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Seasonal variations of phytoplankton phosphorus stress in the Yellow Sea Cold Water Mass

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Abstract

The Yellow Sea is located between the China Mainland and the Korean Peninsula, representing a typical shallow epicontinental sea. The Yellow Sea Cold Water Mass (YSCWM) is one of the most important physical features in the Yellow Sea. The characteristics of vertical profiles and seasonal variations of biogenic elements in the YSCWM may lead the variations of nutrient availability (e.g., phosphorus) and phosphorus stress of phytoplankton. In this study, the authors surveyed the seasonal variations of phytoplankton phosphorus stress with emphasis on the effect of the YSCWM during the four cruises in April and October 2006, March and August 2007. Using both bulk and single-cell alkaline phosphatase activity (APA) assays, this study evaluated phosphorus status of phytoplankton community, succession of phytoplankton community and ecophysiological responses of phytoplankton to phosphorus in the typical region of the YSCWM. With the occurrence of the YSCWM, especially the variations of concentration of dissolved inorganic phosphorus (DIP), the results of bulk APA appeared corresponding seasonal variations. Along Transects A and B, the mean APA in August was the highest, and that in March was the lowest. According to the ELF-labeled assay's results, seasonal variations of the ELF-labeled percentages within dominant species indicated that diatoms were dominant in March, April and October, while dinoflagellates were dominant in August. During the four cruises, the ELF-labeled percentages of diatoms except *Paralia sulcata* showed that diatoms were not phosphorus deficient in April 2006 at all, but suffered from severe phosphorus stress in August 2007. In comparison, the ELF-labeled percentages of dinoflagellates were all above 50% during the four time series, which meant dinoflagellates such as *Alexandrium* and *Scrippsiella*, sustained perennial phosphorus stress.

Key words: alkaline phosphatase, phosphorus stress, phytoplankton, Yellow Sea Cold Water Mass, Yellow Sea

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1 Introduction

Although nitrogen is always considered as the primary limiting factor in marine environment (Currie et al., 1986; Holmboe et al., 1999; Conley, 2000; Sundareshwar et al., 2003), there are more and more evidences that gradually prove phosphorus (P) may control primary production in many areas such as the Sargrasso Sea, the northern Red Sea, the eastern Mediterranean Sea, the central Atlantic and the oligotrophic Pacific gyre (Stihl et al., 2001; Vidal et al., 2003; Lomas et al., 2004; Thingstad, 2005). Phosphorus is an essential nutrient utilized by phytoplankton for energy transport and growth and phosphate availability can be an important factor influencing phytoplankton species composition and abundance. Compared to other bio-limiting nutrient and trace elements, phosphorus is often regarded as the "ultimate" limiting nutrient over long time scales (Redfiled, 1958; Tyrell, 1999; Karl, 2014).

Dissolved inorganic phosphorus (DIP) can be directly taken up by microorganisms, however, and dissolved organic phosphorus (DOP) have to be utilized by ectoenzyme which are membrane-bound or located in the periplasmatic space (Cembella et al., 1984). As an important ectoenzyme, alkaline phosphatase (AP) can cleave the phophate moiety from DOP and the free phosphate can be taken up by cells (Cembella et al., 1984; Chróst, 1991). With the highlight of P limitation or stress in the marine areas, the bioavailability of DOP has become the focus of marine ecology research (Dyhrman, 2005; Huang and Morris, 2005). Alkaline phosphatase activity (APA) is a common indicator of P deficiency and widely used as a marker of P stress in many phytoplankton (Dyhrman and Palenik, 1999), such as diatoms, dinoflagellates, green algae, cyanobacteria, and coccolithophores, which express AP under P-limit (Dyhrman and Palenik, 1997; Riegman et al., 2000; Stihl et al., 2001; Dyhrman et al., 2002; Kruskopf and Plessis, 2004). AP released by phytoplankton is regulated by extracellular and intracellular P concentration, permitting an evaluation of P-status of natural phytoplankton populations based upon APA (Dyhrman and

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Palenik, 1999; Dyhrman et al., 2002).

In the past, APA has been widely measured in freshwater systems (e.g., Pettersson, 1980; Thingstad et al., 1993). Many recent studies have proved that this indicator can also be utilized in some marine systems such as the upwelling regions (Sebastián et al., 2004a, b; Ruttenberg and Dyhrman, 2005), the North Pacific Sub-tropical Gyre (Karl et al., 2001) and the areas where high frequent HABs occurred (Huang et al., 2007). About the China's seas, some research on the areas of the Changjiang (Yangtze) Estuary and the adjacent coastal East China Sea (Hu et al., 1989; Wong et al., 1998; Huang et al., 2007), the upwelling regions of the Taiwan Strait (Ou et al., 2006; Hong et al., 2011) , the Zhujiang (Pearl) Estuary and the north of the South China Sea (Harrison et al., 1990; Yin et al., 2000) had showed that P limit or P stress also happened to some diverse extents. Furthermore, nutrient incubation experiments which imitated the status of phytoplankton *in situ* admitted that phosphorus controlled the primary production in the upper waters (Ou et al., 2008; 2010). However, few focused on the P limit or P stress in the Yellow Sea, much less on the APA as an indicator to express the P-status of the Yellow Sea.

The traditional bulk APA assays have proved that a number of sea areas are P deficiency, for instance, the northern Red Sea, the central Atlantic Ocean, the southern and central Baltic Sea (Li et al., 1998; Vidal et al., 2003; Nausch, 1998, 2004). This method is performed on bulk natural water using colorimetric and fluorogenic substrates (Rengefors et al., 2001). The main limitation of bulk measurements is that it provides little information on differences between specific AP induced by different algal taxa and between groups of planktonic organisms. A crude separation within zooplankton, phytoplankton, and bacteria can only provide ambiguous information as a function of particle size by differential filtration (Rengefors et al., 2001). A novel method called single-cell ELF (Enzyme Labeled Fluorescence) assay can overcome those problems within bulk APA assays (González-Gil et al., 1998). The single-cell APA based upon ELF can determine the P-status at individual taxon level within natural phytoplankton community and allows for *in situ* fluorescent labeling of AP between groups of planktonic organisms (Dyhrman and Palenik, 2001; Dyhrman et al., 2002; Rengefors et al., 2001, 2003; Ou et al., 2006; Huang et al., 2007). However, some investigators pointed that this method could not be adapted for detecting AP in small fragile algae and provide no information on enzyme hydrolytic rates (Rengefors et al., 2001). Due to the advantages and limitations of both methods, field studies generally utilize both methods to obtain the complete view on P-status of phytoplankton community (Ou et al., 2006; Huang et al., 2007). In this study, the authors aimed to evaluate nutrient stress status of phytoplankton community and the variation of phytoplankton community by using both bulk and single-cell APA assays. Furthermore, this study is an important part of the China GLOBEC-IMBER project.

