H. akashiwo* was obtained by injecting *H. akashiwo* culture into rabbits. Although monoclonal antibody technique has been exploited in previous studies (Carrera et al., 2010; Córdova et al., 2002; Gas et al., 2009), polyclonal antibodies raised against the cell

Table 1 The quantity of *Heterosigma akashiwo* cells needed to saturate 1 μg of immuno-magnetic beads (IMB)

Quantity of IMB (μg)	Number of cells attached to IMB ($\times10^6$)	Number of cells needed to saturate 1 μg of IMB conjugate ($\times10^4$)
140	7.91±0.36	5.65±0.26
70	5.4±0.26	7.71±0.37
35	2.2±0.11	6.29±0.31

Table 2 Recovery of *Heterosigma akashiwo* cells by IMB separation

Cell quantity counted by microscopy	130 000	10 000	1 000
Quantity of IMB (μg)	7	7	7
Cell quantity isolated by IMB	119 066±5 928	8 667±382	667±144
Recovery(%)	91.6±4.56	86.67±3.82	66.7±1.44

Table 3 Cross-reactivity of IMB with different red tide algae

	Thalassiosira sp.	*Alexandrium catenella*	*Symbiodinium*s p.	*Cryptomonas* sp.
Cell quantity by microscopy	1×10^4	1×10^4	1×10^4	1×10^4
Quantity of IMB (μg)	7	7	7	7
Cellbi ndingw ithI MB	960±80	530±40	750±70	250±110
Bindingc oefficient(%)	9.6±0.8	5.3±0.4	7.5±0.7	12.5±1.1

Table 4 Comparison of cell densities counted by light microscopy and flow cytometry

Cell count by light microscopy(/mL)($\times10^6$)	Cell count by flow cytometry (/mL) ($\times10^6$)		Recovery (%)
	From unialgal sample	From mixed algal sample	
2	2.17±0.11	1.99±0.09	91.7

