

# Orthogonal design for optimization of pigment extraction from surface sediments of the Changjiang River Estuary

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## Abstract

Using a suitable solvent for extracting pigments from sediment for high performance liquid chromatography (HPLC) analysis is critical for obtaining qualitative and quantitative estimates of phytoplanktonic and benthic algal biomass, as well as community composition. Five methodological factors (sample dehydration, extraction solvent, extraction duration, number of extractions, and ratio of solvent volume: sample weight) were studied using an  $L_9$  ( $3^4$ ) orthogonal design in a sedimentary pigment extraction experiment on samples collected from the Changjiang large-river delta-front estuary (LDE), using HPLC analysis. The results show that the optimal extraction method for sedimentary pigments should include freeze-drying samples prior to extraction. The effects of different factors on sedimentary pigment extraction were separated by the  $L_9$  ( $3^4$ ) orthogonal design experiments and showed that the extraction solvent was the most important, with extraction duration the second most important, and numbers of extraction and ratio of solvent volume: sample weight was the least important. The mixed solvent treatment comprised of acetone, methanol and water (80:15:5, by volume) was best for polar pigment extraction, with 100% acetone better for apolar pigments. For most pigments employed in this study (i.e., peridinin, fucoxanthin, alloxanthin, diatoxanthin, zeaxanthin, pheophytin-a and  $\beta$ -carotene), 3 h was found to be enough time for extraction from these deltaic sediments. However, for chlorophyll-a, the most important pigment used for estimating algal biomass, 12 h was needed. A small amount of solvent (3 ml) with duplicate extractions obtained the greatest amount and diversity of pigments. Unfortunately, no extraction method was found to be suitable for all pigments in sediments. The choice of extraction procedure should be made in accordance with the objective of each study, taking into consideration the properties of sediments and pigments in question.

**Key words:** sedimentary pigments, extraction, orthogonal design, HPLC, Changjiang River Estuary

## 1 Introduction

Sedimentary pigments have been shown to be useful biomarkers of algal and bacterial communities in contemporary estuarine and marine ecosystems (Bianchi, 2007; Jeffrey et al., 1997, and references therein). Pigments have also been used as fossil indicators of past primary productivity (Harris et al., 1996; Repeta, 1993) and historical reconstruction of

environmental change (Dahl et al., 2004; Chen et al., 2001; Bianchi et al., 2000; Schaeffer et al., 1993). In estuarine and coastal zones, which are commonly impacted by anthropogenic activities, sedimentary pigments can be used to reconstruct historical changes in phytoplankton community composition (Bianchi et al., 2002; Chen et al., 2001), effects of eutrophication (Kowalewska, 2005; Bianchi et al., 2000), and to trace

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different sources of organic carbon inputs to sediments (Bianchi et al., 1993). For all applications of pigments mentioned above, it is critical that extracting pigments qualitatively and quantitatively from sediments into suitable solvents for high performance liquid chromatography (HPLC) analysis. Otherwise, pigment concentrations are likely to be overestimated or underestimated.

Despite the wide application of sedimentary pigments as biomarkers in aquatic systems (e.g., Bianchi, 2007; Jeffrey et al., 1997), systematic studies, especially the effects of different factors, on the extraction procedures of sedimentary pigments for HPLC analysis have not been well documented (Szymczak-Zyla et al., 2008; Leavitt and Hodgson, 2001; Buffan-Dubau and Carman, 2000; Wright et al., 1997, and references therein). Based on these studies, an optimal extraction procedure requires the following criteria: high extraction efficiency of all pigments, high fidelity without alteration products of pigments, good replication with high precision, and simplicity for operators (Wright et al., 1997). Several factors, such as the physical characteristics of sediments, chemical properties and relative quantities of pigments in sediments, and practical safety of the employed organic solvents for lab users, are all considered to be important in the extraction of sedimentary pigments (Leavitt and Hodgson, 2001; Wright et al., 1997). To date, a universally applicable extraction method for pigments in sediments has not been adopted because of the wide range of sediment types and sources (Leavitt and Hodgson, 2001). Thus, selection of an appropriate procedure for a wide variety of sedimentary matrices remains an essential step for future studies.

Since a systematic study of the different factors that affect extraction of sedimentary pigments typically requires an extensive array of experimental manipulations, an inclusive statistically-designed experiment that requires relatively fewer experimental trials is necessary. In previous studies, single factor alternate methods (Buffan-Dubau and Carman, 2000; Wright et al., 1997) and factorial designs (Devesa et al., 2007) have been used for such purposes. In most traditional experimental manipulations, while the numbers of required experimental trials increase geometrically with an increasing number of variables, the applications of an orthogonal experimental design have been shown to dramatically reduce this number (Yang et al., 2008; Devesa et al., 2007; Wan et al., 1994). The theory and methodology for an orthogonal

array experimental design, a chemometric method for the optimization of analytical procedures, have been effectively described in other studies (Yang et al., 2008; Wan et al., 1994). Briefly, an orthogonal design is a cost-effective optimization strategy that can be used to assign experimental factors in a series of experimental combinations whose results can then be analyzed using a common mathematical procedure. In an orthogonal array, different combinations of numerals of any two columns have equal appearance frequency; hence, effects of different factors can be separated (Wan et al., 1994).

