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The modification and optimizing of the CHEMTAX running in the South China Sea

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Abstract
In order to determine the phytoplankton community composition, the modification and optimizing of the CHEMical TAXonomy (CHEMTAX) running was carried out through samples grouping, successive run and evaluate the results for HPLC-pigment samples in the South China Sea (SCS). The vertical distribution of the ratio of pigment to total Chl *a* (TChl *a*) exhibited three different patterns, including increasing with depth pattern (e.g., But-Fuco), decreasing with depth pattern (e.g., Zea) and increasing at deep chlorophyll maximum (DCM) pattern (e.g., Hex-Fuco). The vertical profiles for Fuco/TChl *a* and Pras/TChl *a* was higher in coast than in the shelf and basin, and the Zea and Dv-Chl *a* expressed conversely. So the samples in the coastal stations must be separated for the cluster analysis group procedure in the SCS. Successive run was introduced into the CHEMTAX calculation and the output results were evaluated by the convergence of pigment/TChl *a* ratios. Most of the ratios were well converged at the fifth running, except Zea/TChl *a* for *Prochlorococcus* and Chl b/TChl *a* for prasinophytes and so on. To evaluate the fifth running's results, haptophytes_8 and chlorophytes were two phytoplankton groups with much uncertainty. But the fifth estimated value was better than running once was supported by the regression evidence between the measured pigment concentration and calculation values. *Synechococcus* was another component with much mutability, and the CHEMTAX's result should be compared to the flow cytometry's cell abundance.

Key words: phytoplankton, CHEMTAX, pigment/Chl *a* ratio, successive run, South China Sea

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1 Introduction

Phytoplankton is the major primary producer in the ocean ecosystem and supply around 50% of the organic carbon through the photosynthesis (Field et al., 1998). The standing biomass and dynamic variation of phytoplankton was the essential question in both marine biology and biological oceanography studies. In addition, the composition of phytoplankton in natural condition was great variable no matter at the species or community level (Fasham, 2003).

The methods to indentify the species or community composition of phytoplankton were developing through the biological oceanography studies, including the microscopy (Utermöhl, 1958; Johnson and Sieburth, 1979), the chemical biomarker (Zapata et al., 1987; Gieskes et al., 1988), flow cytometry (FCM) (Sieracki et al., 1998) and molecular biology (Saldarriaga et al., 2001). Photosynthetic pigments were analyzed as an indicator for their specificity among different phytoplankton groups (Ston and Kosakowska, 2000). The remarkable superiority for this identification indentify method is the exhibiting of the full size fractional phytoplankton groups.

The calculation from pigments to the Chl *a* biomass of different phytoplankton groups had a trilogy progress which from linear regression (Gieskes and Kraay, 1983) to multiple simultaneous equation (Everitt et al., 1990; Letelier et al., 1993) and to matrix factorization (Mackey et al., 1996). The matrix factorization defining method was the CHEMTAX (Mackey et al., 1996). It is a program to estimate the contribution of different phytoplankton class or group to the total Chl *a* (TChl *a*) biomass, using the pigments data matrix and the individual pigment/TChl *a* ratio matrix (Mackey et al., 1996, 1997). So the choice of the initial pigment/TChl *a* ratio could affect the calculation of the biomass seriously (Goericke and Montoya, 1998, Jeffrey et al., 1999). The artificial grouping in the pigments samples and assessing the output pigment/TChl *a* ratio's reliability might make more trouble generation during the calculation, much less the response of pigment/TChl *a* ratio to the environmental factors (e.g., light and nutrients) (Latasa, 2007). So in this study, we aimed to evaluate the calculation results of CHEMTAX and optimize the rules or essential points which were important for the CHEMTAX running in the SCS.

2 Methods

2.1 *Photosynthetic pigments*

Seawater samples (4–16 L) for pigment analysis were filtered onto Whatman GF/F filters of 47 mm diameter under gentle vacuum (<150 mmHg). The filters were wrapped with aluminum foil and frozen stored in liquid nitrogen until analysis. When tranport-

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ed to the laboratory the frozen samples were displaced in freezer (−80℃). The pigment concentrations were detected using High Performance Liquid Chromatography (HPLC) following standard method (Zapata et al., 2000; Mendes et al., 2007). The frozen filter was soaked in 2 mL N, N-dimethylformamide (DMF) extraction in a freezer (−20℃) for 2 h (Furuya et al., 1998). The extractions were then filtered through Whatman GF/F filters of 13 mm diameter (Swinnex Filter Holder) to clean the debris then mixed with ammonium acetate solution (1 mol/L) (600 μL: 600 μL). Each mixture was partially injected into an Agilent series 1100 HPLC system fitted with a 3.5 μm Eclipse XDB C_8 column (100 mm×4.6 mm; Agilent Technologies). Quantification was confirmed by the standards manufactured by Danish Hydraulic Institute (DHI) Water and Environment, Hørsholm, Denmark. The abbreviation of pigments and affiliation to each phytoplankton groups were listed in Table 1.

