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Research Paper

Selection of protocols for phytoplankton pigment analysis: a comparative study*

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Abstract Pigments are widely used as indices for estimation of phytoplankton biomass and composition, and many protocols have been developed to analyze pigments in phytoplankton. Different protocols were compared using four solvents (methanol, 95% methanol, dimethylformamide, and 90% acetone) and two instruments (fluorometer and high-performance liquid chromatography (HPLC) coupled with diode array detector). Analysis of chlorophyll *a* (Chl *a*) with fluorometer could lead to over- or underestimation due to the interference from its derivatives in all probability. Among the four extractants, 90% acetone had a high recovery for chlorophylls. In contrast, 95% methanol was a poor extractant for chlorophylls due to the degradation of Chl *a*, especially in diatoms. The 95% methanol, however, had high extraction efficiencies for most diagnostic xanthophylls. Therefore, the selection of pigment analytical protocols should follow the specific purpose of phytoplankton study. In addition to fluorometry, an HPLC method with 90% acetone as extractant shall be a good choice for the analysis of Chl *a* to estimate phytoplankton biomass, especially for diatom-dominated samples, while an HPLC method with 95% methanol as extractant be more suitable to characterize different taxa in phytoplankton communities.

Keyword: pigment; fluorometry; high-performance liquid chromatography (HPLC); extraction; phytoplankton

1 INTRODUCTION

Phytoplankton, as important marine primary producer and the basis of marine food web, plays vital roles in the biogeochemical cycle and energy flow of marine ecosystems (Mendes et al., 2011). Pigments in phytoplankton are generally used to capture light energy for photosynthesis. Phytoplankton pigments can be divided into three major categories: chlorophylls (including chlorophyll *a*, *b*, *c*, *d*), carotenoids, and phycobiliproteins. Chlorophyll *a* (Chl *a*) is the major photosynthetic pigment present in almost all phytoplankton taxa, hence acting as a crucial biological parameter for estimating phytoplankton biomass (Falkowski and Kiefer, 1985; Gregg et al., 2003). Some specific carotenoids can serve as biomarkers for different phytoplankton taxa, such as fucoxanthin for diatoms, peridinin for dinoflagellates, and alloxanthin for cryptophytes (Jeffrey and Vesk, 1997).

Since the 1950s, spectrophotometry (Richards and Thompson, 1952), fluorometry (Holm-Hansen et al., 1965), thin-layer chromatography (TLC, Jeffrey, 1974), high-performance liquid chromatography (HPLC, Mantoura and Llewellyn, 1983; Zapata et al., 1987), and liquid chromatography coupled with mass spectrometry (LC-MS, Airs et al., 2001) have been successively applied to determine Chl-*a* content or to separate multiple phytoplankton

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pigments in oceanographic studies. Among all the methods, fluorometry and HPLC are the most widely used methods for pigment analysis. Fluorometry is routinely used for the quantitative analysis of Chl *a*, while the HPLC is mainly used to resolve the complex suit of pigments in phytoplankton for characterization of different phytoplankton taxa based on their diagnostic pigments. Thus, HPLC has become a favorite tool for marine researchers to perform ecological studies and to validate remote sensing data (Bidigare et al., 2002; Moisan et al., 2012; Wang et al., 2018; Kramer et al., 2022). At present, more than 20 HPLC-based methods have been developed for pigment analysis, using a variety of extractants, columns, mobile phases, and gradient programs.

Pigment extraction is the first step to analyze phytoplankton pigments. Both the extraction efficiency of solvents and the stability of pigments could affect the accuracy of experimental results (Suzuki et al., 1993). Organic solvents like methanol, acetone, and dimethylformamide (DMF) are widely used for pigment extraction, among which acetone and methanol are often adopted in the extraction of pigments in marine algae (Wright, 1997). Acetone is superior in the extraction of Chl *a* and causes less artefacts compared with methanol and DMF (Richards and Thompson, 1952; Schagerl and Künzl, 2007). Many countries adopt the fluorometric method using 90% acetone as extractant for chlorophylls, as the standard method for determination of Chl *a* in marine phytoplankton (GB 17378.7-2007, EPA Method 445.0). However, some studies indicate that methanol would be a better solvent than acetone for the extraction of certain chlorophylls and carotenoids (Holm-Hansen and Riemann, 1978; Wright, 1997; Zhu, 2007), but sometimes accompanied by the formation of Chl-*a* derivatives (Mantoura and Llewellyn, 1983). DMF is an excellent solvent for pigment extraction from algae (Neveux, 1988), but it is not recommended due to its severe toxicity.

According to the statistics published in nine international journals within the year 2005, pigment quantification was performed by spectrophotometry (35%), fluorometry (35%), and HPLC (25%), using six solvents at 15 different concentrations (Schagerl and Künzl, 2007). The wide varieties of pigment analytical methods make it difficult to compare pigment analytical data. Pigment analytical methods were compared by several marine research organizations and projects, such as the Joint Global Ocean Flux Study (JGOFS, Latasa et al., 1996) program, the Sea-viewing Wide Field-of-view Sensor (SeaWiFS, Marrari et al., 2006) project, and the Medium Resolution Imaging Spectrometer (MERIS, Ohde et al., 2007) validation team. However, the results are not consistent. The fluorometric method may significantly over- or under-estimate the Chl-*a* concentrations compared to the HPLC method in the same extractants, mostly acetone or 90% acetone. Both MERIS and SeaWIFS found that fluorometry gave higher Chl-*a* data than HPLC, but the opposite was also found in some studies (Bianchi et al., 1995; Yoo et al., 2002; Kumari, 2005). In our previous studies of phytoplankton samples, the fluorometric data of Chl *a* were generally in consistence with the HPLC data, while some samples had significantly higher fluorometric data than HPLC. This inconsistent knowledge seems to indicate different responses of phytoplankton taxa on the above treatments.

In view of the different pigment extractants used for the fluorometric and HPLC methods, and the potential impacts caused by various microalgal groups in phytoplankton samples, the authors compared different protocols in analysis of pigments extracted from various microalgal groups. This study aims to clarify the factors affecting Chl-*a* measurement by fluorometric and HPLC methods, and to provide recommendations for the selection of pigment analytical protocols in oceanographic studies.