The aim of this study was to use an  $L_9 (3^4)$  orthogonal experimental design to determine the effects of the aforementioned primary factors (Leavitt and Hodgson, 2001; Wright et al., 1997) on the extraction of sedimentary pigments. In particular, we attempted to optimize the extraction procedure of pigments in sediments collected from one of the largest deltaic regions in the world—the Changjiang River (Yangtze River) Estuary.

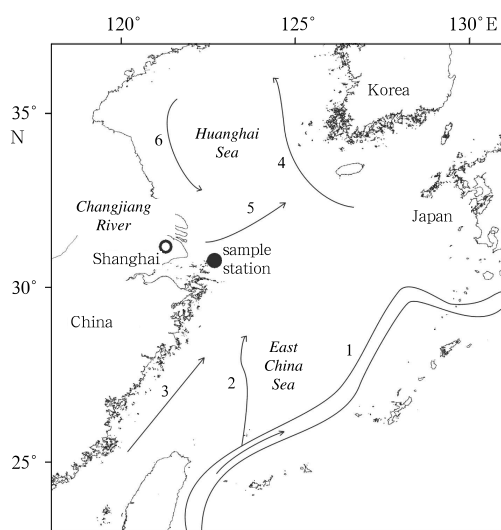
## 2 Materials and methods

### 2.1 Study areas and sediment collection strategy

The Changjiang River is the largest river in China and is the primary source of terrigenous sediments delivered to the continental shelf of the East China Sea (Wang et al., 2008). Large quantities of terrigenous materials are transported by the Changjiang River and deposited within the Changjiang large-river delta-front estuary (LDE) and the adjacent inner-shelf area (Liu et al., 2009). The prolific burial, transportation and remineralization of organic matter within this LDE make it an important interface between continental Asia and the Pacific Ocean (Liu et al., 2009). For example, the magnitude of river sediment and water fluxes from this LDE clearly have a global impact on ocean biogeochemistry, and also provide a natural “recorder” of global environmental change in these deltaic sediments (Bianchi and Allison, 2009). Sedimentary pigments have recently been used to reconstruct historical changes in paleoproductivity, phytoplankton composition and hypoxic events in the Changjiang LDE (Li et al., 2011; Jin et al., 2010). However, further work is needed to determine the optimal pigment extraction procedures in the sediments from this and other dynamic sedimentary regimes.

Sediment samples for pigment analysis were col-

lected onboard the R/V *Dongfanghong II*, in the Changjiang River Estuary ( $30^{\circ}45.9'N$ ,  $122^{\circ}41.4'E$ ) (Fig. 1) using a box-corer in June 2006. Surface sediments were carefully collected using a hand-trowel, homogenized, and stored frozen ( $-20^{\circ}C$ ) prior to pigment analysis—as recommended by Reuss and Conley (2005). The content of total organic carbon (TOC) in surface sediment is 0.82%.



**Fig.1.** Locations where sediment samples were collected from the Changjiang River Estuary ( $30^{\circ}45.9'N$ ,  $122^{\circ}41.4'E$ ) in June 2006 for sedimentary pigment analysis. The summer water current circulation pattern in the East China Sea and Huanghai Sea (Yellow Sea) (Naimie et al., 2001) are shown: 1. Kuroshio, 2. Taiwan Warm Current, 3. East China Sea Coastal Current, 4. Huanghai Sea Warm Current, 5. Changjiang River Plume, and 6. Huanghai Sea Coastal Current.

## 2.2 Single factor experiment for dehydration steps

Chlorophyll and carotenoid pigments are lipophilic and water-insoluble, unlike phycobilin pigments which are hydrophilic (Rowan, 1989). Since this study was focused on water-insoluble pigments, the water content of sediments could alter the suitability of organic solvents for maximizing extraction efficiency of sedimentary pigments. Therefore, it was necessary to dehydrate sediments prior to extracting pigments. The common methods used for dehydration are freeze-drying (Plante-Cuny et al., 1993) and centrifugation (Eckardt et al., 1991). We used a single-factor experiment to determine which dehydration step was