The Yellow Sea is located between the China Mainland and the Korean Peninsula, representing a typical shallow epicontinental sea. Water properties and the general circulation in the Yellow Sea are strongly influenced by external forces in its surroundings, i.e., atmosphere, land and the ocean (Ichikawa and Beardsley, 2002). The wind over the Yellow Sea is monsoonal, northwestward in winter and southeastward in summer. The Changjiang River supplies about 80% of the total discharge of fresh water from rivers around the Yellow Sea, East China Sea and Bohai Sea. Seasonal and interannual changes of various boundary forces cause large variations in the density stratification and horizontal circulation pattern in the Yellow Sea. The Yellow Sea Cold Water Mass (YSCWM) is one of the most important physical features in the Yellow Sea (Oh et al., 2013; Zhang et al., 2008). The characteristics of vertical profiles and seasonal variations of biogenic elements in the YSCWM may lead the variations of nutrient availability (e.g., phosphorus) and nutrients stress of phytoplankton. In this study, in order to obtain comprehensive view on P-status of phytoplankton community, the authors surveyed the spatial and seasonal variations of phytoplankton P stress and phytoplankton community in the Yellow Sea during the four cruises in April and October 2006, March and August 2007. These results can help to know more about ecophysiological responses of phytoplankton to phosphorus in the typical region of the YSCWM and effects of the YSCWM on the succession of phytoplankton community in the Yellow Sea.

2 Materials and methods

2.1 *Cruises and sampling stations*

Field studies were carried on using the R/V *Beidou* during April 10–19 and October 15 to November 4, 2006, and March 14–23 and August 2–9, 2007. The sampling stations on the two transects (A and B) were shown in Fig. 1. Water samples were collected using 2.5 L Niskin bottles on a rosette sampler attached to a Seabird CTD set. At each station, a Seabird 19 CTD was used to measure the water temperature, salinity, density and fluorescence.

2.2 *Nutrient analysis*

Water was filtered through Whatman GF/F filters immediately after sampling and stored at –20 °C. The concentration of dissolved inorganic nitrogen (DIN, including NO_3^-, $NO_2^-$and NH_4^+), reactive silicate and soluble reactive phosphorus were determined colorimetrically using a flow injection analyzer (Technicon AA III autoanalyzer) (Murphy and Riley, 1962; Armstrong et al., 1967; Pai et al., 1991).

2.3 *Chlorophyll a*

The authors utilized the high-performance liquid chromatography (HPLC) method to measure chlorophyll *a* (Chl *a*). Water samples were collected from Niskin into clean polyethylene bottles. Samples were immediately filtered through 47 mm GF/F filters and a vacuum of less than 100 mm Hg. Filters were folded in half twice and wrapped in aluminum foil, labeled, and stored in liquid nitrogen until on-shore analysis. After removal from liquid nitrogen, the pigments were extracted by placing the filters in 5.0 mL 100% acetone for 24 h in the dark at –20°C. For 47 mm GF/F filters, the final extraction solution included 86% acetone and the final extraction volume was 5.8 mL. After extraction, the samples were centrifuged for 5 min to remove cellular debris. Samples and standards were injected into the HPLC system. The chromatogram was then collected on a recording device. The identities of the peaks from the sample extracts were determined by comparing their retention times with those of pure standards and algal extracts of known pigment composition. The HPLC system was calibrated with pigment standards (Sigma) obtained commercially. In order to correct any errors introduced by evaporation or experimental losses, Internal standard (canthaxanthin in acetone, Fluka) was added

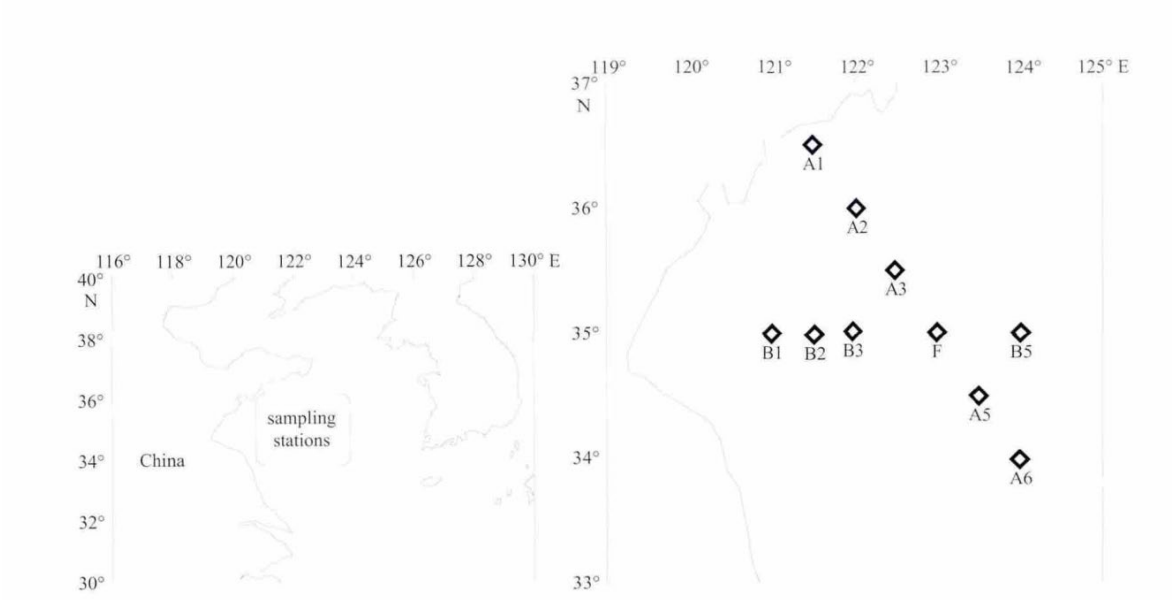

Fig.1. Sampling stations in the zone of the Yellow Sea Cold Water Mass. Two transects (A and B) were studied in four cruises. Station F (35.0°N, 123.0°E) stands for the point of intersection (Stas A4 and B4 were the same station).

to each sample. External standards were also run before each sample set for daily HPLC calibration.