2 MATERIAL AND METHOD

2.1 Chemical and reagent

The pigments examined in this study are listed in Table 1. Internal standard (8′-apo-β, ψ-carotaldehyde) purchased from Sigma was used for quantitative analysis of pigments. All other pigment standards were purchased from DHI Water and Environment, Denmark. The polarity of each pigment is indicated in the table based on its chemical structure and retention time (Table 1). Reagents used in the study, including methanol, acetone, DMF, acetonitrile, pyridine, and acetic acid, were all HPLC grade.

2.2 Algae culture and sample collection

Diatom *Phaeodactylum tricornutum*, chlorophyte *Nannochloris* sp., pelagophyte *Aureococcus anophagefferens*, cryptophyte *Cryptomonas* sp., dinoflagellate *Prorocentrum donghaiense*, and haptophyte *Phaeocystis globosa* were used for the

Table 1 Pigments examined in this study (including chlorophyll-*a* derivatives detected in microalgal samples without standards)

Number	Pigment	Abbreviation	Any available standards?	Polarity
1	Chlorophyll c_3	Chl c_3	√	+ + +
2	Chlorophyllide *a*	Chlide *a*	–	+ + +
3	Magnesium-2,4-divinylpheoporphyrin $a_{5\ m}$onomethyl ester	MgDVP	√	+ + +
4	Chlorophyll c_2	Chl c_2	√	+ + +
5	Methy-chlorophyllide *a*	Me-Chlide *a*	–	+ + +
6	Peridinin	Peri	√	+ +
7	19′-butanoyloxyfucoxanthin	But-fuco	√	+ +
8	Fucoxanthin	Fuco	√	+ +
9	Neoxanthin	Neox	√	+ +
10	Prasinoxanthin	Pras	√	+ +
11	Violaxanthin	Viol	√	+ +
12	Astaxanthin	Asta	√	+ +
13	Diadinoxanthin	Diad	√	+ +
14	Alloxanthin	Allo	√	+ +
15	Diatoxanthin	Diat	√	+ +
16	Zeaxanthin	Zeax	√	+ +
17	Lutein	Lute	√	+ +
18	Trans-beta-apo-8′-carotenal	IS	√	
19	Chlorophyll *b*	Chl *b*	√	+
20	Chlorophyll-*a* allomers	Chl *a*-a	–	+
21	Chlorophyll *a*	Chl *a*	√	+
22	Chlorophyll-*a* epimers	Chl *a*-e	–	+
23	*α*, *β*-carotene	Acar	√	+
24	*β*, *β*-carotene	Bcar	√	+

The "+" symbolizes the intensity of pigment polarity, with a greater number of "+" signs indicating a stronger polarity. – means no data.

experiment. The separation information of the algal species is given in Supplementary Table S1. The microalgae were cultivated in L1 medium prepared with sterilized natural seawater, with a salinity of about 32. The cultivation temperature was 20±1 °C and the light intensity was 100 μE/(m²·s) with a light:dark cycle of 14 h:10 h. *Phaeodactylum tricornutum* and *Nannochloris* sp., which are common microalgae in aquaculture industry, are often used for commercial pigment production. *Prorocentrum donghaiense*, *A*. *anophagefferens*, and *P. globosa* are typical causative species of harmful algal blooms in the coastal waters. *Cryptomonas* sp. is widely distributed in the coastal waters around the world. The selection of these microalgae could cover most of the pigments in natural phytoplankton samples.

For each microalgal species, 10–20 mL of culture in exponential phase was collected and filtered on 25-mm Whatman GF/F fiberglass filters for pigment analysis. Natural phytoplankton samples were collected from Huiquan Bay of Qingdao by filtering 1 000-mL seawater through a GF/F membrane for each sample. In addition, 10–20 mL of *P. tricornutum* and *Cryptomonas* sp. cultures in exponential and stationary growth phases were collected, individually, to test the difference in Chl *a* determined by fluorometric and HPLC methods. All the pigment samples were carried out in triplicate for different extractants and frozen at -80 °C until analysis.

2.3 Pigment analysis method

2.3.1 Fluorometric Chl-*a* analysis

The extraction and measurement of Chl *a*

followed the protocol of China's National Standard GB 17378.7-2007. Filters collected previously were placed into brown vials and soaked in 10 mL of 90% aqueous acetone overnight (about 24 h) at 4 °C in darkness. Chl-*a* content was calculated according to the fluorescence intensity measured before and after acidification (10% hydrochloric acid) using a calibrated Turner Designs fluorometer (Arar and Collins, 1997).

2.3.2 HPLC pigment analysis

The analytical procedure followed the protocol developed by Zapata et al. (2000), with some modifications (Kong et al., 2012). The frozen filter was scissored into small pieces and extracted with 1 400 μL of organic solvent in a vial, to which 100 μL of 8′-apo-β, ψ-carotaldehyde (750 μg/L in 90% acetone) was added as internal standard. The vial was then placed in an ice bath and sonicated for 5 min. The extract was filtered through 0.22-μm PTEE syringe filters to remove debris. An aliquot of filtrate (800 μL) was collected and mixed with 160-μL Milli-Q water in a vial, then 100-μL pigment extract was injected into the HPLC system. The total time of pigment extraction should not exceed 4 h. Pigments were separated using a Waters Symmetry C8 column connected to a Waters E2695 HPLC system with binary gradient elution. Mobile phase A was methanol∶acetonitrile∶aqueous pyridine solution (50∶25∶25, V∶V∶V), mobile phase B was methanol∶acetonitrile∶acetone (20∶60∶20, V∶V∶V). Absorption spectra at 300–750 nm were recorded using a Waters 2998 diode array detector. Pigments were identified by co-chromatography with pigment standards and their respective absorption spectra, and subsequently quantified by calibration curves previously established for each pigment to internal standard. Due to the lack of standards for Chl-*a* derivatives, such as chlorophyllide *a* (Chlide *a*), methyl-chlorophyllide *a* (Me-Chlide *a*), chlorophyll-*a* allomers (Chl *a*-a), and chlorophyll-*a* epimers (Chl *a*-e), their contents were calculated based on the ratio of their specific extinction coefficient to Chl *a*. Chl *a*-a and Chl *a*-e use the same specific extinction coefficient as Chl *a*, while Me-Chlide *a* uses the same specific extinction coefficient as Chlide *a* (Jeffrey et al., 1997).