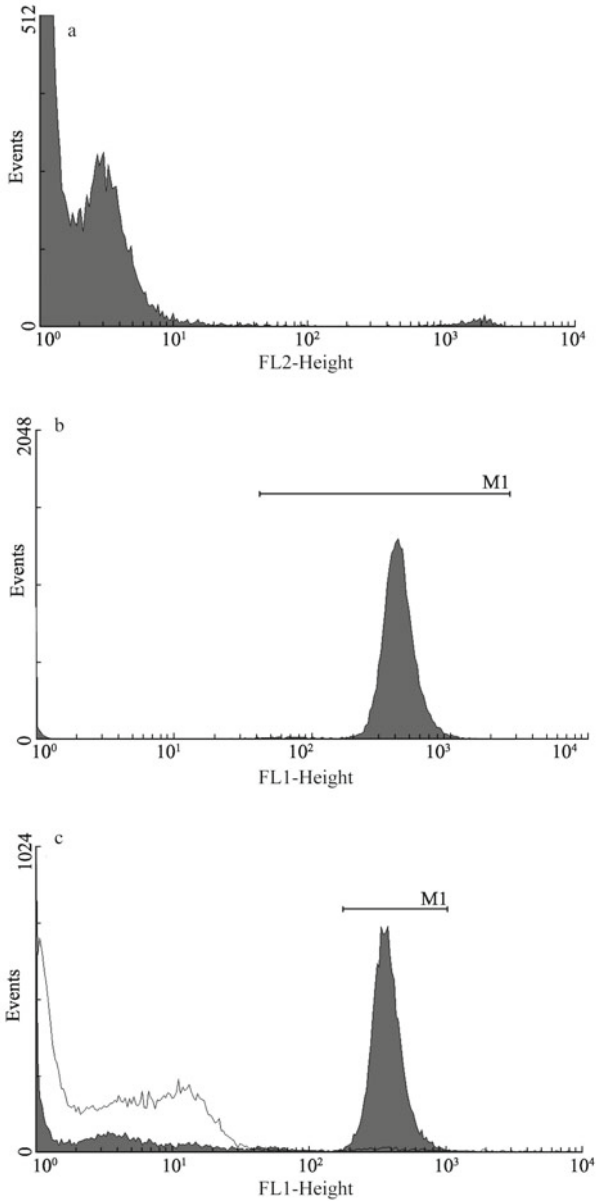

Fig.1 Flow cytometry histograms

a. auto-fluorescence signals of *Heterosigma akashiwo*; b. *H. akashiwo* labeled with FITC-conjugated antibodies from a unialgal cell sample; c. *H. akashiwo* labeled with FITC-conjugated antibodies from a mixed algal sample containing *H. akashiwo*, *Symbiodinium* sp., *Alexandrium catenella*, *Thalassiosira* sp., *Cryptomonas* sp., and *Alexandrium tamarense*. M1 designated the cell counting region in the chart. FL1 is the fluorescence gate used to monitor the signal from FITC; excitation wavelength = 488 nm; emission wavelength = 530 nm. FL2 is the fluorescence gate used to monitor the signal from the algal pigments; excitation wavelength = 488 nm; emission wavelength = 585 nm.

surface as introduced in this study have the advantages of simplicity and specificity, which has also been highlighted in other reports (Mendoza et al., 1995; Costas and Lopez-Rodas, 1996).

The immuno-magnetic separation technique has been widely used in medical studies to purify and characterize a wide range of cell types, such as tumor cells (Vredenburgh et al., 1991) and lymphoid cells (Durcová et al., 1998; Babatz et al., 2003; Yan et al., 2004). Results of our study indicate that this technique is also applicable for detecting HAB species. High recovery (86.67%–91.6%) was achieved when the treated microalgae were at a high cell density. As the target cell density decreased, the recovery (66.7%) decreased accordingly. Costas and Lopez-Rodas (1996) used magnetic beads labeled with secondary antibodies to separate and enumerate *Alexandrium minutum* from natural samples with a high recovery rate (61%–92%), a recovery rate comparable to the present study, which demonstrates the applicable of the procedure. Aguilera et al. (1996) produced monoclonal antibodies and compared separation approaches using magnetic beads labeled with primary and secondary antibodies and concluded that both techniques were equally effective for isolating *Alexandrium fundense* from the clonal culture in their study. When tested in natural samples, the primary antibody labeling method was slightly less effective (80% recovery) than the secondary antibody labeling method, however, a comparative recovery ratio was achieved in their study. In our study, the primary polyclonal antibody was labeled with magnetic beads, which was a simple method that shortened the time required for the procedure without the need for a secondary antibody; therefore, it could minimize loss of target cells. Work is ongoing to modify the processing steps to minimize cell loss so that this method can be used to enumerate *H. akashiwo* in the field.

Immuno-magnetic separation of a single algal species from a sample containing mixed phytoplankton and detritus is simple, rapid, and quite reliable (Aguilera et al., 1996). Weak cross-reactivity of *H. akashiwo* IMB with other HAB microalgae was observed in our study. This may be due to the specificity of the raised polyclonal antibody or to some parameters of the protocol, such as the elution time and strength. As cell loss undoubtedly occurs during the washing steps (Aguilera et al., 1996; Córdova et al., 2002), it is difficult to quantify microalgae at low density. Therefore, pre-concentration of natural samples, either by centrifugation or filtration, is necessary for analyzing water samples with a low density of target cells. However, such concentration steps have the undesirable effect of concentrating other co-occurring organisms and detritus of similar size.