2.4 *Bulk alkaline phosphatase activity assay*

Bulk APA was determined fluorometrically as the release of 4-methylumbelliferyl phosphate (MUP, Sigma), according to a modified method based on Hoppe (2003), Huang et al. (2007) and Ou et al. (2010). APA was measured in unfractionated samples in the four cruises. The water samples in triplicate with MUP substrate (final concentration 150 μmol/L) were incubated during 1–4 h at field temperature in the dark. When incubation was end, hydrolyzation of AP was terminated by 8 mmol/L $HgCl_2$ solution. The fluorescent product, 4-methylumbelliferone (MUF, Sigma), with 3-(cyclohexylamino)-1-propanesulfonic acid (CAPS, Sigma) buffer (pH 10.3), was detected using a Shimadzu (RF-5301PC) spectrofluorometer with Em=450 nm and Ex=365 nm. Control blanks in triplicate were consisted of *in situ* water samples without substrates and incubated the same as water samples. When incubation was end, MUP substrate was added in each control blank after adding 8 mmol/L $HgCl_2$ solution, and then was immediately detected by spectrofluorometer the same as water samples. Calibration was performed with standard solutions of 4-methylumbelliferone (MUF, Sigma) in range of 0–1 μmol/L. Bulk APA was expressed as nmol of MUF released L^{-1} h^{-1}.

2.5 *Single-cell alkaline phosphatase activity assay*

One to thirty liters surface seawater (according to the phytoplankton biomass *in situ*) was concentrated using 10 μm meshes and resuspended. The concentrated samples were processed for ELF labeling by centrifuging for 4 min at 3 500 *g*. The supernatant was discarded and the cell pellets were transferred to a 1.5 mL microfuge tube. According to Dyhrman and Palenik (1999) and Ou et al. (2006), the authors proceeded as following: at first, the cell pellets were incubated for 30 min with 100 μL 70% ethanol solution at 4°C in the dark. Second, the tubes were centrifuged 4 min at 3 500 *g* and the supernatant was discarded. And then, 5 μL ELF reagent (Molecular Probes) and 95 μL 0.22 μm filtered sterile *in situ* seawater was added to the cell pellets and the cells were incubated for 1 h at 4°C in the dark. After incubation, the cells were rinsed three times using sterile seawater to remove residual ELF substrate. Finally, the cell pellets were suspended in 100 μL sterile seawater and stored at 4°C in the dark before analysis by epifluorescent microscopy. The samples were observed under Leica epifluorescent microscopy with a DAPI (4′6′-diamidino-2-phenyl-indole) filter and a 100 W mercury lamp. All samples were counted using standard light (a Tungsten lamp) alternated with mercury light to check for ELF activity. The percentage of ELF-labeled cells for a given taxon was determined as the fraction of fluorescently labeled cells relative to the total number of cells counted, and was used as an index of algal sensitivity to P stress.

2.6 *Data analysis*

The statistical significance of differences in environmental parameters and APA was evaluated using Tukey's studentized range test. Correlations between APA and environmental parameters were tested by linear regression. All tests were performed using the software SPSS 13.0.

3 Results

3.1 *Environmental parameters of the field area*

The distribution of temperature, salinity, Chl *a*, DIP, DIN and salinity along Transects A and B of the field area during the four cruises in April and October 2006, March and August 2007 were shown in the Table 1. According to Fig. 1, two transects (A and B) were located in the southern Yellow Sea. In the warm seasons (August and October), the YSCWM generally existed in the north of latitude 34° and the west of longitude 122.5° in the field area (Liu, 2012), where the temperature was lower than 10°C, and salinity was higher than 33.5. In the cold seasons (March and April), the distribution of temperature, salinity and DIP from the surface to the bottom were consistent, while serious vertical stratification occurred in the warm seasons (August and October) (Figs 2–4). High-temperature and high-salt water in

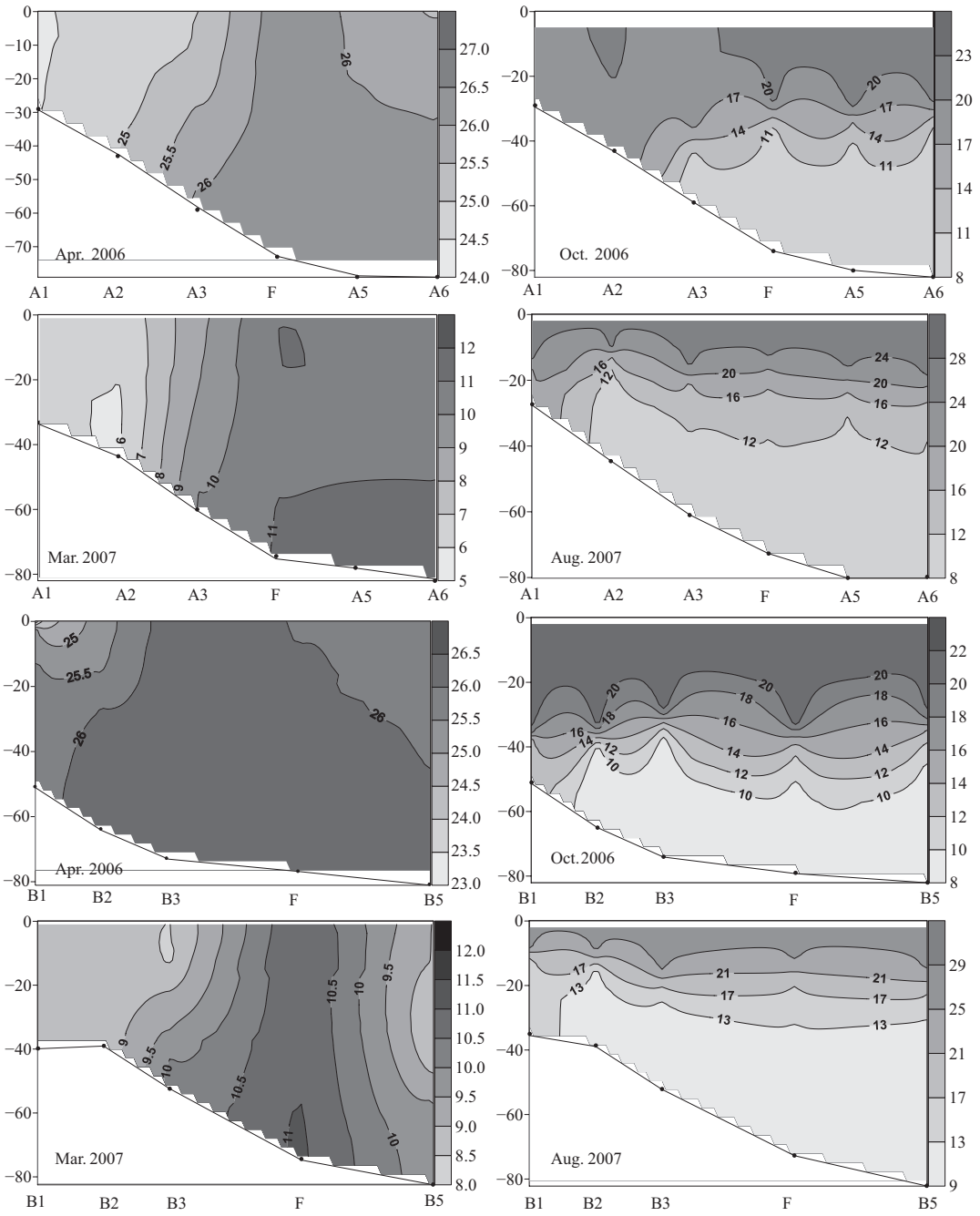

Fig.2. Vertical variations of temperature (°C) on Transects A and B during April 2006, October 2006, March 2007, and August 2007.

the central region of the Yellow Sea was observed in April (Figs 2 and 3).