2.4 Experimental design

The schematic diagram of experimental design for this study is shown in Fig.1. The experiment consists of two main parts stated in the two subsections below.

2.4.1 Pigment extraction efficiency of different solvents

The extraction efficiency (extracted pigment content) of four solvents (100% methanol, 95% methanol, DMF and 90% acetone) for chlorophylls and carotenoids in six microalgal samples and natural phytoplankton sample was compared using the HPLC method (Table 2). In addition, an extra

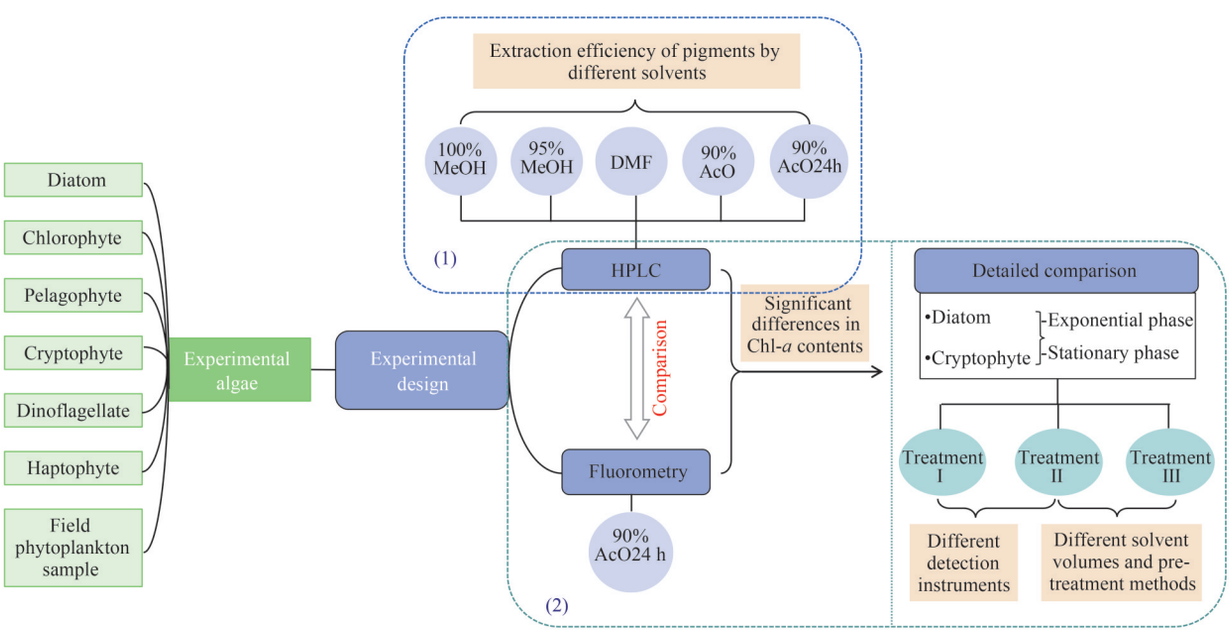

Fig.1 Schematic diagram of experimental design

100%MeOH: 100% methanol; 95%MeOH: 95% methanol; DMF: dimethylformamide; 90%AcO: 90% acetone; 90%AcO24h: extracted in 90% acetone for 24 h.

Table 2 Solvents and time used to extract phytoplankton pigments for HPLC analysis

Sample	Solvent and extraction time	Abbreviation
Diatom *Phaeodactylum tricornutum*	100% methanol, 4 h	100%MeOH
Chlorophyte *Nannochloris* sp. Pelagophyte *Aureococcus anophagefferens*	95% methanol, 4 h	95%MeOH
Cryptophyte *Cryptomonas* sp.	DMF, 4 h	DMF
Dinoflagellate *Prorocentrum donghaiense* Haptophyte *Phaeocystis globosa*	90% acetone, 4 h	90%AcO
A phytoplankton sample collected from Jiaozhou Bay	90% acetone, 24 h	90%AcO24h

group was set up to evaluate the effect of extraction time using 90% acetone to extract pigments for 24 h. Except for the extractant and the extraction time, the other procedures processes were all identical.

2.4.2 Comparison of chlorophyll-*a* analyses by fluorometric and HPLC methods

Chlorophyll *a* in six species of microalgae and a sample collected from Jiaozhou Bay were extracted and determined by fluorometry (extracted with 10-mL 90% acetone for 24 h) and HPLC (extract with 1.4-mL 90% acetone within 4 h), respectively. The fluorometric and HPLC procedures used here are relatively common for pigment analysis, and the primary objective of the comparison is to evaluate the comparability of Chl-*a* data obtained by these two main methods.

Two species of microalgae, *P. tricornutum* and *Cryptomonas* sp., with dramatic differences in Chl-*a* content determined by the two methods, were further compared. Samples were collected in exponential phase and stationary phase, respectively, to assess the effect of physiological status of microalgae on Chl-*a* determination. Three groups were set up to test the effects of instrument, extractant volume and sample pretreatment on the determination of Chl *a* (Table 3).

2.5 Statistical analysis

The bar chart, pie chart, and heat map were generated using Origin 2021. Difference between groups was tested using one-way analysis of variance or independent sample *t*-test, and the difference was considered statistically significant when $P<0.05$.

3 RESULT

3.1 Pigment extraction efficiency of different solvents

3.1.1 Extraction efficiency of Chl-*a* pigment by different solvents

Extraction efficiency of four commonly used organic solvents for Chl *a* in six species of microalgae and one phytoplankton sample by HPLC methods are shown in Fig.2. Both Chl *a* and its derivatives were detected by HPLC, including Chlide *a*, Me-Childe *a*, Chl *a*-a, and Chl *a*-e. In diatom *P. tricornutum* (Fig.2a), Chlide *a* was the most abundant Chl-*a* derivative (689–1 118 μg/L), far more than Chl *a* (98–440 μg/L). Chlide *a* (63–72 μg/L) was also high in cryptophyte *Cryptomonas* sp. (Fig.2d), only slightly lower than Chl *a* (82–118 μg/L). In the other three microalgal species and the phytoplankton sample, the content of Chl-*a* derivatives was much lower.