Immunofluorescence is the most frequently used technique to identify algal cells. Cell counting with immunofluorescence is performed on a flow cytometer, which allows the rapid enumeration of small cells, and size measurements of a large numbers of cells. The combination of size with the autofluorescent properties of individually detected cells can be used in cluster analyses (Peperzak et al., 2000). Vrieling et al. (1994a) found that monoclonal antibody-labeled cells of the ichthyotoxic dinoflagellate *Gyrodinium aureolum* could be identified via flow cytometry. However, when compared with counts made with a light microscope, quantification was poor due to loss of cells during sample processing. Despite the cell loss problem, in the summer of 1991 at two stations in the central North Sea, *Gyrodinium aureolum* could be readily identified during formation and decline of a bloom using the flow cytometry method (Vrieling et al., 1994b, 1995), however, no quantitative data were provided. Sako et al. (1996) also reported problems with quantification resulting from cells loss when they attempted to quantify *Alexandrium* spp. in field samples by using monoclonal antibodies. In our study, *H. akashiwo* was successfully isolated from the mixed microalgae sample using the immunofluorescence FCM technique, indicating the potential applicability of this technique for analysis of field samples. This technique was also highlighted by Costas and Lopez-Rodas (1996) using another red tide alga *Alexandrium minutum* and a comparable recovery ratio was obtained. However, the quantity of algal cells counted from the mixed sample was 8.3% lower than that counted from unialgal sample. This small difference could have been caused by cross-reactivity of other species with the antibody, which was demonstrated in the IMB separation results. Thus, considerable effort is still needed to take antibody probes from the developmental stage to direct application with natural populations.

5C ONCLUSION

In this study, immuno-labeling technique was developed to quantitatively detect *H. akashiwo*, which is an important HAB species. The technique involves two major steps: obtaining the polyclonal antibody with high titers against *H. akashiwo* and counting *H. akashiwo* cells. IMB or FCM techniques were used to selectively isolate and count *H. akashiwo* cells.Both antibody-involved IMB and antibody-based FCM were effective for detecting and quantifying *H. akashiwo*. The IMB technique exhibited a high recovery of *H. akashiwo* cells (66.7%–91.6%). With the FCM technique, 91.7% of the targeted cells could be sorted out from mixed microalgae samples within several minutes. These immuno-based techniques are promising tools for separating and enumerating HAB species, but procedures such as enumeration and cell sorting of target species using IMB and FCM must be perfected for field application.

References

Anderson D M. 2009. Approaches to monitoring, control and management of harmful algal blooms (HABs). *Ocean and Coastal Management*, **52**: 342-347.

Aguilera A, Gonzalez-Gil S, Keafer B A, Anderson D M. 1996. Immunomagentic separation of cells of the toxic dinoflagellate *Alexandrium fundyense* from natural plankton samples. *Marine Ecology Progress Series*, **143**: 255-269.

Babatz J, Röllig C, Oelschlägel U, Zhao S, Ehninger G, Schmitz M, Bornhäuser M. 2003. Large-scale immunomagnetic selection of $CD14^+$ monocytes to generate dendritic cells for cancer immunotherapy: a phase I study. *Journal of Hematotherapy and Stem Cell Research*, **12**:515- 523.

Bates S S, Leger C, Keafer B A, Anderson D M. 1993. Discrimination between domoic acid producing and non-toxic forms of the diatom *Pseudonitzchia pungens* using immunofluorescence. *Marine Ecology Progress Series*, **100**:185- 195.

Carrera M, Garet E, Barreiro A, Garcé E, Pérez D, Guisande C, González-Fernández Á. 2010. Generation of monoclonal antibodies for the specific immunodetection of the toxic dinoflagellate *Alexandrium minutum* Halim from Spanish waters. *Harmful Algae*, **9**:272- 280

Caron D A, Dennett M R, Moran D M, Schaffner R A, Lonsdale D A, Gobler C J, Nuzzi R, McLean T I. 2003. Development and application of a monoclonal-antibody technique for counting *Aureococcus anophagefferens*, an alga causing recurrent brown tides in the Mid-Atlantic United States. *Applied and Environmental Microbiology*, **69**: 5492- 5 502.

Chan L L, Hodgkiss I J, Lam P K S, Wan J M F, Chou H N, Lum J H K, Lo M G Y, Mak A S C, Sit W H, Lo S C L. 2005. Use of two-dimensional gel electrophoresis to differentiate morphospecies of *Alexandrium minutum*, a paralytic shellfish poisoning toxin-producing dinoflagellate of harmful algal blooms. *Proteomics*, **5**: 1 580-1 593.

Cho E S, Costas E. 2004. Rapid monitoring for the potentially ichthyotoxic dinoflagellate *Cochlodinium polykrikoides* in Korean coastal waters using fluorescent probe tools. *Journal of Plankton Research*, **26**:175- 180.