The mean temperature of surface water in March and April was significantly lower than that in August and October (p<0.05). The mean salinity of the surface water in March and April was significantly higher than that in August and October (p<0.05). The concentration of Chl *a* in the surface water in April was significantly higher than that in August and October (p<0.05) and was the highest among four cruises. The distribution of DIP and DIN in the surface water showed the similar trend and the highest value was in April and the lowest in October, however, there was no significant difference among four cruises (p>0.05). The concentration of silicate in the surface water in March was significantly higher than that in April (p<0.01).

3.2 *Bulk alkaline phosphatase activity*

In the cold seasons, surface total APA ranged 2.57–19.12 nmol/(L·h), with the mean value of (11.52±5.31) nmol/(L·h) during April in 2006, while surface total APA ranged from below the detection limit to 10.11 nmol/(L·h), with a mean value of

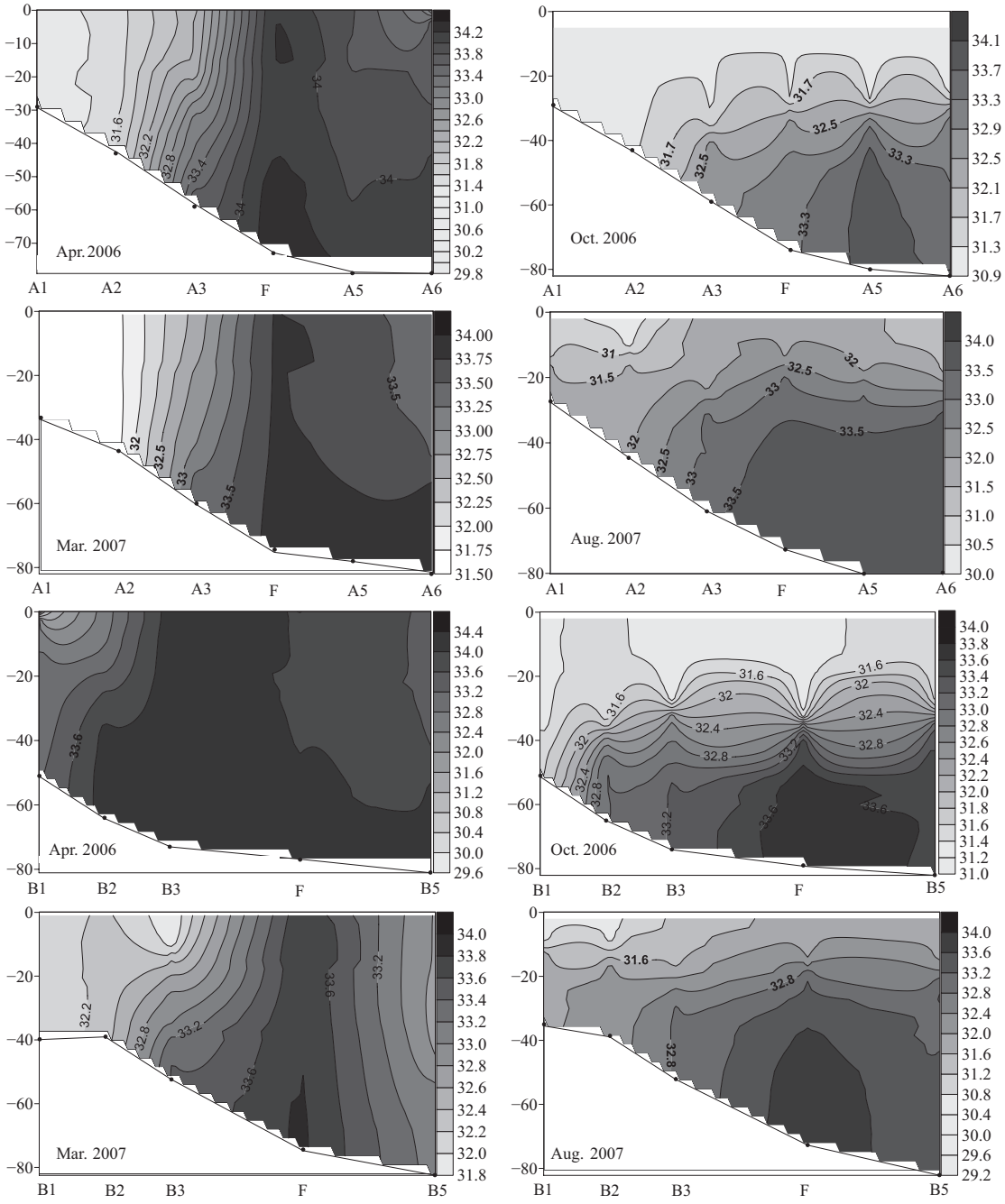

Fig.3. Vertical variations of salinity on Transects A and B during April 2006, October 2006, March 2007, and August 2007.

(3.09±3.21) nmol/(L·h) during March in 2007. In the warm seasons, surface total APA ranged 1.30–19.53 nmol/(L·h), with the mean value of (9.60±5.78) nmol/(L·h) during October in 2006, while surface total APA ranged 4.78–93.89 nmol/(L·h), with the mean value of (39.97±31.83) nmol/(L·h) during August in 2007. According to correlation tests (Table 2), total APA at surface showed fine negative correlations with salinity, DIP and DIN, and fine positive correlations with temperature and Chl *a*.