The extraction efficiency of Chl *a* with 90% acetone was the highest in almost all the samples tested, while that with 95% methanol was the lowest (except for *P. tricornutum*). The extraction efficiency of DMF is only slightly lower than that of 90% acetone. In diatom *P. tricornutum*, the extraction efficiency of 100% methanol was the lowest for Chl *a*, followed by 95% methanol, which were equivalent to 22% and 49% of the extraction efficiency of 90% acetone. In dinoflagellate *P. donghaiense*, the extraction efficiency of the 95% methanol was only a quarter of the 90% acetone. In chlorophyte *Nannochloris* sp., cryptophyte

Table 3 Four experimental groups to analyze the Chl-*a* difference between fluorometry and HPLC methods

Sample	Group	Solvent	Extraction time (h)	Extractant volume (mL)	Sample preparation	Instrument
Phaeodactylum tricornutum—exponential phase	I	90% acetone	24	10	/	Fluorometer
Phaeodactylum tricornutum—stationary phase	II	90% acetone	24	10	/	HPLC
Cryptomonas sp.—exponential phase *Cryptomonas* sp.—stationary phase	III	90% acetone	24	1.4	Scissored and sonicated	HPLC

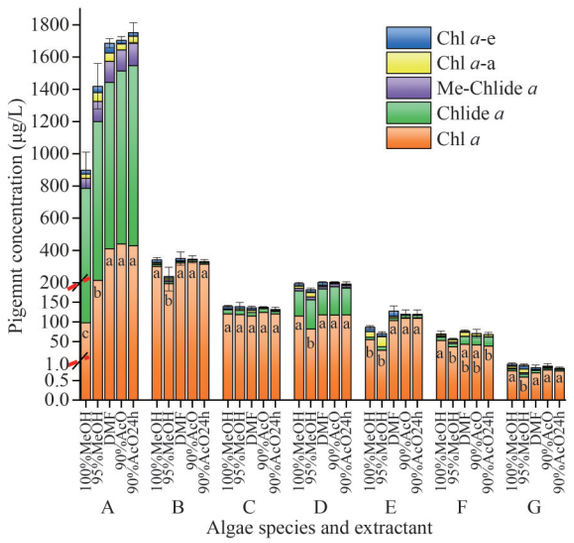

Fig.2 Extraction efficiency of chlorophyll *a* and its derivatives by different extractants from different algae

Different letters indicate statistically significant differences (P<0.05) between treatments. A: *Phaeodactylum tricornutum*; B: *Nannochloris* sp.; C: *Aureococcus anophagefferens*; D: *Cryptomonas* sp.; E: *Prorocentrum donghaiense*; F: *Phaeocystis globosa*; G: natural phytoplankton sample. 100%MeOH: 100% methanol; 95% MeOH: 95% methanol; DMF: dimethylformamide; 90%AcO: 90% acetone; 90%AcO24h: extracted in 90% acetone for 24 h.

Cryptomonas sp. and the phytoplankton sample from Jiaozhou Bay, the extraction efficiencies of the 95% methanol for Chl *a* were also much lower, but there were no significant differences among 100% methanol, 90% acetone and DMF (P>0.05). In pelagophyte *A. anophagefferens* and haptophyte *P. globosa*, there was no significant difference among the four solvents (P>0.05), although extraction efficiency of the 95% methanol was slightly lower.

For pigments extracted from diatom *P. tricornutum*, both the content of Chl-*a* derivatives and their proportions to total Chl-*a* pigment (Chl *a* and its derivatives) were significantly higher in 100% and 95% methanol than in 90% acetone and DMF (P<0.05; Fig.3), suggesting that the use of methanol as extractant may promote the degradation of Chl *a* to its derivatives. Extraction of Chl *a* with 90% acetone for a prolonged period of 24 h resulted in a slight decrease in Chl-*a* content, but the difference was not significant (P>0.05) (Fig.2). The content of Chl-*a* derivatives extracted with 90% acetone for 24 h from the diatom *P. tricornutum* increased slightly, but this phenomenon was not evident in other samples.

3.1.2 Extraction efficiency of other chlorophylls and carotenoids with different solvents

Pigment composition of the six microalgal species and the phytoplankton sample collected from Jiaozhou Bay were analyzed (Supplementary Fig.S1). Besides Chl *a*, carotenoids and other chlorophylls were also detected in the six microalgal species. The chlorophylls detected included Chl *b*, MgDVP, Chl c_2, and Chl c_3. Carotenoids included the carotenes Acar and Bcar, and a variety of diagnostic xanthophylls, such as Fuco for diatoms, Peri for dinoflagellates, Lute for chlorophytes, But-fuco for pelagophytes and haptophytes, and Allo for cryptophytes. Various pigments were detected in the natural phytoplankton sample, including MgDVP, Chl c_2, Peri, Fuco, Neox, Pras, Viol, Allo, Diad, Diat, Zeax, Lute, Chl *b*, and Bcar, but their contents were very low.

The extraction efficiency for various pigments in the six microalgal species using four solvents is generally consistent (Fig.4a–f). Similar to Chl *a*, Chl *b* also had the lowest content in 95% methanol, about half of those in DMF and 90% acetone. Components of Chl *c* (MgDVP, Chl c_2, and Chl c_3) with high polarity also had the lowest, even undetectable, contents in methanol (100% and 95% methanol), and their contents were the highest in DMF. The contents of the non-polar carotenes Acar and Bcar were significantly lower in 95% methanol than in 90% acetone and DMF (P<0.05). However, for

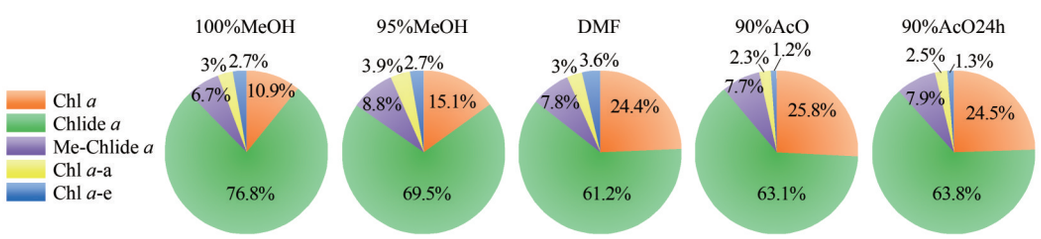

Fig.3 The ratio of Chl-*a* derivatives to total Chl-*a* pigment (Chl *a* and its derivatives) in different extractants of *Phaeodactylum tricornutum*

100%MeOH: 100% methanol; 95%MeOH: 95% methanol; DMF: dimethylformamide; 90%AcO: 90% acetone; 90%AcO24h: extracted in 90% acetone for 24 h.