best for pigment extraction. Frozen sediment was first homogenized and divided into two groups. One was freeze-dried with a LABCONCO (Freezone-6, USA) system and another was centrifuged (2 770 g, TDL-40B, ANKE, China) to remove water. Both groups were divided into triplicate sub-samples (i.e.,  $n=3$ ). Each sub-sample (ca. 1 g dry weight) was flushed with 4 ml 100% acetone, mixed by vortex stirrer (MS 3 basic, IKA, Germany), sonicated (SK250H, KUDOS, China) in ice-water bath for 5 min, placed in a freezer ( $-20^{\circ}C$ ) for 3 h, centrifuged, and the supernatant decanted into a glass tube. The extraction process was repeated again (the duration in a freezer is 0 h for the second time). The extracts were combined, evaporated to dryness with nitrogen, twice re-dissolved with 250  $\mu$ l 95% methanol (i.e., 500  $\mu$ l in all), filtered with a syringe filter (0.45  $\mu$ m PTFE, Whatman, UK), and placed into a 1.5 ml amber glass vial prior to HPLC analysis. The whole extraction procedure was performed in dark or dim light, and under low temperature conditions (Leavitt and Hodgson, 2001).

## 2.3 Selection of factors for orthogonal design experiment

Based on the primary factors considered to be important when extracting sedimentary pigments (Leavitt and Hodgson, 2001; Wright et al., 1997), four factors were selected for the  $L_9$  ( $3^4$ ) orthogonal experiment (shown in Table 1). The design was focused on determination of an optimal extraction method—using the most efficient dehydration step. The  $L_9$  ( $3^4$ ) matrix is a three-level orthogonal design, in which three conditions are selected for each factor. The numbers of trials are nine, which is far fewer than factorial design (Devesa et al., 2007). The four factors (with three conditions for each one) connected with pigment extraction examined in this study were: A. extraction duration (0, 3 and 12 h); B. extraction solvents [the most common solvent 100% acetone (ACE) (Reuss and Conley, 2005; Airs et al., 2001; Chen et al., 2001), mixture solvent recommended by Leavitt and Hodgson (2001): acetone: methanol: water=80:15:5 (v/v/v) (MIX), and 95% methanol (MET) which is usually used to extract algal pigments from water column (Yao et al., 2006)]; C. numbers of extraction (once, twice and three times); D. ratios of solvent volume to sample weight (v/w, sample weights were set as ca. 1 g dry weight, and solvent volumes as 2, 3, 4 ml based on pre-experimental trials).

It should be noted that while these combinations

**Table 1.** L<sub>9</sub> (3<sup>4</sup>) orthogonal experiment designed for the extraction of sedimentary pigments

Test No.	A: Extraction duration/h	B: Extraction solvent <sup>1)</sup>	C: Numbers of extraction	D: Ratio of v/w/ml·g <sup>-1</sup>
1	A <sub>1</sub> 0	B <sub>1</sub> ACE	C <sub>1</sub> 1	D <sub>1</sub> 2
2	A <sub>1</sub> 0	B <sub>2</sub> MIX	C <sub>2</sub> 2	D <sub>2</sub> 3
3	A <sub>1</sub> 0	B <sub>3</sub> MET	C <sub>3</sub> 3	D <sub>3</sub> 4
4	A <sub>2</sub> 3	B <sub>1</sub> ACE	C <sub>2</sub> 2	D <sub>3</sub> 4
5	A <sub>2</sub> 3	B <sub>2</sub> MIX	C <sub>3</sub> 3	D <sub>1</sub> 2
6	A <sub>2</sub> 3	B <sub>3</sub> MET	C <sub>1</sub> 1	D <sub>2</sub> 3
7	A <sub>3</sub> 12	B <sub>1</sub> ACE	C <sub>3</sub> 3	D <sub>2</sub> 3
8	A <sub>3</sub> 12	B <sub>2</sub> MIX	C <sub>1</sub> 1	D <sub>3</sub> 4
9	A <sub>3</sub> 12	B <sub>3</sub> MET	C <sub>2</sub> 2	D <sub>1</sub> 2

Notes: <sup>1)</sup> ACE represents 100% acetone, MIX mixture solvent of acetone: methanol: water=80:15:5 (v/v/v), and MET 95% methanol.

of different extraction regimes were not exhaustive for all possible solvents used in extraction of pigments from the past studies (e.g., 90% acetone, dimethyl formamide [DMF], dimethyl sulphoxide [DMSO], dimethyl acetamide, ethanol, chloroform, benzene, tetrahydrofuran [THF]) (Louda et al., 2000; Wright et al., 1997, and references therein), they were chosen by considering their extractability of a broad diversity of pigments, safety to the lab-users, and represent enough variability for an orthogonal design experiments with these types of sediments.

## 2.4 HPLC pigment analysis

All pigments were analyzed with an Agilent 1100 HPLC system equipped with a quaternary pump (G1311A), an on-line vacuum degasser (G1379A), a manual sampler (100  $\mu$ l loop), a column compartment (G1316A), a photodiode array detector (PDA, G1315B), and a fluorescence detector (G1321A) (Agilent Technologies, USA). The injector was connected via a guard-column to a reverse-phase C<sub>18</sub> column (5  $\mu$ m particle size, 250 mm×4.6 mm i.d., Spherisorb ODS2, Waters, USA) which was thermostated at 25 °C. The HPLC gradient program was a modification of Wright et al. (1991), as described by Chen et al. (2001). The injection volume was 100  $\mu$ l and the flow rate was 1.00 ml/min.