Table 1. List of abbreviation of pigments and affiliation to each phytoplankton groups (adapted and modified from Jeffrey et al. (1997)

Pigments	Abbr.	Dinoflagellates Dino	Diatoms Diat	Haptophytes_8 Hapt_8	Haptophytes_6 Hapt_6	Chlorophytes Chlo	Cryptophytes Cryp	*Prochlorococcus* Proc	*Synechococcus* Syne	Prasinophytes Pras
Chlorophylls										
Monovinyl chlorophyll *a*	Chl *a*	•	•	•	•	•	•		•	•
Divinyl chlorophyll *a*	Dv-Chl *a*							•		
Total chlorophyll *a*	TChl *a*									
Monovinyl chlorophyll *b*	Chl *b*					•				•
Divinyl chlorophyll *b*	Dv-Chl *b*						•			
chlorophyll c_1+c_2	Chl c_1+c_2	•	•	•	•		•			
chlorophyll c_3	Chl c_3			•	•					
Xanthophylls										
Alloxanthin	Allo						•			
19'-Butanoyloxyfucoxanthin	But-Fuco			•	•					
Diadinoxanthin	Diadino	•	•	•	•					
Diatoxanthin	Diato	•	•	•	•					
Fucoxanthin	Fuco		•	•	•					
19'-Hexanoyloxyfucoxanthin	Hex-Fuco				•					
Lutein	Lut					•				•
Neoxanthin	Neo					•				•
Peridinin	Peri	•								
Prasinoxanthin	Pras									•
Violaxanthin	Viol					•				•
Zeaxanthin	Zea					•		•	•	

2.2 *CHEMTAX running*

The chemical taxonomy program, CHEMTAX, was applied under MATLAB (The MathWorks, Inc., Natick, Massachusetts) platform to acquire the relative contributions of taxa to TChl *a*. Thirteen pigment markers were introduced to quantify each fraction of the TChl *a* pool of nine phytoplankton classes, including dinoflagellates (Dino), diatoms (Diat), haptophytes_8 (Hapt_8), haptophytes_6 (Hapt_6), chlorophytes (Chlo), cryptophytes (Cryp), *Prochlorococcus* (Proc), *Synechococcus* (Syne) and prasinophytes (Pras). The ratios of initial inputting pigment to Chl *a* (F_{input}) followed the processes using in previous studies (Table 2) with the addition of Dv-Chl *a* for *Prochlorococcus* for the SCS (Table 3) (Mackey et al., 1996). According to the rule of running CHEMTAX mentioned by Latasa (2007), successive runs were necessary to gain the convergence between input and output ratio (F_{output}).

2.3 *Statistic analysis*

Figures were drawn by OriginPro 8.5 (OriginLab Corporation, Northampton, MA, USA). The independent-samples T-test, One-Way ANOVA and Duncan's multiple range test was deal by PASW® Statistics 17.0 software.

3 Results

3.1 *Samples' group dividing*

Three vertical distribution patterns of pigment/TChl *a* ratio were concluded, including the increasing pattern (IP), increasing with depth (Fig. 1a); decreasing pattern (DP), decreasing with depth (Fig. 1b); and biomass pattern (BP), increasing at the DCM (Fig. 1c).

For IP, But-Fuco, Fuco, Allo and Chl c_3 were classified to the pigment/TChl *a* ratio's depth increasing. The increasing mainly happened in the euphotic layer and had the increasing tendency but drastic variation under the euphotic layer (around 100 m). In the euphotic zone, almost all the four pigments' ratio maximum was at the 75 m layer. There was 5.5-fold for But-Fuco, 1.8-fold for Allo and 1.4-fold for Chl c_3 ratio increasing which was compared between 75 m and the surface, respectively. The Fuco/TChl *a* ratio was slightly different from the other three pigments above, as the ratio's minimum was at the 50 m layer and 1.5-fold higher at 75 m.

For DP, the ratio decreasing happened on the Zea, Peri, Viol and Diat. The bottom of euphotic zone was also chosen as the boundary because of the variation at deep layer. The decreasing percentage was 85% for Zea, 41% for Peri, 54% for Viol and 70% for Diat at 75 m relative to the surface maximum, respectively.

The Hex-Fuco, Pras, Chl *b* and Dv-Chl *a* followed the BP distribution structure. The ratio increased 1.9-fold for Hex-Fuco, 4.1-fold for Pras, 2.7-fold for Chl *b* and 1.2-fold for Dv-Chl *a* comparing between DCM and surface, respectively.