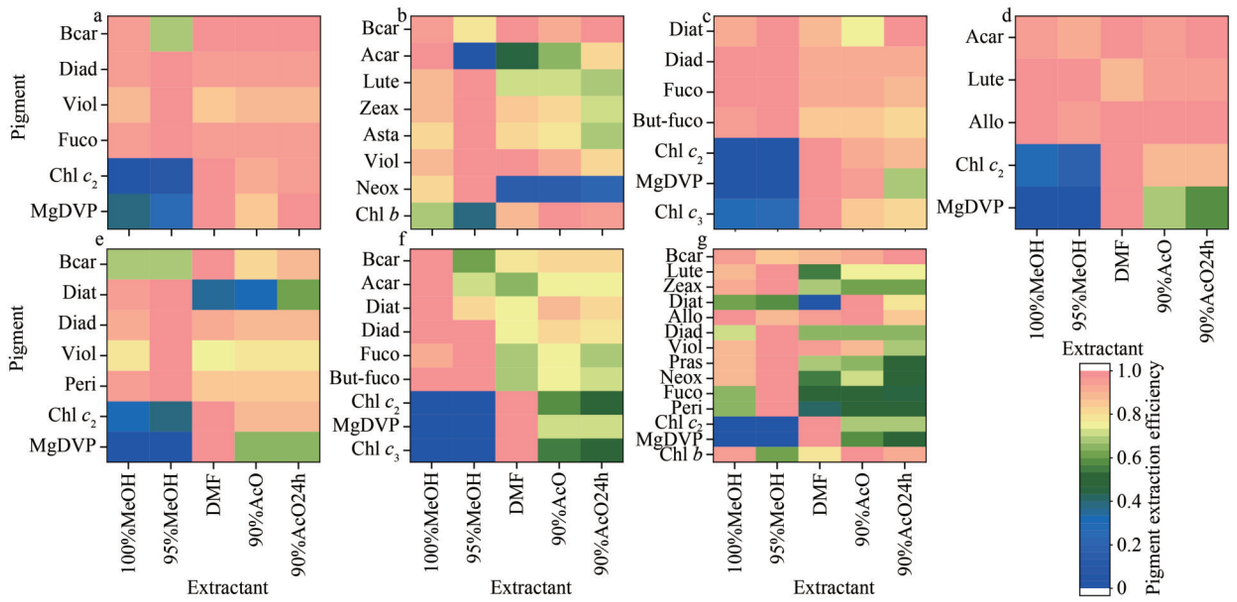

Fig.4 Extraction efficiency of other chlorophylls and carotenoids by different extractants from different algae

a. *Phaeodactylum tricornutum*; b. *Nannochloris* sp.; c. *Aureococcus anophagefferens*; d. *Cryptomonas* sp.; e. *Prorocentrum donghaiense*; f. *Phaeocystis globosa*; g. natural phytoplankton sample. Pigment extraction efficiency of 0–1: for each pigment, the highest value obtained by different extractants was taken as 100%, and the ratio of the values obtained by other extractants to the highest value is taken as the pigment extraction efficiency of this extractant. 100%MeOH: 100% methanol; 95% MeOH: 95% methanol; DMF: dimethylformamide; 90%AcO: 90% acetone; 90%AcO24h: extracted in 90% acetone for 24 h.

xanthophylls like Fuco, Peri, But-fuco, Viol, Lute, Diad, Zeax, Neox, and Asta, the extraction efficiency was the highest in 95% methanol. The contents of Fuco and Peri in 95% methanol were 1.4–2.4 times of those in DMF and 90% acetone. Unlike most xanthophylls, the extraction efficiency of Allo was the lowest in 95% methanol. Pigments extraction from the natural phytoplankton sample showed a similar pattern to that of cultured microalgae (Fig.4g), except that Diat was not detected in DMF extracts due to its low content. The contents of carotenoids and other chlorophylls extracted with 90% acetone for 24 h were slightly lower than those for 4 h, but the differences were not significant (P>0.05).

3.2 Comparison of Chl-*a* analyses by fluorometric and HPLC methods

The Chl-*a* content in different samples determined by fluorometric (extracted with 10-mL 90% acetone for 24 h) and HPLC (extracted with 1.4-mL 90% acetone within 4 h) methods were compared (Table 4). The absolute Chl *a* of HPLC was significantly lower than that of fluorometry in six cultured algae and natural phytoplankton sample (P<0.05), and the total Chl *a* (Chl *a*+Chlide *a*+Me-Childe *a*+Chl *a*-a+Chl *a*-e) of HPLC was still lower, except for *P. tricornutum* and *P. globosa*.

The differences between the two methods varied with algal samples. In diatom *P. tricornutum*, the absolute Chl-*a* content determined by fluorometry was almost three times higher than that determined by HPLC, but the total Chl *a* determined by HPLC was 1.4 times higher, which may be related to its large amount of Chl-*a* derivatives. In cryptophyte *Cryptomonas* sp., the absolute Chl *a* determined by fluorometry was 2.2 times higher than that determined by HPLC. For dinoflagellate *P. donghaiense*, chlorophyte *Nannochloris* sp., pelagophyte *A. anophagefferens*, and haptophyte *P. globosa*, the differences were smaller than the above algae but still significant (P< 0.05), and the fluorometry results were about 1.1–1.6 times higher than the HPLC results. The difference between the two methods in natural phytoplankton samples containing multiple algae species is intermediate, with the fluorometric result being about 2.1 times higher than HPLC.