Cho E S, Choi B D, Cho Y C, Kim T G, Kim H G. 2001a. Discrimination of three highly toxic *Alexandrium tamarense* (Dinophyceae) isolates using FITC-conjugated lectin probes. *Journal of Plankton Research*, **23**:89- 95.

Cho E S, Kodaki Y, Park J G. 2001b. The comparison of two strains of toxic *Pseudo-nitzschia multiseries* (Hasle) and non-toxic *Pseudo-nitzschia pungens* (Grunow) Hasle isolated from Chinhae Bay, Korea. *Algae*, **16**:275- 285.

Cho E S, Seo G M, Lee S G, Kim H G, Lee S J, Rhodes L L, Hong Y K. 1998. Application of FITC-conjugated lectin probes for the recognition and differentiation on some Korean coastal red tide microalgae. *Journal of Fisheries Science and Technology*, **1**:250- 254.

Córdova J L, Jamett A, Lembeye G S. 2002. Immunolocalization of theca antigens on *Alexandrium catenella* and their use to isolate cells from fixed phytoplankton samples. *Harmful Algae*, **1**:351- 360.

Costas E, Rodas V L. 1994. Identification of marine dinoflagellates using fluorescent lectins. *Journal of Phycology*, **30**:987- 990.

Costas E, Zardoya R, Bautista J, Garrido A, Rojo C, Rodas V L. 1995. Morphospecies vs genospecies in toxic marine dinoflagellates: an analysis of *Gymnodinium catenatum*/*Gyrodinium impudicum* and *Alexandrium minutum*/*A*. *lusitanicum* using antibodies, lectins and gene sequences. *Journal of Phycology*, **31**:801- 807.

Costas E, Lopez-Rodas V. 1996. Enumeration and separation of the toxic dinoflagellate *Alexandrium minutum* from natural samples using immunological procedures with blocking sntibodies. *Journal of Experimental Marine Biology and Ecology*, **198**:81- 87.

Durcová G, Yamaguchi M, Takahashi S, Imai H. 1998. Immunomagnetic isolation of mouse embryonic Stem cells from heterogeneous cell population. *Journal of Reproduction and Development*, **44**:85- 89.

Gas F, Pinto L, Baus B, Gaufres L, Crassous M P, Compere C, Quéméneur E. 2009. Monoclonal antibody against the surface of *Alexandrium minutum* used in a whole-cell ELISA. *Harmful Algae*, **8**: 538-545.

Hallegraeff G M, Anderson D M, Cembella A D. 1995. Manual on Harmful Marine Microalgae IOC Manuals and Guides. UNESCO Publisher, Paris.

Hou J J, Huang B Q, Lai H Y. 2005. Application of PNA (peptide nucleic acid) probes biotechniques in detection on red tide microalgae. *Chinese Journal of Public Health*, **21**: 1 123-1 125. (in Chinese with English abstract)

Jiao X M, Xu H, Hua W H, Sheng J M. 2010. The red tide organisms in Haizhou Bay. *Journal of Aquaculture*. **5**: 25-29. (in Chinese with English abstract)

Khan S, Arakawa O, Onoue Y. 1997. Neurotoxins in a toxic red tide of *Heterosigma akashiwo* (Raphidophyceae) in Kagoshima Bay, Japan. *Aquaculture Res*., **28**:9- 14.

Long H, Zhou Y, Yu J, Hu Y F, Fu G J. 2008. Analyses on harmful algal blooms in Zhejiang coastal waters from 2001 to 2007. *Marine Environmental Science*, **27**: 1-4. (in Chinese with English abstract)

MacKenzie L. 1991. Toxic and noxious phytoplankton in Big Glory Bay, Stewart Island, New Zealand. *J*. *Appl*. *Phycol*., **3**:19- 34.

Mendoza H, Lopez-Rodas V, Gonzalez-Gil S, Aguilera A, 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 *Gymnodinium* and *Alexandrium*. *J*. *Exp*. *Mar*. *Biol*. *Ecol*., **186**:103- 115.