Even though the remarkable vertical distribution patterns for different pigments, the group dividing could not proceed according to the depth or light attenuation directly due to the greater variation was exhibiting among the different horizontal scale (Fig. 2). The distribution pattern in the coast was somehow different from what in the shelf and basin. The Fuco/TChl *a* ratio was higher in

the coast than in the shelf and basin obviously (Fig. 2a), and Pras/TChl *a* ratio also had the same status. But the other pigment/TChl *a* ratio was nearly higher in the shelf and basin than in the coastal zone (Figs 2b and c). It followed that when dealing with the group dividing in the SCS, the coast sample must be isolated from the samples in shelf and basin. For the latter, grouping according to the depth or light attenuation might be suitable for CHEMTAX (six groups, 0–10 m, 11–30 m, 31–60 m, 61–90 m, 91–130 m and 131–200 m). But the samples in the coast should be processed alone and divided into sub-grouping under the principle of cluster analysis.

Table 2. Initial inputting pigment/Chl *a* ratio for the Southern Ocean (Mackey et al., 1996)

	Peri	But-Fuco	Fuco	Hex-Fuco	Neo	Pras	Viol	Allo	Lut	Zea	Chl *b*	Chl *a*
Pras (T3)					0.15	0.32	0.06		0.01		0.95	1.00
Dino	1.06											1.00
Cryp								0.23				1.00
Hapt (T3)				1.70								1.00
Hapt (T4)		0.25	0.59	0.54								1.00
Chlo					0.06		0.06		0.20	0.01	0.26	1.00
Syne										0.35		1.00
Diat			0.75									1.00

Table 3. Initial inputting pigment/Chl *a* ratio for the SCS

	Peri	But-Fuco	Fuco	Hex-Fuco	Neo	Pras	Viol	Allo	Lut	Zea	Chl *b*	Dv-Chl *a*	Chl *a*
Dino	1.06												1.00
Diat			0.75										1.00
Hapt (T8)		0.25	0.59	0.54									1.00
Hapt (T6)				1.70									1.00
Chlo					0.06		0.06		0.20	0.01	0.26		1.00
Cryp								0.23					1.00
Proc										0.37	0.68	1.00	
Syne										0.35			1.00
Pras					0.15	0.32	0.06		0.01		0.95		1.00

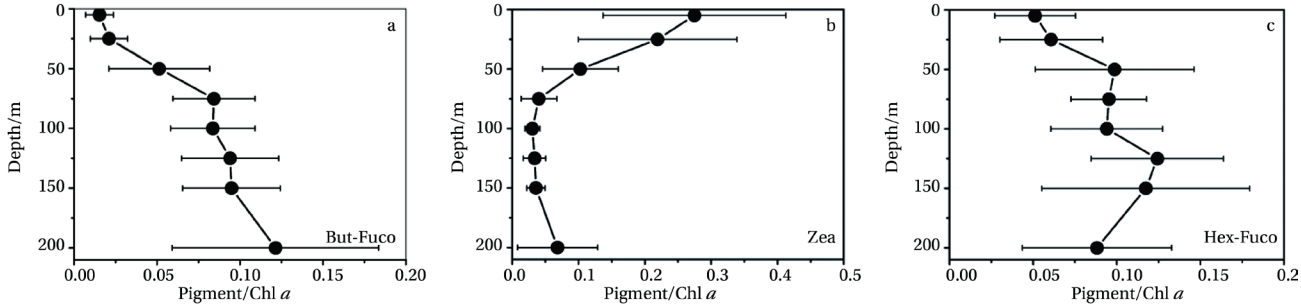

Fig. 1. Three vertical distribution pattern of Pigment/TChl *a* in the SCS. a. But-Fuco/TChl *a*, b. Zea/TChl *a* and c. Hex-Fuco *a*/TChl *a*.

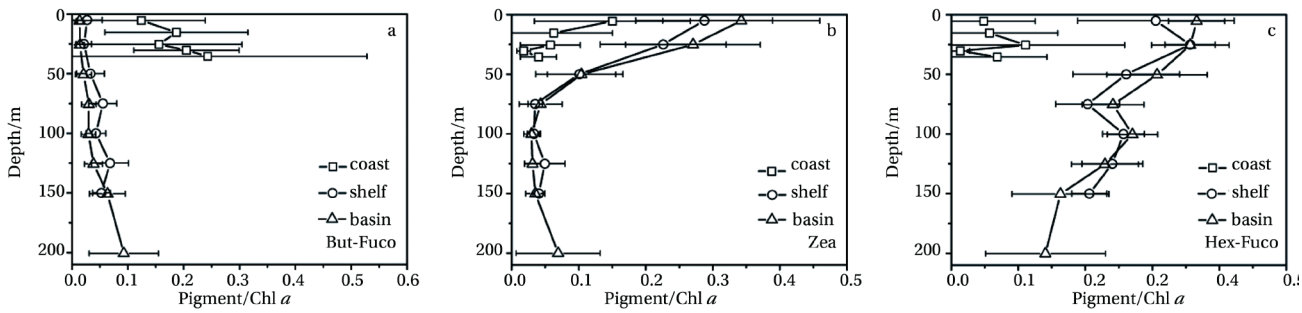

Fig. 2. Pigment/TChl *a* ratios distribution pattern in the SCS coast, shelf and basin. a. Fuco/TChl *a*, b. Zea/TChl *a* and c. Dv-Chl *a*/TChl *a*.