HPLC pigment chromatograms were integrated

using ChemStation software. Pigments were identified based on absorbance spectra and retention time by comparing with authentic pigment standards obtained from DHI Water & Environment, Denmark. Eight diagnostic pigments, peridinin (Perid, Dinoflagellates), fucoxanthin (Fuco, Diatoms), alloxanthin (Allo, Cryptophytes), diatoxanthin (Diato, Diatoms and Dinoflagellates), zeaxanthin (Zea, Cyanobacteria), chlorophyll-a (Chl-a, total algal abundance), pheophytin-a (PHTin-a, degradation product of Chl-a) and  $\beta$ -carotene ( $\beta$ -Car, total algal abundance) with different polarities and stabilities, were employed (Table 2). While these pigments represent only a fraction of diversity of chlorophyllous and carotenoid pigments found in natural sediments, they do represent some of the most important pigments representative of dominant phytoplankton classes found in coastal environments (Bianchi, 2007; Jeffrey et al., 1997, and references therein). All analyses were conducted using the areas of the chromatographic peaks (AU·s) directly rather than normalizing the data to  $\mu$ g/g or nmol/g organic carbon or dry weight sediment. Statistical analysis of the dataset was conducted by Statistical Package for the Social Science (SPSS) software.

## 3 Results and discussion

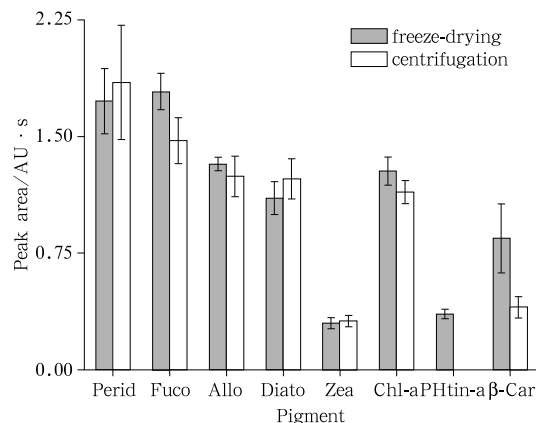
### 3.1 Sediment dehydration

Peak areas of pigments extracted from sediment

**Table 2.** Summary of eight diagnostic pigments characteristics

Pigment	Abbreviation	Retention time/min	$\lambda_{\max}$ /nm	Polarity	Stability <sup>1)</sup>
Peridinin	Perid	10.3	474	polar	4
Fucoxanthin	Fuco	11.9	446	polar	2
Alloxanthin	Allo	18.6	454 483	polar	1
Diatoxanthin	Diato	19.8	454 482	polar	2
Zeaxanthin	Zea	21.8	457 481	polar	1
Chlorophyll-a	Chl-a	26.1	431 662	apolar	3
Pheophytin-a	PHTin-a	31.1	411 662	apolar	1
$\beta$ -Carotene	$\beta$ -Car	33.2	457 481	apolar	1

Notes: <sup>1)</sup> Relative degree of chemical stability is ranked from most (1) to least (4) stable (Leavitt and Hodgson, 2001).



**Fig.2.** Extracted pigment contents (mean $\pm$ 1 SD,  $n=3$ ) in surface sediment determined from freeze-dried and centrifuged samples (ca. 1 g dry weight) extracted in 4 ml 100% acetone twice for 3 h.

samples, dehydrated by both freeze-drying and centrifugation, are shown in Fig. 2. Most of the pigments showed no significant difference in concentration ( $t$ -test,  $p > 0.10$ ) from freeze-dried versus centrifuged treatments. Only Fuco, PHtin-a and  $\beta$ -Car showed a significant difference ( $t$ -test,  $p < 0.05$ ), whereby freeze-dried samples yielded higher pigment concentrations than centrifuged samples (Fig. 2), supporting previous experimental results (Buffan-Dubau and Carman, 2000). In that study, it was shown that most sedimentary pigment extraction efficiencies were signif-

icantly lower in natural wet sediments compared to freeze-dried samples. As expected, water content of sediments can change the extraction efficiency of pigments. From a logistical perspective, it also required significantly more time to evaporate hydrated extracts from centrifuged sediments compared to freeze-dried sediments. Another consideration of this longer evaporated period is that labile pigments may be degraded, creating more extraction artifacts in the data. Thus, freeze-drying samples, as recommended by Buffan-Dubau and Carman (2000) and Leavitt and Hodgson (2001) was chosen as the dehydration step in this study.