Along Transect A (Fig. 5), the vertical maximum APA occurred at Sta. A3 at 10 m layer (34.60 nmol/(L·h)) and the minimum occurred at Sta. A6 at surface (2.57 nmol/(L·h)) during April in 2006. During October in 2006, the vertical maximum APA occurred at Sta. F at 10 m layer (22.00 nmol/(L·h)) and the minimum occurred at Sta. A5 at 50 m layer (0.03 nmol/(L·h)). During March in 2007, the vertical maximum existed at Sta. F at 30 m layer (15.22 nmol/(L·h)) while minimum APA existed at Sta. A3 at surface (below the detection limit). During August in 2007, the vertical maximum lay on Sta. F at surface (93.89 nmol/(L·h)) while minimum APA lay on Sta. A3 at 58 m layer (below the detection limit).

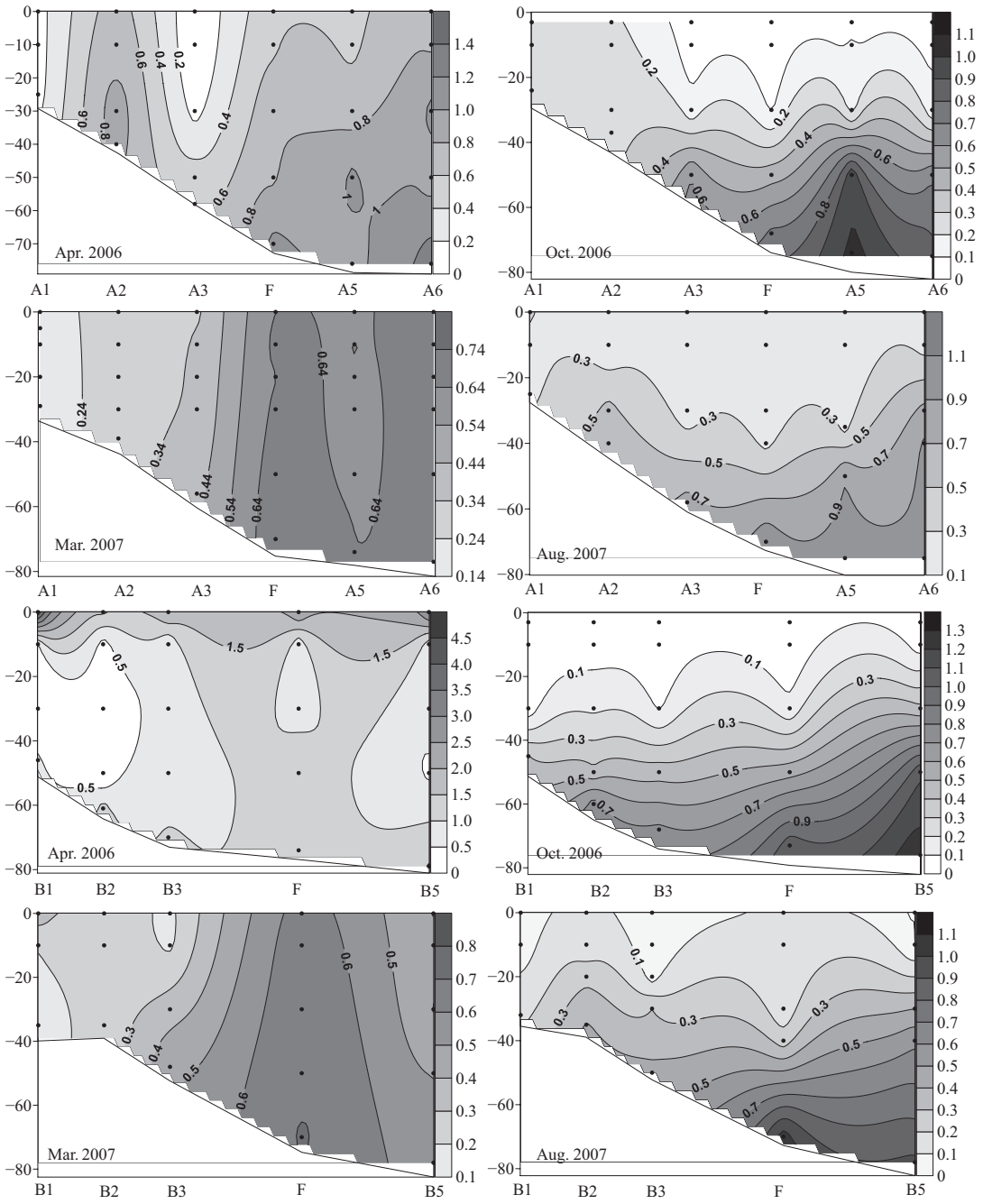

Fig.4. Vertical variations of concentration of dissolved inorganic phosphate (μmol/L) on Transects A and B during April 2006, October 2006, March 2007, and August 2007.

Along Transect B (Fig. 5) the vertical maximum APA occurred at Sta. B2 at surface (29.91 nmol/(L·h)) and the minimum occurred at Sta. B3 at 70 m layer (4.61 nmol/(L·h)) during April in 2006. During October in 2006, the vertical maximum APA occurred at Sta. F at 30 m layer (35.02 nmol/(L·h)) and the minimum occurred at Sta. B2 at 10 m layer (below the detection limit). During March in 2007, the vertical maximum APA existed at Sta. F at 30 m layer (15.22 nmol/(L·h)) while minimum APA existed at Sta. B3 at 10 m layer (0.26 nmol/(L·h)). During August in 2007, the vertical maximum APA lay on Sta. B1 at 10 m layer (114.61 nmol/(L·h)) while minimum APA lay on Sta. B5 at 40 m layer (7.52 nmol/(L·h)).

3.3 *Single-cell alkaline phosphatase activity*

During April in 2006, October in 2006, and March in 2007 (Table 3), diatoms such as *Coscinodiscus* spp., *Chaetoceros* spp., *Paralia sulcata*, *Thalassiosira* spp., and *Pseudo-nitzschia* spp. dominated the phytoplankton community at surface in the Yel-

low Sea (Table 3), and in contrast, the diversity of dinoflagellates and chrysophyta were very low, except that chrysophyta (such as *Dictyocha fibula*) was still one of the dominant species. During August in 2007, the dominant species were replaced by dinoflagellates (such as *Scrippsiella trochoidea*) at surface (Table 3).

According to Table 3, different species labeled with ELF showed different percentages of ELF straining. The percentage of ELF-labeled cells of dinoflagellates was higher than that of chrysophyte or diatoms, although differences still existed among the dinoflagellate species. The results of ELF straining presented seasonal variations during the four time intervals (Table 3). The percentages of total ELF-labeled cells during August in 2007 were higher than those during other three time intervals, except *Paralia sulcata* and *Dictyocha fibula*, and this result corresponded favorably with bulk APA assay. The percentages of ELF straining *Paralia sulcata* did not change significantly among the four time intervals. The percentage of ELF straining *Dictyocha fibula* during August in 2007 was lower than that during October in 2006. For diatoms, the percentage of ELF straining was mostly lowest during April in 2006. However, for some dinoflagellates, such as *Scrippsiella trochoidea* and *Alexandrium* spp., the percentages of ELF straining were all above 50% during four time intervals, and above 90% during August in 2007.