3.2 *Successive running*

Ten times successive run of CHEMTAX was applied in the SCS to evaluate the variation of F_{output} from F_{input} under the rules of Latasa (2007). As the using of group dividing processing mention-

ed above, different group had the same response or could be distinguished for each F_{output} (Fig. 3).

Three convergence patterns were acquired from the running. The first one was well converged no matter how many times' running, for examples, the Allo/TChl *a* for Cryp (Fig. 3a) and Zea/TChl *a* for Chlo (Fig. 3b). This pattern always occurred in the pigment whose specificity was intense and rarely shared by different phytoplankton groups, like Peri, Zea, Chl *b* and Allo. The second pattern included the Hex-Fuco/TChl *a* for Hapt_8 (Fig. 3c), Pras/TChl *a* for Pras (Fig. 3d) and But-Fuco/TChl *a* for Hapt_8 which the ratio could access convergence after successive running. The last one was those ratio hard to be converged, like Zea/TChl *a* for Proc (Fig. 3e), Viol/TChl *a* for Chlo and Chl *b*/TChl *a* for Pras. These pigments were shared by different phytoplankton groups, like Chl *b* for Chlo and Pras. The Fuco/TChl *a* for Diat was more complex as most of groups was well converged but a dramatic deviation occurred in the group of 31–60 m. This condition was also found in the Peri/TChl *a* for Dino and Hex-Fuco/TChl *a* for Hapt_6 in the same group which might be the results of the variation of the pigment concentration and the fluctuation of the DCM.

The status of convergence among groups also showed some distinction. The surface group did not have any change in each ratio. But the deeper groups had more deviation from the initial F_{input} even though they could be converged finally. Most of the groups could get the steady ratio at the fourth or fifth running, and there were no evidence for the better F_{output} when running more times.

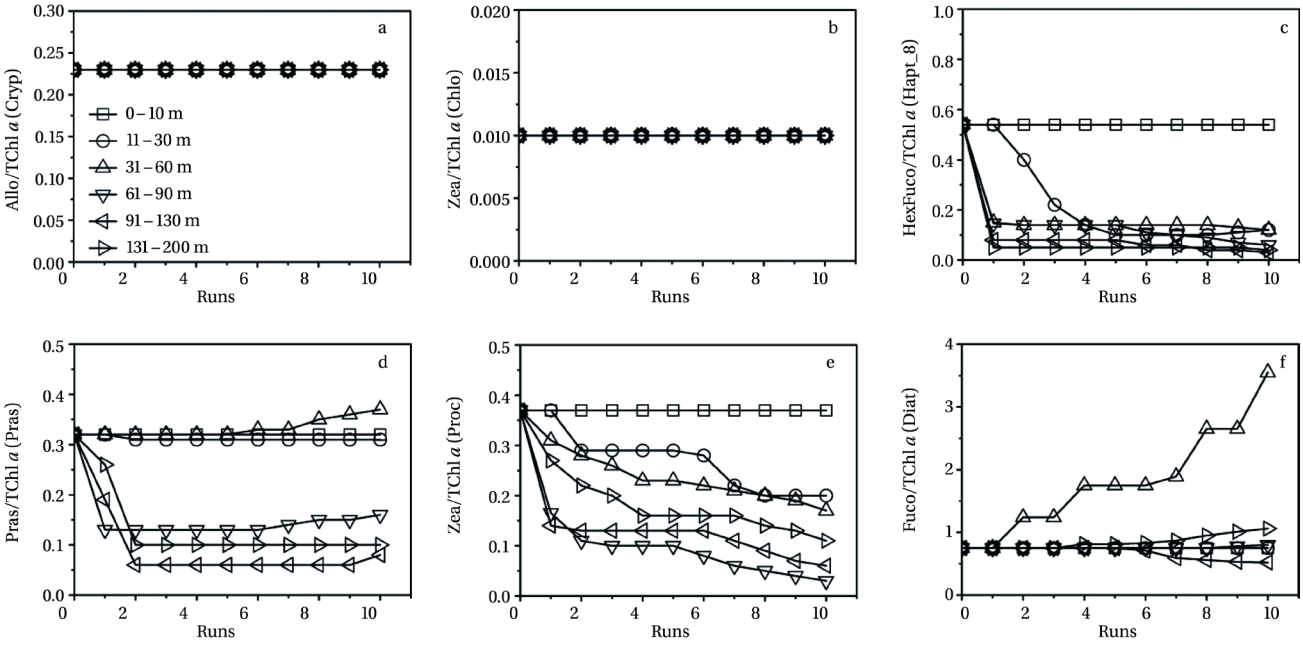

Fig. 3. Pigment/TChl *a* ratios after successive running of CHEMTAX in the SCS. a. Allo/TChl *a* for Cryp, b. Zea/TChl *a* for Chlo, c. Hex-Fuco/TChl *a* for Hapt_8, d. Pras/TChl *a* for Pras, e. Zea/TChl *a* for Proc, and f. Fuco/TChl *a* for Diat.