### 3.2 Physical properties as factors affecting pigment extraction in sediments

Test results of the  $L_9$  ( $3^4$ ) orthogonal experiment are presented in Table 3. Since only a local optimal extraction condition could be selected based on the results shown in Table 3, further orthogonal analysis was needed for global optimization. Hence, the  $K$  (average of certain factor and level, can be used to determine the global optimal extraction condition) and  $R$  (range of  $K$ , can show the effect of a certain factor on the extraction efficiency) values were calculated and are shown in Table 4. Here:  $K_i^X = (\sum \text{the amount of target pigments at } X_i) / 3$ ,  $R^X = \max\{K_i^X\} - \min\{K_i^X\}$  with  $X$  (factor)=A, B, C or D, and  $i$  (level)=1, 2 or 3.

**Table 3.** Extracted pigment contents of  $L_9$  ( $3^4$ ) orthogonal experiment (mean $\pm$ 1 SD,  $n=3$ ) (unit: AU·s)

Test No.	Perid	Fuco	Allo	Diato	Zea	Chl-a	PHtin-a	$\beta$ -Car
1	0.956 $\pm$ 0.121	1.217 $\pm$ 0.187	0.778 $\pm$ 0.102	0.852 $\pm$ 0.103	0.291 $\pm$ 0.032	1.126 $\pm$ 0.148	0.189 $\pm$ 0.034	0.629 $\pm$ 0.078
2	1.392 $\pm$ 0.101	1.578 $\pm$ 0.104	1.519 $\pm$ 0.194	1.445 $\pm$ 0.108	0.340 $\pm$ 0.052	0.895 $\pm$ 0.156	—	0.465 $\pm$ 0.078
3	1.435 $\pm$ 0.134	1.571 $\pm$ 0.174	1.477 $\pm$ 0.107	1.456 $\pm$ 0.172	0.406 $\pm$ 0.042	0.601 $\pm$ 0.174	—	0.506 $\pm$ 0.051
4	1.728 $\pm$ 0.208	1.790 $\pm$ 0.116	1.321 $\pm$ 0.042	1.102 $\pm$ 0.106	0.298 $\pm$ 0.037	1.276 $\pm$ 0.091	<b>0.356<math>\pm</math>0.031</b>	<b>0.845<math>\pm</math>0.222</b>
5	<b>1.713<math>\pm</math>0.084</b>	<b>1.882<math>\pm</math>0.032</b>	<b>2.221<math>\pm</math>0.065</b>	<b>1.758<math>\pm</math>0.064</b>	0.410 $\pm$ 0.004	0.883 $\pm$ 0.055	—	0.596 $\pm$ 0.193
6	1.584 $\pm$ 0.062	1.643 $\pm$ 0.078	1.480 $\pm$ 0.056	1.459 $\pm$ 0.080	<b>0.441<math>\pm</math>0.019</b>	0.505 $\pm$ 0.139	—	0.554 $\pm$ 0.035
7	1.499 $\pm$ 0.056	1.807 $\pm$ 0.052	1.411 $\pm$ 0.053	1.155 $\pm$ 0.063	0.306 $\pm$ 0.037	<b>1.425<math>\pm</math>0.015</b>	0.270 $\pm$ 0.029	0.573 $\pm$ 0.041
8	1.435 $\pm$ 0.085	1.634 $\pm$ 0.097	1.801 $\pm$ 0.089	1.466 $\pm$ 0.017	0.376 $\pm$ 0.013	1.307 $\pm$ 0.053	—	0.467 $\pm$ 0.073
9	1.502 $\pm$ 0.333	1.563 $\pm$ 0.330	1.294 $\pm$ 0.313	1.416 $\pm$ 0.320	0.386 $\pm$ 0.088	0.723 $\pm$ 0.157	—	0.321 $\pm$ 0.240

Note: The local optimal extraction conditions are in bold.

#### 3.2.1 Ranking order of factors

The effects of different factors on different characteristic pigments were separated by the  $L_9$  ( $3^4$ ) orthogonal experiment. Based on the  $R$  values, the effect orders of the four factors were not the same:  $A > C > D > B$  for Perid and Fuco;  $B > A > C > D$  for Allo, Diato and Zea; and  $B > A > D > C$  for Chl-a and  $\beta$ -Car (Table 4). The extraction solvent was found to be the most important determinant for extraction efficiency in most

of the tested pigments, and the extraction duration was the second most important. Comparatively, number of extraction and the ratio of v/w were not found to have significant effects. As for the labile pigments (e.g., Perid and Fuco) (Leavitt and Hodgson, 2001), the influence of extraction duration was greater than solvent type.