Due to the significant difference in Chl *a* determined by the fluorometric and the HPLC methods, a further experiment was carried out to explain the difference using the diatom *P. tricornutum* and the cryptophyte *Cryptomonas* sp. (Fig.5). Pigments extracted from microalgae using the same protocol (10-mL 90% acetone for 24 h)

Table 4 Chlorophyll-*a* content (μg/L) determined by fluorometric and HPLC method in different algae using 90% acetone as extractant

Experimental algae	Chl *a*-fluorometry	Chl *a*-HPLC	Total Chl *a*-HPLC
Phaeodactylum tricornutum	1 260±26* (*n*=3)	439±109* (*n*=2)	1 703* (*n*=2)
Nannochloris sp.	372±9.6* (*n*=3)	329±61* (*n*=3)	345* (*n*=3)
Aureococcus anophagefferens	158±1.4* (*n*=3)	124±10* (*n*=3)	137* (*n*=3)
Cryptomonas sp.	257±2.0* (*n*=3)	118±6.6* (*n*=3)	201* (*n*=3)
Prorocentrum donghaiense	154±2.7* (*n*=3)	111±2.4* (*n*=3)	120* (*n*=3)
Phaeocystis globosa	67±0.7* (*n*=3)	42±2.0* (*n*=3)	71* (*n*=3)
Natural phytoplankton sample	1.4±0.1* (*n*=3)	0.8±0.1* (*n*=3)	0.9* (*n*=3)

*: $P<0.05$.

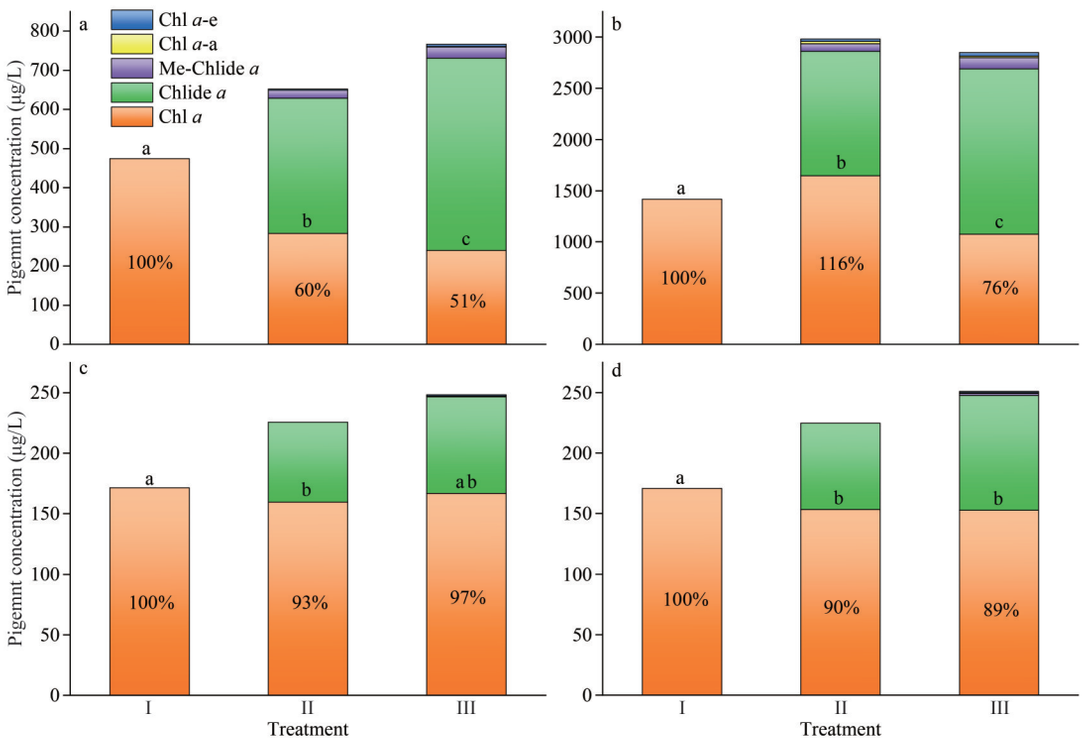

Fig.5 Chlorophyll *a* and its derivatives extracted and measured by fluorometric or HPLC method in different algal growth stages

Different letters indicate statically significant differences ($P<0.05$) between treatments. a. *Phaeodactylum tricornutum*—exponential phase; b. *Phaeodactylum tricornutum*—stationary phase; c. *Cryptomonas* sp.—exponential phase; d. *Cryptomonas* sp.—stationary phase. See Table 3 for the specific treatment of the four groups (I, II, III). The percentage represents the proportion of Chl-*a* content of other three groups relative to that of fluorometric method.

were determined by both fluorometric and HPLC methods (the first and the second column of each graph in Fig.5). The Chl-*a* contents of *P. tricornutum* and *Cryptomonas* sp. collected at both exponential and stationary phases were also compared using HPLC methods. For the diatom *P. tricornutum*, the Chl-*a* content measured by fluorometer was remarkably higher (1.4 times) than the HPLC method at the exponential phase. The total Chl-*a* pigment measured by HPLC, however, was always higher than the Chl *a* measured by fluorometer, indicating that the fluorometric detection could be interfered by Chl-*a* derivatives.

The effect of pretreatment on Chl-*a* determination was also tested (the second and third column of each graph in Fig.5). A lower volume of extractant (1.4-mL 90% acetone) and sonication treatment led to a decrease in Chl-*a* content and a corresponding

increase in Chlide *a* in the diatom *P. tricornutum* collected at both phases, while it had little effects on the cryptophyte *Cryptomonas* sp. It seems that Chl-*a* extraction and determination in *P. tricornutum* was strongly influenced by the physiological status compared to *Cryptomonas* sp.

4 DISCUSSION

4.1 Effect of solvents and pigment properties on the analysis of phytoplankton pigment

Results of the present study showed that the extraction efficiency of chlorophylls, carotenes, and xanthophylls in different organic solvents varied widely, which appears to be related to their polarity matching degree. Chlorophylls and carotenoids contain numerous compounds with different functional groups and carbon chain sizes, resulting in different polarity (Saini and Keum, 2018). Chlorophylls are the main photosynthetic pigments with a similar phyrin macrocycle centered with a magnesium atom. Chl *a* and Chl *b* differ slightly in the side-chain at C7 of the tetrapyrrole, while Chl *c* has greatly enhanced polarity with no hydrophobic phytol side-chain at C17. Carotenoids can be classified into carotenes and xanthophylls. Carotenes (e.g., Acar and Bcar) are unsaturated hydrocarbon compounds with weak polarity, whereas xanthophylls (e.g., Fuco, Peri, Zeax) have moderate polarity containing at least one functional group containing oxygen. According to the chemical structure and elution order, the polarity of pigments decreases sequentially from Chl *c*, most xanthophylls, Chl *b*, Chl *a*, and carotenes without polar functional groups (Table 1).