4 Discussion

4.1 *Effect of YSCWM on P stress of phytoplankton*

The YSCWM is a unique hydrographic phenomenon in the

Table 1. Variations of the environmental parameters at the surface layer among April 2006, October 2006, March 2007, and August 2007

Parameter	Apr. 2006		Oct. 2006		Mar. 2007		Aug. 2007	
	Range	Mean	Range	Mean	Range	Mean	Range	Mean
T/°C	8.1–13.5	10.4±1.9	19.5–22.8	20.9±1.1	6.2–11.0	9.0±1.6	25.7–27.9	26.8±0.6
S	29.7–34.2	32.2±1.6	30.9–31.5	31.2±0.2	31.6–33.8	32.6±0.8	29.8–31.9	31.2±0.7
Chl *a*/mg·m^{-3}	0.52–5.66	1.80±1.61	0.04–0.81	0.40±0.23	0.50–1.19	0.80±0.22	0.05–3.22	0.46±0.93
DIP/μmol·L^{-1}	0.07–0.77	0.48±0.23	0.03–0.22	0.10±0.06	0.13–0.71	0.41±0.21	0–0.32	0.13±0.08
DIN/μmol·L^{-1}	8.83–20.94	15.07±3.55	1.40–4.25	2.43±0.99	1.95–10.47	5.99±3.25	2.06–10.44	5.66±3.53
Silicate/μmol·L^{-1}	0–5.78	2.51±2.05	3.16–7.73	4.60±1.32	0.13–12.68	5.89±4.49	1.71–5.71	3.70±1.26
Bulk APA/nmol·L^{-1}·h^{-1}	2.57–19.12	13.17±7.76	1.30–19.53	12.49±10.32	BD–10.11	8.74±12.32	4.78–93.89	38.25±26.63

Note: BD represents below the detection limit.

Table 2. Correlations of bulk APA with different parameters at the surface layer on Transect A and B, and the significance level (*P*) during April 2006, October 2006, March 2007, and August 2007

Parameter	Apr. 2006		Oct. 2006		Mar. 2007		Aug. 2007	
	R	*P*	*R*	*P*	*R*	*P*	*R*	*P*
T/°C	0.241		0.478	*	0.604	**	0.249	
S	–0.066		–0.418	*	–0.614	**	–0.490	*
DIP/μmol·L^{-1}	–0.169		–0.475	*	–0.663	**	–0.433	*
DIN/μmol·L^{-1}	–0.531	**	–0.408	*	–0.124		–0.065	
Chl *a*/mg·m^{-3}	0.573	**	0.463	*	0.133		0.471	*

Notes: *R* represents correlation coefficient, + and – positive and negative correlation between bulk APA and specific parameter, * $P<0.05$, and ** $P<0.01$.

Table 3. Summary of phytoplankton taxa at the surface layer in the Yellow Sea and single-cell enzyme-labeled fluorescence (ELF) labeling during April 2006 October 2006, March 2007, and August 2007

Taxa	Apr. 2006		Oct. 2006		Mar. 2007		Aug. 2007	
	Cell count	ELF/%	Cell count	ELF/%	Cell count	ELF/%	Cell count	ELF/%
Bacillariophyta								
Coscinodiscus spp.	632	0	962	51	2 147 (*)	39	168	74
Chaetoceros spp.	532	0	1 560 (*)	31	456	28	543	59
Skeletonema spp.	572	1	316	8	711	10	—	—
Paralia sulcata	1 158	0	1 171 (*)	9	2 693 (*)	15	360	13
Pseudo-nitzschia spp.	404	0	1 232 (*)	23	—	—	134	40
Thalassiosira spp.	1 966 (*)	1	282	57	621	23	—	—
Chrysophyta								
Dictyocha fibula	—		1 101 (*)	71	—	—	318	58
Pyrrophyta								
Alexandrium spp.	1 451	63	478	79	489	65	414	95
Scrippsiella trochoidea	951	50	317	63	332	64	847 (*)	97

Notes: * represents dominant species and — no detection.