#### 3.2.2 Extraction duration

The range of extraction durations were either

**Table 4.** Further analysis of L<sub>9</sub> (3<sup>4</sup>) orthogonal experiment results (unit: AU·s)

Pigments		A	B	C	D
Perid	<i>K</i> <sub>1</sub>	1.261	1.394	1.325	1.390
	<i>K</i> <sub>2</sub>	<b>1.675</b>	<b>1.513</b>	1.541	1.492
	<i>K</i> <sub>3</sub>	1.479	1.507	<b>1.549</b>	<b>1.533</b>
	<i>R</i>	0.414	0.119	0.224	0.143
Fuco	<i>K</i> <sub>1</sub>	1.455	1.604	1.498	1.554
	<i>K</i> <sub>2</sub>	<b>1.771</b>	<b>1.698</b>	1.643	1.676
	<i>K</i> <sub>3</sub>	1.668	1.592	<b>1.753</b>	<b>1.665</b>
	<i>R</i>	0.316	0.106	0.255	0.122
Allo	<i>K</i> <sub>1</sub>	1.258	1.170	1.353	1.431
	<i>K</i> <sub>2</sub>	<b>1.674</b>	<b>1.847</b>	1.378	1.470
	<i>K</i> <sub>3</sub>	1.502	1.417	<b>1.703</b>	<b>1.533</b>
	<i>R</i>	0.416	0.677	0.350	0.102
Diat	<i>K</i> <sub>1</sub>	1.251	1.036	1.259	1.342
	<i>K</i> <sub>2</sub>	<b>1.439</b>	<b>1.556</b>	1.321	1.353
	<i>K</i> <sub>3</sub>	1.346	1.444	<b>1.456</b>	<b>1.341</b>
	<i>R</i>	0.188	0.520	0.197	0.012
Zea	<i>K</i> <sub>1</sub>	0.345	0.298	0.369	0.362
	<i>K</i> <sub>2</sub>	<b>0.383</b>	0.375	0.341	0.362
	<i>K</i> <sub>3</sub>	0.356	<b>0.411</b>	<b>0.374</b>	<b>0.360</b>
	<i>R</i>	0.038	0.113	0.033	0.002
Chl-a	<i>K</i> <sub>1</sub>	0.874	<b>1.276</b>	0.979	0.911
	<i>K</i> <sub>2</sub>	0.888	1.028	0.964	0.942
	<i>K</i> <sub>3</sub>	<b>1.152</b>	0.610	<b>0.970</b>	<b>1.061</b>
	<i>R</i>	0.278	0.666	0.015	0.150
$\beta$ -Car	<i>K</i> <sub>1</sub>	0.533	<b>0.683</b>	0.550	0.515
	<i>K</i> <sub>2</sub>	<b>0.665</b>	0.509	0.543	0.531
	<i>K</i> <sub>3</sub>	0.454	0.460	<b>0.559</b>	<b>0.606</b>
	<i>R</i>	0.211	0.223	0.016	0.091

Notes:  $K_i^X = (\sum \text{the amount of target pigments at } X_i) / 3$ ;  
 $R^X = \max\{K_i^X\} - \min\{K_i^X\}$ ;  $X$  is A, B, C or D;  $i=1, 2$  or  $3$ .

The global optimal extraction conditions are in bold.

from 0 to 24 h (Engstrom et al., 2006; Patoine and Leavitt, 2006; Leavitt and Hodgson, 2001), or not performed for an exact amount time (Reuss and Conley, 2005; Airs et al., 2001; Chen et al., 2001) in different studies. Three different durations (0, 3 and 12 h) were selected in the orthogonal experiment based on a single factor pre-experiment of different duration (0, 1, 3, 6 and 12 h). Our results showed that the extracted contents of most pigments (except for Chl-a) from sediment samples for 3 h were higher than 0 and 12 h (Table 4). This likely occurred because 0 h was clearly too short to extract pigments and 12 h appeared too long, especially for labile pigments (e.g. Perid) which may have started to degrade. The amount of extracted Chl-a increased significantly with extraction duration. This likely resulted from the presence of “bound” Chl-a (non-extractable by acetone) which took a longer time to release into “free” pool

(acetone extractable) (Sun et al., 1993). Therefore, interpretation of the percent of Chl-a, such as a measure of eutrophication (Kowalewska, 2005) or relative abundance of chloropigments (Chen et al., 2003) should be done with caution—a suitable extraction duration should be taken into consideration. Otherwise, the degradation degree of Chl-a could be overestimated or underestimated.

### 3.2.3 Extraction solvents

The approaches to selecting solvents for pigment extraction can be quite varied, such as numerous organic solvents [ACE (Reuss and Conley, 2005; Airs et al., 2001; Chen et al., 2001), MIX (Engstrom et al., 2006; Leavitt and Hodgson, 2001), buffered methanol (2% 0.5 mol/L ammonium acetate) (Brotas and Plante-Cuny, 2003; Wright et al., 1991), 90% acetone (Lucas and Holligan, 1999), DMF (Fietz et al., 2005), DMSO (Devesa et al., 2007), etc.]. In this study, ACE, MIX and MET were chosen to test the effect of solvents on sedimentary pigment extraction based both on their extraction efficiency and safety to the users. The results show that the extracted contents of Perid, Fuco, Allo and Diato (polar pigments) were greatest in MIX, while ACE was the best extraction solvent for Chl-a, PHTin-a and  $\beta$ -Car (apolar pigments) (Tables 3 and 4). Both Chl-a and PHTin-a were extracted with higher efficiency by ACE than MIX or MET. This indicates that PHTin-a extracted by ACE was indeed representative of the natural sediment sample rather than any artifact produced during the extraction process.