3.3 *Assessment of the output results*

Comparison was introduced between the results of the fifth running and only once to find the variation during the successive run (Fig. 4). Most of the phytoplankton groups could get well linear regression between the two dealing except the haptophytes_8 (Fig. 4c) and chlorophytes (Fig. 4f). It implied that these two phytoplankton groups were the major instability components in CHEMTAX running. For the haptophytes_8, scattered points implied that both overestimate and underestimate values were generated for any running. But the result of once running for chlorophytes was underestimated remarkably in contrast to the fifth running. To indentify whether the results of the fifth running was credible, regression was applied between the measured pigment concentration and the reverse deduced one from the fifth running's results (Fig. 5). The latter was just the concentration of the measured TChl *a* multiply by the fifth F_{output}. Both of the But-Fuco (Fig. 5a) and Chl *b* (Fig. 5b) obtained relatively better regression relationship for the fifth running than once (R^2=0.742, p<0.010 for But-Fuco and R^2=0.805, p<0.001 for Chl *b*).

Synechococcus was another trouble maker in the successive running, as it had two clusters in the regression within a remarkable overestimated branch in once running's result even though most of the samples matched well. The same analysis method as above also showed the fifth running's result (R^2=0.921, p<0.001) was better than running once (R^2=0.864, p<0.001).

4 Discussion

The pigment/TChl *a* ratio was the core question in the CHEMTAX processing as it could affect the calculation directly (Mackey et al., 1996, 1997, 1998). In the studies for the different region, including the estuary (Lewitus et al., 2005), bay (Hashihama et al., 2008), lagoons (Sarmento and Descy, 2008) and even freshwater (Guisande et al., 2008), there were many adjustments in the chosen of the pigment matrix and the initial ratio. The idealized scheme was the isolation and incubation of the phytoplankton components of the *in situ* water in lab, and then got their each pigment/TChl *a* ratio. But it was so complex to acquire the ratio for every species. The regulatory initial ratio was applied for most of the CHEMTAX running based on the ratio given by Mackey et al. (1996). So the grouping and F_{output} convergence impacted the calculation result together.