Nakanishi K, Karube I, Hiroischi S, Uchida A, Ishida Y. 1996. Detection of the red tide-causing plankton *Chattonellamarina* using a piezoelectric immunosensor. *Analytica Chimica Acta*, **325**:73- 80.

O'Halloran C, Silver M W, Holman T R, Scholin C A. 2006. *Heterosigma akashiwo* in central California waters. *Harmful Algae*, **5**:124- 132.

Peperzak L, Vrieling E G, Sandee B, Rurtten T. 2000. Immuno flow cytometry in marine phytoplankton research. *Scientia Marina*, **64**:165- 181.

Rodas V L, Costas E. 1997. Characterization of morphospecies and strains of *Microcystis* (cyanobacteria) from natural populations and laboratory clones using cell probes (lectins and antibodies). *Journal of Phycology*, **33**: 446-454.

Sako Y, Murakami T, Adachi M, Uchida A, Ishida Y, Yamaguchi M, Takeuchi T. 1996. Detection of the toxic dinoflagellate *Alexandrium* species by flow cytometry using a monoclonal antibody. *In*: Yasumoto T, Oshima Y, Fukuyo Y eds. Harmful and Toxic Algal Blooms. IOC-UNESCO, Sendai. p.464-466.

Sun N B, Sui Z H, Bao Z M, Zhang D B. 2008. Study of three red tide algae using immuno-fluorescent technique. *Acta Oceanologica Sinica*, **6**: 132-139. (in Chinese with English abstract)

Vrieling E G, Peperzak L, Gieskes W W C, Veenhuis M. 1994a. Detection of the ichthyotoxic dinoflagellate *Gyrodinium* cf. *aureolum* and morphologically related *Gymnodinium* species using monoclonal antibodies: a specific immunological tool. *Marine Ecology Progress Series*, **103**:165- 174.

Vrieling E G, Gieskes W W C, van Zeijl W J M, Veenhuis M. 1994b. Detection of *Gyrodinium aureolum* in Dutch coastal waters by particle analysis based on immno-flow cytometry. *Netherlands Journal of Aquatic Ecology*, **28**: 249-254.

Vrieling E G, Gieskes W W C, Rademaker T W M, Vriezekolk G, Peperzak L, Veenhuis M. 1995. Flow cytometric identification of the ichthyotoxic dinoflagellate *Gyrodinium aureolum* in central North Sea. *In*: Lassus P, Arzul G, Erard E, Gentien P, Marcaillou C eds. Harmful Marine Algal Blooms. Lavoisier, Paris. p.743-748.

Vredenburgh J J, Simpson W, Memoli V A, Ball E D. 1991. Reactivity of anti-CD15 monoclonal antibody PM-81 with breast cancer and elimination of breast cancer cells from human bone marrow by PM-81 and immunomagnetic beads. *Cancer Research*, **51**: 2 451-2 455.

Xiang J J, Ling Q J, Lv S H, Deng N, Zhu X J, Tang Y, Yang H Y, Xie L C. 2005. The preparation and characterisation analysis of polyclonal antibodies against the 4 kinds red-tide algae. *J. Jinan University* (*Natural Science*), **26**(5): 700-704. (in Chinese with English abstract)

Yan G H, Xing D, Tan S, Chen Q. 2004. Rapid and sensitive immunomagnetic-electrochemiluminescent detection of p53 antibodies in human serum. *Journal of Immunological Methods*, **288**:47- 54.

Zhang B Y, Wang G C, Qi Y Z, Zou J Z, Tseng C K. 2005. Identification of two species of dinoflagellate using fluorescence in situ hybridization. *High Technology Letters*, **15**: 101-105. (in Chinese with English abstract)

Zhang D, Sui Z H, Wang C Y, Bao Z M. 2010. Morphological and molecular identification of a marine Nano-diatom. *Acta Oceanologica Sinica*, **32**(2): 168-173. (in Chinese with English abstract)

Zhang N G, Wang C S, Wang J T, Zhu D D, Shi X Y, Wang X L. 2008. The preliminary analysis of nutrients in harmful algal blooms in the East China Sea in the spring and summer of 2005. *Acta Oceanologica Sinica*, **30**: 153-159. (in Chinese with English abstract)