### 3.2.4 Number of extractions and ratios of v/w

The extracted contents of most pigments increased both with numbers of extraction and ratios of v/w, but the ranges were smaller (Table 4). Hence, extracting sedimentary pigments under 3 ml/g (v/w) twice is recommended, taking into consideration the simplicity for operators. Interestingly, in past studies, when freeze-drying was used, the numbers of extractions are almost never mentioned (Reuss and Conley, 2005; Chen et al., 2001; Leavitt and Hodgson, 2001; Bianchi et al., 2000; Buffan-Dubau and Carman, 2000). In contrast, when using centrifugation, the numbers of extraction are reported, typically ranging from twice to three times (Kowalewska, 2005; Naylor and Keely, 1998; Eckardt et al., 1991). The solvent

volumes and sample weights also vary enormously in different studies (Reuss and Conley, 2005; Leavitt and Hodgson, 2001; Buffan-Dubau and Carman, 2000; Naylor and Keely, 1998; Eckardt et al., 1991) typically ranging from ca. 3 ml/g dry weight to over 50 ml/g. In this study, we chose once, twice and three times as three levels of numbers of extraction, and 2, 3 and 4 ml/g as three levels of ratios of v/w for the orthogonal experiment. Generally, small amount of solvent with multiple numbers of extractions should obtain higher levels of pigment content by using less solvent, which can shorten the time needed by the following nitrogen blow-drying process. Also, less labile pigments might be degraded and fewer artifacts should be produced during this process allowing for assured fidelity.

### 3.3 Optimization of sedimentary pigments extraction for the Changjiang LDE

Based on the above analysis, an ideal extraction

method was not found, even for the limited number of sedimentary pigments analyzed in this study because of the differences in the properties of pigments (e.g., polar vs. apolar, labile vs. stable, easy vs. hard to be extracted). Therefore, the choice of sedimentary pigments extracting procedure should be made in accordance with the objective of each study, taking into consideration the properties of pigments. For example, if a study is focused on phytoplankton production or biomass and its historical change, or on eutrophication, the employed biomarker pigments (Chl-a and its degradation products) are mostly apolar; if a study is focused on characterizing planktonic and benthic algal community compositions and their changes, the used diagnostic pigments (Perid, Fuco, Allo, Diato, Zea, etc) are mostly polar. In most previous studies, unfortunately, it seems that the properties of pigments were not considered—one extracting procedure for all pigments was usually used (Table 5).

**Table 5.** Extraction procedures of sedimentary pigments from different estuaries

Study areas	Pre-treatment <sup>1)</sup>	Extraction solvent <sup>2)</sup>	Extraction duration	Numbers of Extraction <sup>3)</sup>	v/w per time	Pigments <sup>4)</sup>	References
Changjiang Estuary, China	A	ACE	12 h	2	3 ml/1 g	apolar: Chl-a, PHTin-a, $\beta$ -Car	this study
Changjiang Estuary, China	A	MIX	3 h	2	3 ml/1 g	polar: Perid, Fuco, Diato, Allo, Zea	this study
Mississippi River Estuary, USA	A	ACE	overnight	—	3 ml/1–2 g	apolar: Chl-a, PHTin-a, SCEs, CCEs, etc polar: BChls, BPHTins, Fuco, Zea, etc	Chen et al. (2003, 2001)
Four Northern European estuaries	A	ACE	overnight	—	2 ml/0.1–0.5 g	apolar: Chl-a, $\beta$ -Car, PHTin-a, etc polar: Fuco, Diato, Allo, Lutein, Zea, etc	Reuss et al. (2005)
Cocodrie, LA, USA	A	ACE	3 h	—	5 ml/0.5 g	apolar: Chl-a, PHTin-a, $\beta$ -Car polar: Fuco, Zea, Lutein, PHide-a	Buffan-Dubau and Carman (2000)
Westerschelde Estuary, Netherlands	A	90% acetone	0	—	10 ml/0.5 g	apolar: Chl-a, $\beta$ -Car, PHTin-a, etc polar: Chl-c, Perid, Fuco, Diato, Lutein-Zea, PHide-a, etc	Lucas and Holligan (1999)
Tagus Estuary, Spain	A	Buffered methanol	15 min	—	2–3 ml/1 g	apolar: Chl-a, $\beta$ -Car, etc polar: Chl-c, Fuco, Allo, Lutein, Zea, etc	Brotas and Plante-Cuny (2003)
Estuaries of Wisla and Odra, Poland	B	ACE	0	3	15 ml/0.5–10 g	apolar: Chl-a, PHTin-a, pPHTin-a, SCEs, etc polar: Chl-c	Kowalewska (2005)