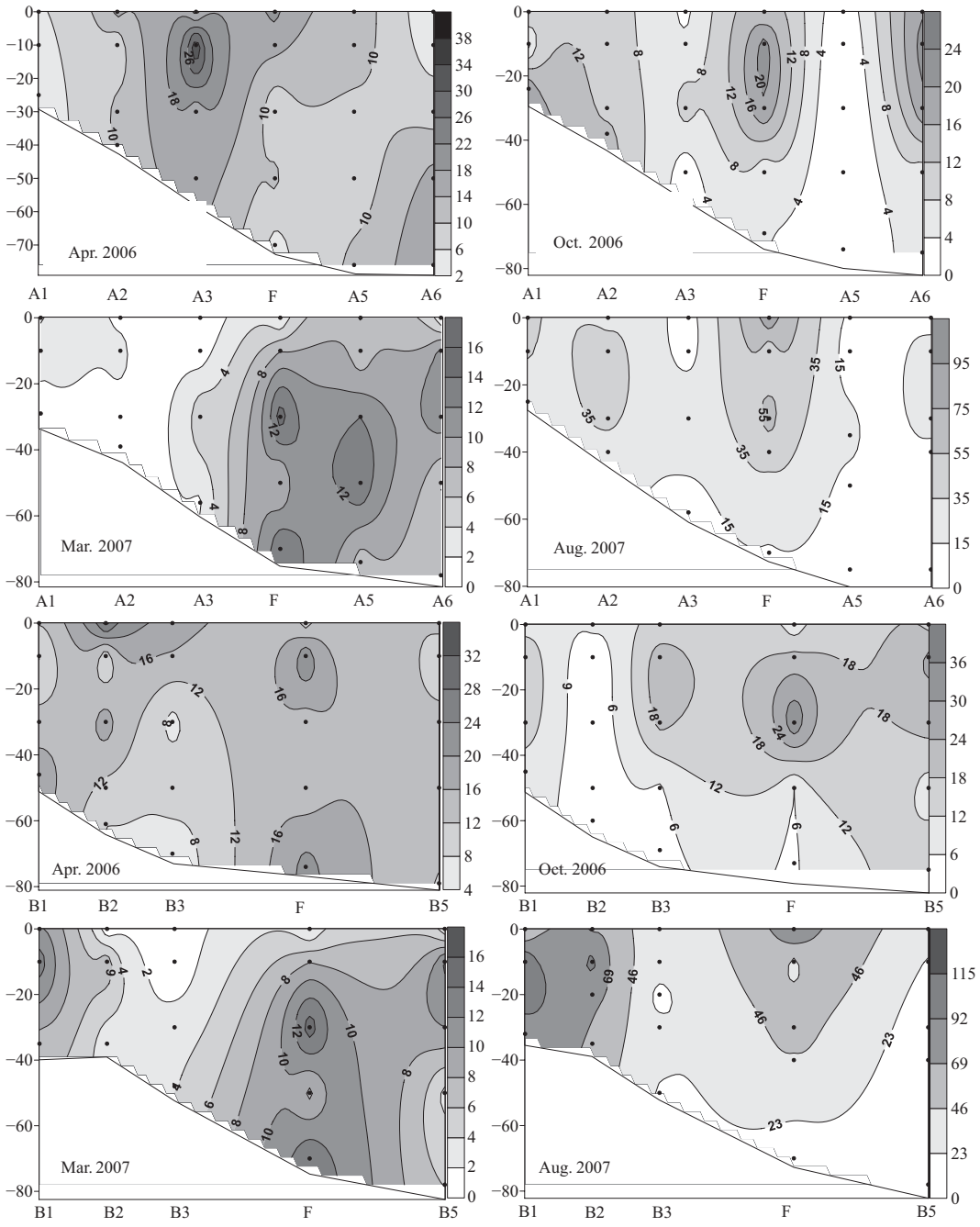

Fig.5. Vertical variations of bulk APA (nmol/(L·h)) on Transects A and B during April 2006, October 2006, March 2007, and August 2007.

Yellow Sea. Yu et al. (2006) indicated that the Qingdao Cold Water Mass, Ren-Can Off-Shore Cold Water Mass, and the bottom cold water in the northern East China Sea are embodied in the YSCWM. The special hydrographic environment has significant influence on the distribution and ecological function of nutrients in the Yellow Sea. The seasonal stratification in the central Yellow Sea occurs from spring to late autumn, which makes the nutrients in the surface water deplete in summer and accumulate the nutrients in the deep water below the thermocline, however, the nutrients in the bottom water will recruit back to upper water again in the cold seasons because of vertical mixing. In fact, the results of this study had proved that (Figs 2–4). During March and April, temperature and salinity distributed evenly because water body mixed vertically very well, so that the concentration of DIP from surface to bottom was distributed almost evenly. Conversely, DIP was accumulated in the bottom during August and October due to the stratifications, so that the bottom water in the central Yellow Sea became a large

"bank" of nutrients. What is more, the authors found that the most serious stratification phenomenon occurred in August. The YSCWM was a typical water mass with low temperature, high salinity and high nutrients in summer in the central Yellow Sea. Those above corresponded with the results of bulk APA (Fig. 5) and single cell APA assays and their seasonal variations (Table 3).

According to previous studies, the YSCWM brews in spring, blooms in summer, decays in autumn, and disappears in winter (Yakahashi and Yanagi, 1995; Ren and Zhan, 2005). There are two recognized reasons to accelerate the formation of the YSCWM: one is the formation of thermocline, the other is the movement of mixed tidal currents (Ren and Zhan, 2005). In spring, the nutrients of the Changjiang River diluted water were inputted to the Yellow Sea. The waters with high concentrations of DIN, phosphate and silicate covered the whole coastal areas from 26°N to 35°N, and extended the southwest of the Cheju Island at about 126°E, but the concentrations of nutrients in the northern and eastern Yellow Sea were very low (Beardsley et al., 1985). In summer, the Changjiang River diluted water could cover more than three quarters of the Yellow Sea and the extending range of phosphate in water is much smaller than that of DIN and silicate (Wang, 2003). In autumn, the prevailing northeastern wind and the low discharge confined the influence of the Changjiang River water to a narrow band southward along the coast (Beardsley et al., 1985), so the waters with high concentrations of DIN, phosphate, and silicate were found in the southern Yellow Sea. Because of the influence of terrestrial runoff from the Korea Peninsula, the eastern coastal water was also rich in DIN and phosphate, but nitrogen-deficient in the northwest of Yellow Sea. In winter, the Yellow Sea warm current water went northwestward to enter the Yellow Sea and the YSCWM absolutely disappeared. The distribution pattern of nutrients in winter is quite similar to that in autumn. For this study, the most serious stratification phenomenon occurred in August (Figs 2–4). The northern boundary was extended to Sta. A2 and the western boundary was extended to Sta. B2. The YSCWM with low temperature, high salinity and high phosphate mainly stayed below 30 m in August and 45 m in October (Figs 2–4). P-deficient often occurred in the euphotic layer when the YSCWM existed (Fig. 4).

In the case of Sta. F, the seasonal variations of DIN:DIP ratio were showed in the Fig. 6. In cold seasons, although DIN:DIP ratios at surface were higher than other water layers, there were no sign of thermocline and P-limit. In contrast, stratification phenomenon occurred between 30 m and 40 m in warm seasons, so that there was an obvious difference for DIN:DIP ratio between upper water and deep water. In warm seasons, obvious P-limit existed from surface water to 30 m layer; however, there were no sign of nutrients limitation below 40 m. According to Fig. 4, P-deficiency often occurred in the euphotic layer when the YSCWM existed in warm seasons, which agreed to the results of Fig. 6. Therefore, the bottom water in the central Yellow Sea was exactly a large "bank" of nutrients, which could supply the growth of phytoplankton community and alleviate nutrients deficiency in the euphotic layer in the next spring. It was said that, if N:P ratio was greater than 30 *in situ*, the field area was under P-limit, and if less than 5, it was under nitrogen limitation (Beardall, Berman et al., 2001). However, some researchers proposed that the determination of nutrient concentration and N:P ratio could not fully illustrate the nutrients limitation *in situ* (Beardall, Yong et al., 2001). As a single index, it was hard for N:P ratio to avoid its shortcoming and bias when it evaluated nutrients limitation.