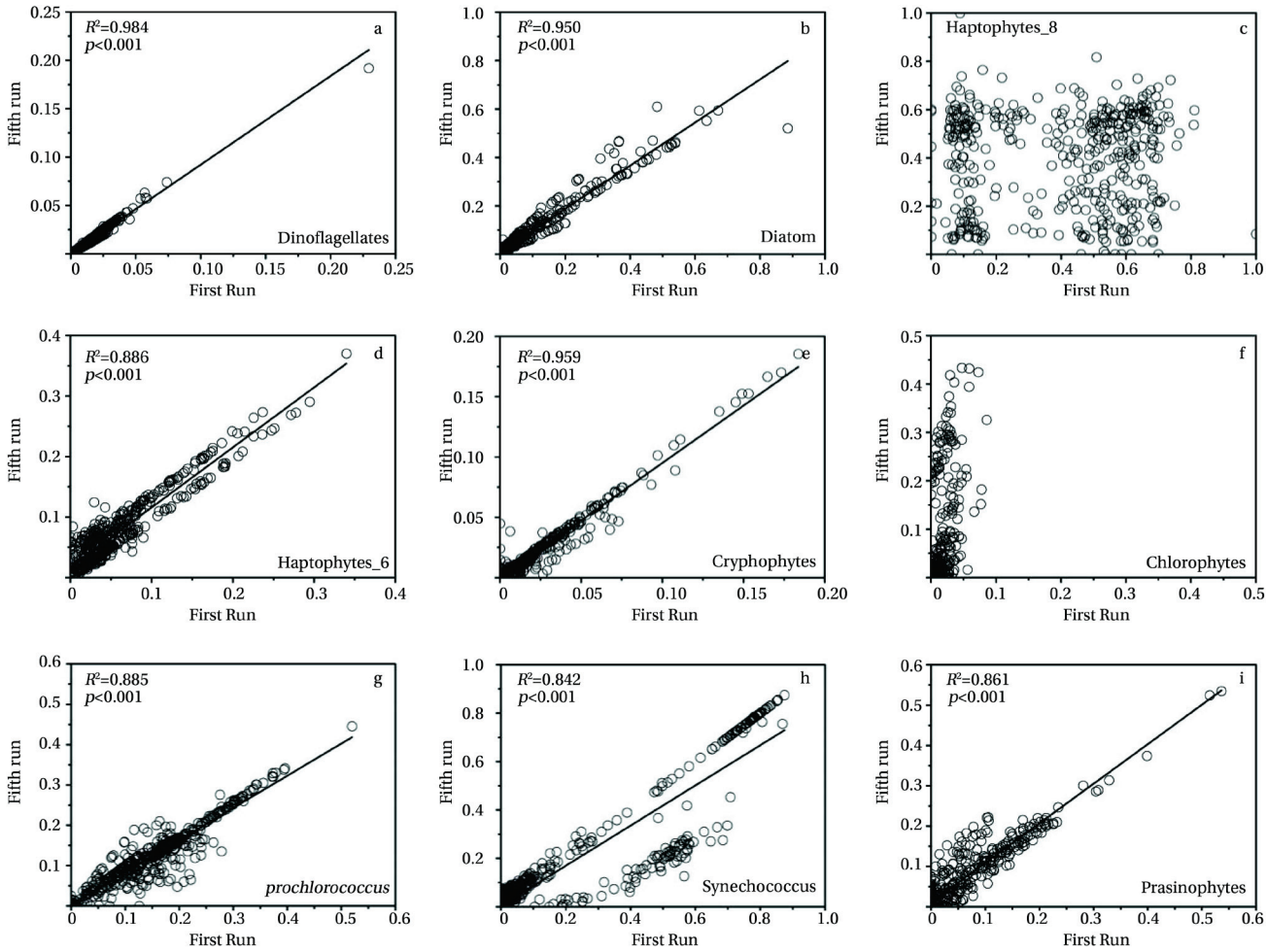

Fig. 4. Comparison of the CHEMTAX results between the first and the fifth running. The scale of the axis was the fraction of each phytoplankton group. a. Dinoflagellates, b. Diatoms, c. Haptophytes_8, d. Haptophytes_6, e. Cryptophytes, f. Chlorophytes, g. *Prochlorococcus*, h. *Synechococcus*, and i. Prasinophytes.

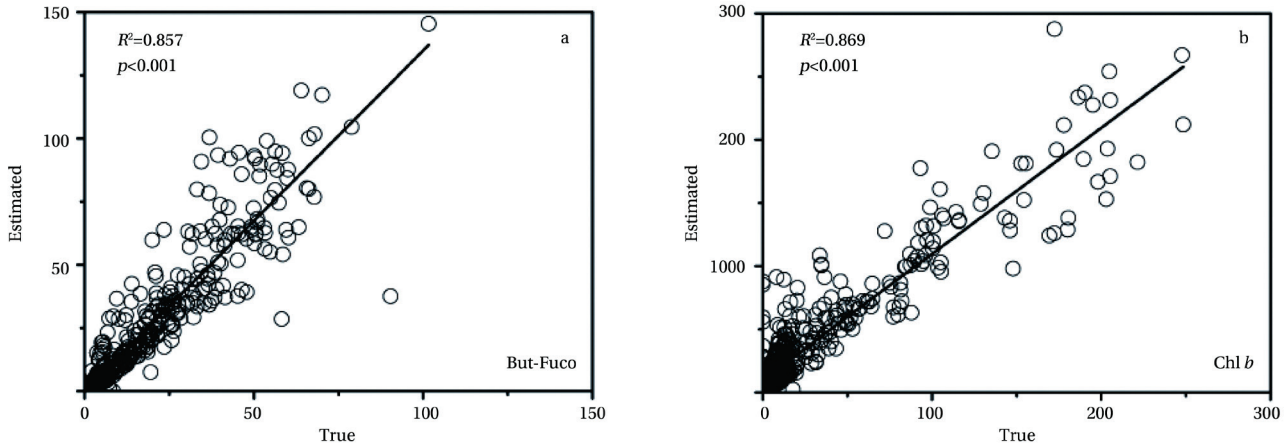

Fig. 5. Comparison of the pigment concentration (ng/L) between the measured value (True) and the results of the fifth running (estimated). a. But-Fuco and b. Chl *b*.

4.1 *Grouping*

The vertical distribution of the pigment/TChl *a* ratio decided the function of the grouping bands. First, the samples in the coastal region in the SCS must be managed independently. The comparison was conducted between the groups that whether or not containing the coastal stations in the SCS (Fig. 6). The differences between the two grouping procedure were so distinct, especially for those station in shelf and basin. If the coastal samples were included, diatoms percentages were overestimated frequently. The richness of nutrients in the coast enhanced the superiority of diatoms, dinoflagellates and prasinophytes, so their pigment/TChl *a* ratio was induced to be higher than in the shelf and basin (Latasa et al., 2010). Cluster analysis was introduced to the group dividing in the study of the bloom and post-bloom in the northw-

estern Mediterranean (Latasa et al., 2010). Hashihama et al. (2008) divided the samples to 100%, 50% and 20% light of the surface and compared the F_{output} to F_{input} between each group. As the Sagami Bay was affected by the Kuroshio, the samples had more oceanic characters than our study in the SCS. Second, the vertical distribution of the pigment/TChl *a* ratio must be taken as the reference to make samples grouping. The ratios increasing with depth was prevailing for But-Fuco, Hex-Fuco, Neo and Peri to TChl *a*, and dreasing for Zea, Chl *b* and Viol (Mackey et al., 1998). In the SCS, the profiles of pigment/TChl *a* ratio were different for Hex-Fuco, Pras and Peri. It might be caused by the different status of the nutrient and stratification compared to the equatorial Pacific or the Southern Ocean. The latter had deeper euphotic depth and it implied the grouping for the upper layers suffered less distraction than in the SCS. The vertical profiles for Chl *b* and Pras had accordance with other studies (Moore et al., 1995; Goericke and Montoya, 1998), as the ratio was higher in the DCM under low light condition.