Acetone is usually more efficient in extracting low-polarity pigments, while ethanol is usually more efficient in extracting high-polarity pigments, with methanol in between (Wright, 1997; Hagerthey et al., 2006). Among the solvents used in this study, acetone has a lower polarity compared to methanol and DMF. Accordingly, the extraction efficiency of weakly polar pigments such as Chl *a*, Chl *b*, and the non-polar carotenes Acar and Bcar, was significantly higher in acetone than in methanol. For highly polar chlorophylls, such as MgDVP, Chl c_2 and Chl c_3, the extraction efficiency was still lowest in methanol but highest in DMF, which is obviously not only explained by the polarity matching degree. It is known that the absorption spectrum of chlorophylls (Chl *a*, Chl *b*, Chl c_1+c_2, and Chl *d*) is affected by different organic extractants, their absorption peaks are lower and broader in methanol and ethanol than in acetone, so chlorophylls are often underestimated in methanol (Ritchie, 2008). This may also be because methanol can promote trans-esterification and allomerization of chlorophylls, which not only decrease chlorophyll level in extracts, but also interfere with their determination (Osório et al., 2020). In a field investigation of *A. anophagefferens* bloom, the extraction of phytoplankton pigments with methanol failed to detect any chlorophyll *c*, which might be caused by the extractant (Kong et al., 2012).

In contrast to chlorophylls, xanthophylls with moderate polarity have the highest extraction efficiency in methanol, especially in 95% methanol. The xanthophyll group is the most complex carotenoid group, and varies in chain length, functional group or configuration, and solubility in solvents. Most xanthophylls contain hydroxyl groups, making them more soluble in alcohols such as methanol. Studies also found that the extraction efficiency of methanol for Fuco, Lute, and other xanthophylls from *Phaeocystis* sp., *Pyramimonas* sp. and *P. tricornutum* was higher than acetone, accompanied by less carotenoid derivatives (Wright, 1997; Van Leeuwe et al., 2006; Soares et al., 2016). Unlike most xanthophylls, Allo has the lowest extraction efficiency in 95% methanol. Allo is a type of natural acetylenic carotenoid, the only carotenoid with two triple bonds found in photosynthetic organisms (West et al., 2016). It is interesting that for Allo and Zeax, which differ only in the triple or double bond at the C7-C8 and C7′-C8′ positions, have such different extraction efficiencies in 95% methanol. Acyclic double bonds adjacent to the triple bond were found to have a strong tendency to cis-configure (Cheng et al., 1974). We speculate that aqueous methanol may lead to the formation of more Allo cis-isomer with different absorption spectra, e.g. manixantin, resulting in an underestimation of Allo.

4.2 Effect of algal taxa and physiology on the analysis of phytoplankton pigment

Pigment extraction from algae is a more complex process than direct solvent-solute interaction (Wright, 1997). The cell wall architecture and metabolic process of algae also affect the efficiency of pigment extraction. The resistance of cell wall will affect the penetration of organic solvents (Pasquet et al., 2011). Some silicified benthic diatoms, armored dinoflagellates, thick-walled freshwater blue-green

algae are notoriously difficult to extract their pigments (Porra, 1991). In this study, diatom *P. tricornutum* and dinoflagellate *P. donghaiense* have siliceous or cellulosic cell walls, whereas pelagophyte *A. anophagefferens* and cryptophyte *Cryptomonas* sp. have naked cells. Corresponding to the difference in cell wall architecture, *P. tricornutum* and *P. donghaiense* showed the greatest difference in Chl-*a* extraction efficiency between methanol, DMF, and 90% acetone, while the other four algal species showed little difference. DMF is a strong cell-penetrating agent with high extraction efficiency of pigments from cyanobacteria and clustered green algae (Neveux, 1988). However, the cell walls of the six algal groups in this experiment were not particularly thick, so the advantages of DMF were not highlighted.

Phytoplankton pigments are susceptible to chemical or enzymatic degradation reactions. The stability of Chl *a* is mainly affected by chlorophyllase (chlorophyll-chlorophyllide hydrolase), a glycoprotein located in the photosynthetic membrane of higher plants and algae (Terpstra, 1981). Chlorophyllase can encounter Chl *a* during aging, death or mechanical disruption of algal cells, promoting the degradation of Chl *a* (Karboune et al., 2005). Under the catalysis of chlorophyllase, phytol is first removed from Chl *a* to form Chlide *a*, then Mg is removed, and finally the phyrin macrocycle is cleaved to form nonfluorescent catabolites (Suzuki et al., 2005; Hörtensteiner, 2009). Jeffrey and Hallegraeff (1987) studied 113 strains of unicellular algae belonging to 93 species from 10 classes. They found that the activity of chlorophyllase varied greatly between species and even between strains, and that about one third of the diatoms had high activity of the acetone-activated chlorophyllase. Diatom *P. tricornutum*, is commonly used as a source for chlorophyllase purification due to its high chlorophyllase activity. It was found that Chlide *a* in *P. tricornutum* could account for 37.8% of the total Chl-*a* pigment when extracted in 90%–95% acetone, much higher than in other algae (Louda et al., 1998). In this study, it was found that Chlide-*a* content in *P. tricornutum* was 3–5 times of Chl *a*. The specific chlorophyllase activities of the other five algae used in this study were not clearly investigated, but their closely related species such as *Prorocentrum micans*, *Nannochloris atomus*, *Cryptomonas maculata*, *Phaeocystis pouchetii*, and *Pelagococcus subviridis* were all found to have low chlorophyllase activity (Jeffrey and Hallegraeff, 1987). Thus the proportion of Chl-*a* derivatives to total Chl *a* in the five experimental algae is not as large as in the *P. tricornutum*.