Notes: <sup>1)</sup> A represents Frozen and freeze-dried before analysis and B Remove water by centrifugation. <sup>2)</sup> ACE represents 100% acetone, MIX mixture solvent of acetone: methanol: water=80:15:5 (v/v/v). <sup>3)</sup> — represents not mentioned. <sup>4)</sup> Chl-a represents chlorophyll-a, Chl-c chlorophyll-c, PHTin-a pheophytin-a, PHide-a pheophorbide-a, pPHTin-a pyropheophytin-a,  $\beta$ -Car  $\beta$ -carotene, Perid peridinin, Fuco fucoxanthin, Diato diatoxanthin, Allo alloxanthin, Zea zeaxanthin, SCEs steryl chlorin esters, CCEs carotenol chlorin esters, BChls bacteriochlorophylls, and BPHTins bacteriopheophytins.

As for the Changjiang LDE, freeze-drying samples was superior to centrifugation and natural wet samples for sedimentary pigments extraction, which is similar to most previous studies (Table 5). However, the results here are different from the previous studies in that these results are based on an  $L_9 (3^4)$  orthogonal design experiment, with the consideration of criteria required by an optimal extraction procedure (Wright et al., 1997). So in conclusion, if the aim of a study was focused on changes of phytoplankton production or biomass (using Chl-a and its degradation products, e.g., pheophytin-a, pheophorbide-a, steryl chlorin esters, carotenol chlorin esters, as biomarkers) (Villanueva and Hastings, 2000), the recommended sedimentary pigments extraction procedure should use 3 ml/g ACE to extract the samples twice for 12 h. If the objective was to characterize planktonic and benthic algal community compositions and their changes (using diagnostic carotenoids, e.g., Perid, Fuco, Allo and Diato, as biomarkers) (Bianchi, 2007; Jeffrey et al., 1997, and references therein), the recommended procedure should use 3 ml/g MIX to extract the samples twice for 3 h. If both phytoplankton production and community composition were key objectives of research, the suggested method should use 3 ml/g MIX to extract the samples for 3 h for the first phase followed by 3 ml/g ACE to extract the samples for 9 h in the second phase.

According to the comparisons of extraction procedures of sedimentary pigments from other estuaries and the Changjiang River Estuary in this study (Table 5), we can find that the procedures used in other studies may not be the optimal conditions for sediment samples from the Changjiang River Estuary based on the results of our orthogonal experiment. For example, when the extraction duration was not long enough, more numbers of extractions and/or higher ratios of v/w were needed (Kowalewska, 2005; Brotas and Plante-Cuny, 2003; Lucas and Holligan, 1999); and when the sediment samples were extracted only one time, higher ratios of v/w were needed (Reuss et al., 2005; Chen et al., 2003, 2001; Buffan-Dubau and Carman, 2000; Lucas and Holligan, 1999), otherwise the extraction efficiency for sedimentary pigments would be decreased.

It is noted here, that the optimal sedimentary pigments extraction conditions were concluded based on the samples collected from Changjiang River Estuary (clay based sediment), and it might be not the best conditions for other based sediments [e.g., carbonate

marls, Louda et al. (2000)]. However, the effects of different factors on different characteristic pigments were separated by orthogonal experiment, which should be valuable for optimizing extraction of pigments from samples collected from various sediment matrixes.

## 4 Conclusions

Based on the results of the  $L_9 (3^4)$  orthogonal design experiment with the consideration of aforementioned criteria for optimal extraction (Wright et al., 1997), we found that the extraction procedure should be made in accordance with the objective of each study, taking into consideration the properties of sediments and pigments in question (e.g., polar vs. apolar, labile vs. stable, easy vs. hard to be extracted). We recommend that the optimized extraction method for sedimentary pigments include freeze-drying samples prior to extraction as opposed to centrifugation. The effects of different factors on sedimentary pigment extraction revealed that extraction solvent was the most important, extraction duration is the second most important, and numbers of extraction and ratios of solvent volume: sample weight are the least important. The mixed solvent of acetone, methanol and water (80:15:5, by volume) is optimal to extract polar pigments, and for apolar pigments, 100% acetone is better. For most pigments employed in this study (e.g., peridinin, fucoxanthin, alloxanthin, diatoxanthin, zeaxanthin, pheophytin-a and  $\beta$ -carotene), 3 h was found to be enough time for extraction from these deltaic sediments. However, chlorophyll-a, the most important pigment used for estimating algal biomass, 12 h was needed. Small amounts of solvent with multiple numbers of extraction (3 ml/g $\times$ 2) obtained the greatest amount and diversity of pigments.

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