4.2 *Phosphorus stress on the natural phytoplankton community*

With the occurrence of the YSCWM, the results of total APA displayed corresponding seasonal variations. Along Transect A and B, the mean value of APA in August 2007 was much higher than other time, and that in March 2007 was the lowest. In August, maximum APA along two transects lay on Sta. F at surface (93.89 nmol/(L·h)) and Sta. B1 at 10 m layer (114.61 nmol/(L·h)) while minimum APA lay on Sta A3 at 58 m layer (below the detection limit) and Sta. B5 at 40 m layer (7.52 nmol/(L·h)). Therefore, phytoplankton in the euphotic layer suffered from much more serious P stress than that in the deep layers. In this study, the authors counted the total APA as bulk APA, but did not measure the relative levels of soluble and cell-bound APA (Li et al., 1998). Using polycarbonate filter to fractionate different size of organisms, there are still some defects: (1) AP is extracellular phosphatase and generally locates outside the cell membrane (Cembella et al., 1984). It is a "slow" natural process to release the dissolved enzymes (Hoppe, 2003). Moreover, it is too difficult to control strength of filtration, hereby it may force some AP on the cell dissolving into the ambient medium (Bruckmeier et al., 2005), so that the results will underestimate cell-bound APA while overestimate soluble APA; (2) There are still some picophytoplankton or bacteria which are below 2 μm size, so the results may oversimplify differentiate several groups of organisms by 0.2, 2 or 3 μm size and are ambiguous to count the different size-fractions. Nedoma et al. (2006) analyzed saturation kinetics of different size-fractions revealed the kinetic heterogeneity of APA. Their results found that high (<2 μm size-fraction) and low

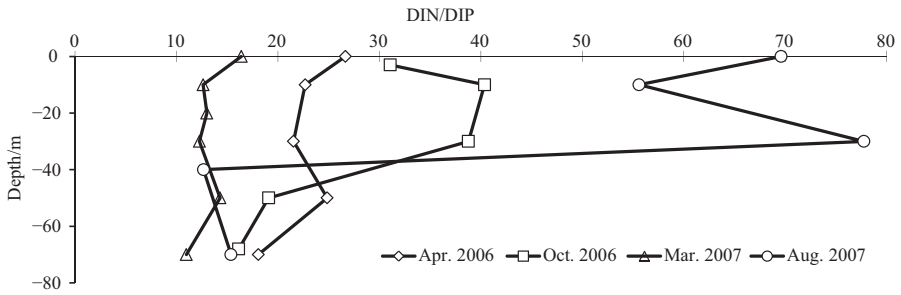

Fig.6. Seasonal variations of DIN:DIP ratio on Sta. F.

(>2 μm size-fraction) affinity components represented independent pre-existing enzymes, or groups of kinetically similar enzymes, mostly probably located at different organisms. Using the same substrate (MUP), the results of total (unfractionated) APA in this study stood for the complex sum of algal and bacterial enzymes activities. Thereby, this result may overlook the independent nature of heterogeneity of APA. Due to including the bacterial APA, the results of total APA on Transect A and B did not always show obvious negative correlations with the concentration of DIP (Tables 1 and 2, Figs 4 and 5). Some previous studies had proved that the mechanisms of regulation of bacterial phosphatases remained obscure and seemed that bacteria induced AP to hydrolysis P compounds to supply the organic moiety for their C and N demands but not for P (Benitez-Nelson and Buesseler, 1999; Hoppe, 2003).

4.3 *P stress on species-specific phytoplankton*

Although the traditional bulk APA bioassays can detect the nutrient status of a natural phytoplankton community, the methods provide little information on differences among specific AP induced by different algal taxa or groups of planktonic organisms. In fact, the bulk APA is an average response to P-status of the whole phytoplankton community, and counting the different size-fractions has no clear information on the P-status of different algal taxa. In contrast, the ELF assays aims at the detection of AP at the single cell level in freshwater and seawater (González-Gil et al., 1998; Dyhrman and Palenik, 1999; Rengefors et al., 2001). The ELF-97 substrate (Molecular Probes) reacts with AP which locates on cell surface and forms a fluorescent precipitate at the site of APA. Using epifluorescence microscopy, it is possible to recognize ELF-labeled AP sites according to fluorescent signal. Hereby, this method can easily verdict the P-status of species-specific phytoplankton.

According to the ELF-labeled assay's results (Table 3), seasonal variations of the ELF-labeled percentages within dominant species indicated that diatoms were dominant in March, April and October, while dinoflagellates were dominant in August. During the four cruises, the ELF-labeled percentages of diatoms except *Paralia sulcata* showed that diatoms were almost not P-deficient in April 2006, but suffered from severe P-stress in August 2007. In comparison, the ELF-labeled percentages of dinoflagellates were all above 50% during the four time series, which meant dinoflagellate such as *Alexandrium* and *Scrippsiella* sustained perennial P stress. In August 2007, the ELF-labeled percentage of dominant species (*Scrippsiella trochoidea*) was up to 97%, which was the highest during the four cruises. Whereas, for some species, such as *Paralia sulcata* and *Dictyocha fibula*, there was no distinct seasonal variations. The highest ELF-labeled percentages of diatoms and dinoflagellates were corresponding to the highest total APA in August. For dinoflagellates, they were poor competitors for phosphate compared to diatoms; they might utilize AP to hydrolyze DOP in order to satisfy their P demand, even when phosphate is available (Rengefor et al., 2003). Dyhrman and Palenik (1999) also suggested that AP of some dinoflagellates was possibly a kind of constitutive phosphatases. Whether or not constitutive phosphatase for dinoflagellates was hardly to resolve in this field experiment. Some studies thought dinoflagellates might develop some unique mechanisms or strategies to compete for nutrients, such as storing surplus nutrients in the internal nutrient pool, switching to utilize dissolved organic nutrients, nutrient retrieval migrations, or mixotrophic nutrition tendencies (Nygaard and Tobiesen, 1993; Anderson et al., 2002; Smayda and Reynolds, 2003; Huang et al., 2007; Ou et al., 2008). Otherwise, the authors found that single cells in the same population did not display the same physiological and morphological status in the ELF-labeled assay. The possible explanations for these phenomenon referred to heterogeneity in the particular microclimate, individual differences in nutritional history, life cycle stage, growth rate, and genetic make-up (Dyhrman and Palenik, 2001; Rengefor et al., 2003). In this study, ELF-labeled assay focused on the specific phytoplankton population (>10 μm fraction), and therefore bacterial cells and other phytoplankton were easy to be visually distinguished from true algal cells. At present, the ELF-labeled assay is still a qualitative or semi-quantitive method, so the authors suggested that this method should be combined with bulk APA assay to evaluate the P stress qualitatively and quantitatively. Using the both methods, researchers can assess inducible APA of phytoplankton from a natural community level down to an individual taxa. Nowadays, some researchers are trying to involve flow cytometry, as well as other immunological assays like microscopical measurement, into ELF assays to integrate a novel quantitative method (González-Gi et al., 1998; Dignum et al., 2004; Bruckmeier et al., 2005; Dyhrman, 2005). Further studies are being carried out to actually deduct the above errors and ambiguity in order to integrate several methods.

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