The enzyme activity of chlorophyllase could be activated by organic solvents, resulting in the formation of Chlide *a* and other degradation products (Mfnguez-Mosquera et al., 1994). The stability of Chl *a* is much lower in methanol than in acetone, and the loss of chlorophyll and carotene is greater when methanol is used as solvent (Tett et al., 1975; Latasa et al., 2001). Chen et al. (2005) found that the activity of chlorophyllase is much higher in ethanol and methanol than in acetone and DMF. Our study also showed that in most algae, the proportion of Chlide *a* to total Chl-*a* pigment was higher in methanol than in 90% acetone, especially in *P. tricornutum*.

4.3 Suggestion of pigment analysis in phytoplankton study

Pigment analysis with fluorometer and HPLC are two main approaches to obtain Chl-*a* data in marine phytoplankton studies. Although HPLC is recommended as the most reliable method for the determination of Chl *a* (Pinckney et al., 1994), fluorometry is still used on most cruises because it is faster, easier to use and cost less expensive. In this study, the Chl-*a* content determined by the fluorometric method was much higher than the absolute Chl *a* determined by HPLC when using the same extractant. However, the fluorometric method can either over- or underestimate the Chl-*a* content in different algae compared to the HPLC-derived total Chl *a*. Many comparative studies between the two methods have yielded similar results and attribute the erroneous estimation of fluorometry to interferences from Chl *b*, Chl *c*, and chlorophyll derivatives (Bianchi et al., 1995; Marrari et al., 2006). With the development of fluorometric technology, Chl *a*, *b*, and *c* could be accurately distinguished from each other, but some Chl-*a* derivatives (e.g., Chl *a*-a, Chl *a*-e, Chlide *a*, and Me-childe *a*) with fluorescence spectra similar to Chl *a* are still indistinguishable (Gibb et al., 2001; Neveux et al., 2012). Different Chl-*a* derivatives interfere with chlorophyll fluorescence to different extents, and therefore the amount and proportion of these derivatives greatly affects the degree of difference between fluorescence and HPLC. The difference between the two methods can be further widened by the choice of the extractant. The standard fluorometric method uses 90% acetone as extractant, whereas the

HPLC method has a variety of extractants, of which methanol and acetone are the most commonly used. The extraction efficiency of organic solvents for phytoplankton pigments varied depending on the type of pigment, and no single solvent is suitable for all pigments from a variety of algae (Kuczynska et al., 2015).

Due to the significant impact of extractants and detection instruments on the results of pigment analysis, appropriate extraction and detection methods should be selected according to the purposes of marine phytoplankton studies. (1) If the research focuses on estimation of phytoplankton biomass instead of the content of pigment types or phytoplankton species composition, the fluorometric method is a reasonable choice in most cases. However, in most coastal phytoplankton communities dominated by diatoms, a large amount of Chl-*a* derivatives with different fluorescence characteristics may interfere with the fluorometric results, leading to an over- or underestimation of total Chl *a* and a serious overestimation of the absolute Chl *a*. In this case, the HPLC method with 90% acetone as extractant is a better choice, which can achieve more accurate quantification of Chl *a* and its derivatives. Given the increasing availability of commercial standards for Chl-*a* derivatives (such as Chlide *a*), the HPLC method will have greater application potential for accurate estimation of phytoplankton biomass. (2) If the research focuses on the distinction of phytoplankton groups, such as analyzing phytoplankton community structure, determining the causative species of algal blooms, and tracking phytoplankton succession, the HPLC method with 95% methanol as extractant is the best choice. The method has high extraction efficiency for various diagnostic xanthophylls. Moreover, methanol is compatible with the mobile phases of most HPLC pigment analyses, which can reduce the adverse interaction among solute, solvent and mobile phase (Torres et al., 2014). To compensate for the underestimation of total Chl-*a* pigment caused by methanol, a conversion relationship between chlorophylls extracted with 90% acetone and 95% methanol could be established in the future through detailed comparative analysis of more laboratory cultured algae and field phytoplankton samples, or a mixed solvent of intermediate global polarity could be developed to obtain the most exhaustive pigment composition profile for phytoplankton community.

CHEMTAX is a useful tool for estimating the abundance of different phytoplankton taxa based on diagnostic pigments determined by HPLC (Mackey et al., 1996). This analysis depends on an initial data matrix of the most appropriate ratios of diagnostic pigments to Chl *a* (Kozlowski et al., 2014). Currently, the most commonly referenced CHEMTAX matrix (Mackey et al., 1996) is based on the absolute Chl *a*, and the pigment data were obtained from multiple studies using different pigment analysis methods. The underestimation of Chl *a* by methanol extraction would lead to the serious deviation of CHEMTAX calculation from the real situation, especially in phytoplankton communities dominated by algal groups with a high proportion of chlorophyll derivatives (such as diatoms). Thus, the initial ratios of pigment: Chl *a* for the phytoplankton classes should be adjusted according to different pigment extractants, and it should be considered whether total Chl-*a* pigment is more appropriate for determining the initial pigment ratio.

5 CONCLUSION

In this experiment, the fluorometric method and the HPLC method with different solvents in determination of phytoplankton pigments were compared. The fluorometric method significantly over- or underestimated the total Chl *a*, which could be three times higher than the absolute Chl *a* detected by HPLC in the diatom *P. tricornutum* due to the presence of Chl-*a* derivatives. The HPLC method with methanol or 95% methanol as extractant had high extraction efficiency for most of diagnostic xanthophylls (e.g., Fuco and Peri were 1.4–2.4 times higher than DMF and 90% acetone), but it had very low extraction efficiency for Chl *a*, *b*, *c* and promoted the production of Chl-*a* derivatives, which is less than half of 90% acetone in diatoms with high chlorophyllase activity. The lack of consistent and appropriate extraction and detection methods leads to incomparable pigment results across studies. For phytoplankton communities dominated by diatoms, it is suggested that the HPLC method with 90% acetone as extractant is a good choice to estimate phytoplankton biomass. The HPLC method with 95% methanol as extractant would be the best choice to resolve phytoplankton composition. There is still a need for more detailed comparisons in the future to establish conversion relationships between different extractants and detection methods, or to develop more functional extractants for a range of pigments.

6 DATA AVAILABILITY STATEMENT

The datasets analyzed during the current study are available from the corresponding author on reasonable request.

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Electronic supplementary material

Supplementary material (Supplementary Table S1 and Fig.S1) is available in the online version of this article at https://doi.org/10.1007/s00343-024-4025-9.