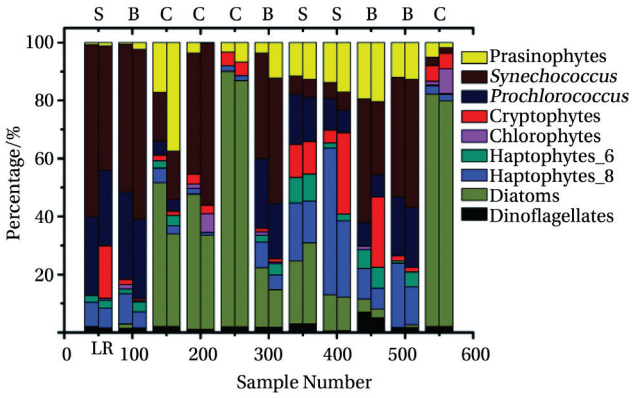

Fig. 6. Comparison of CHEMTAX results between different grouping procedure in 11 random samples in the SCS. The letters on the top axes implied the samples region, with coast (C), shelf (S) and basin (B). The two stack columns for each sample were distinguished as that the left column (L) was the group containing the coastal stations and the right column (R) was grouped by isolating coastal samples alone.

4.2 *Successive run*

The improvement of CHEMTAX procedure through successive run was proposed by Latasa (2007) which was aimed to acquire more credible results of the phytoplankton contribution to the TChl *a* and it was followed by the studies recently (Latasa et al., 2010; Mendes et al., 2011; Eker-Develi et al., 2012; Liu et al., 2012). In this study, most of the pigment/TChl *a* ratios were well converged except the Zea/TChl *a* for Proc, Viol/TChl *a* for Chlo and Chl *b*/TChl *a* for Pras. And the convergence was obviously acquired in the fourth to fifth running which was similar as the result of Latasa et al. (2010) whose best times was the third to fourth running. Mendes et al. (2011) got the best convergence at the sixth running, but also the tenth running results were selected as the final values in some other studies (Eker-Develi et al., 2012; Liu et al., 2012). If we compared the tenth running results to the fifth ones, it was distinct that the excursion happened for both of pig/TChl *a* ratios and community percentages, especially for those minor phytoplankton groups (e.g., chlorophytes and cryptophytes). Although the dominant phytoplankton groups suffered less, ±5% fluctuation was still revealed frequently, and so did the regression between the measured pigments concentration and estimating values.

In this study, only the haptophytes_8 and chlorophytes was deviated between the fifth and once running. The haptophytes_8 had both of the overestimation and underestimation situation betzween the fifth and once running. For the haptophytes_8, which was marked as haptophytes T4 in Latasa (2007), it was the minor group in contrast to the diatoms and haptophytes T3 (haptophytes_6 in this study) and was described as the "troublesome" group because of the sharing pigments with diatoms and haptophytes T3, e.g., the Fuco and Hex-fuco. So even though the converged status was achieved by these two pigments' ratios, the output fraction of haptophytes_8 could also be deviated. It must be evaluated by the contrasting between the measured pigments concentration and the calculation ones (Latasa, 2007). And the same procedure should be taken to the underestimated chlorophytes at running once.

4.3 *Synechococcus*

The regression between the fifth and once running's result expressed two branches of relationship for *Synechococcus*, in which one was well fitted and another was remarkable underestimated by running once. In the previous study, the *Synechococcus*, cryptophytes and haptophytes_8 was underestimated in the results of running once (Latasa, 2007). But the former two groups could get well regression after the tenth running in Latasa (2007). In this study, about one third of the total samples could not land on the regression line. Through the residual analysis for the Zea and Fuco (Fig. 7), the standardized residual (δ) was very low for the Fuco, but it was higher for Zea at about 160 individual samples ahead. And these samples belonged to the 0–10 m and 11–30 m groups. So it could surely tell that the Zea had large fluctuation in the upper mixed layer even though it was the most abund-ant. *Synechococcus* was the dominant phytoplankton in the surfa-ce layer in the SCS (Liu et al., 2007) and the estimation of its biomass through CHEMTAX might produce deviation from the natural status. And the better solution should be comparing the results of CHEMTAX to flow cytometry, which could be dealing with the zeaxanthin content per cell that combining with the *Synechococcus* and *Prochlorococcus* (Kana and Glibert, 1987; Cailliau et al., 1996; Latasa et al., 2010).

5 Conclusions

In this study, the evaluation of CHEMTAX running's results were applied through samples grouping, successive run and the residual analysis for indentifying the phytoplankton community composition by HPLC-pigments method in the SCS. For the grouping, the coast samples should be separated from the ones in the shelf and basin. The other samples could be divided by the depth or light attenuation. Generally, the processing for group should be as meticulous as the samples amount permitted. Successive run expressed good behaviors to some extent, but there were also some inflexible components no matter in pigment/TChl *a* ratios nor in phytoplankton groups. The haptophytes_8 could be overestimated or underestimated when it was a minor group in the previous study. But as a dominant group in the SCS, the haptophytes_8 biomass could also be misjudged. This situation might be induced by the character of the sharing pigments, e.g., Fuco and Hex-Fuco, but not affected by its biomass. Chlorophytes could also be misjudged, but the successive run still revealed its advantage. The calculation for *Synechococcus* could not only evaluate by the Zea/TChl *a* ratio for Syne, as the steady ratios could also lead to the deviation. Upper mixed layer groups were the major uncertain components analyzed by residual. And it implied the comparative procedure with FCM was necessary.

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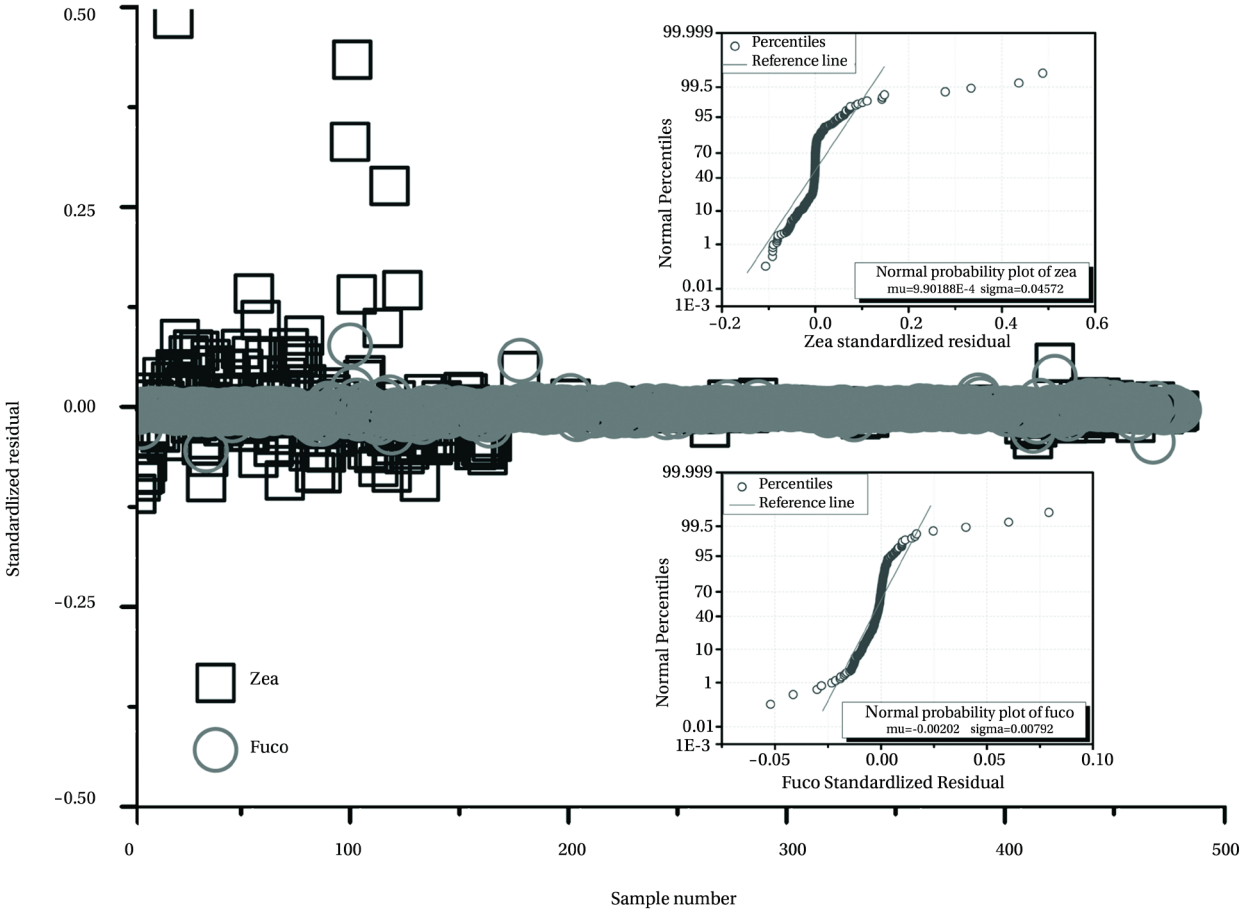

Fig.7. The residual analysis of Zea and Fuco for all the samples (n=487).

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