

NOTE

Extraction of benthic microalgal pigments for HPLC analyses**Evelyne Buffan-Dubau*, Kevin R. Carman**

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ABSTRACT: Studies of microphytobenthic communities often require the use of HPLC pigment analysis. The first step of such chromatographic analyses consists of extracting pigments. Three methodological points relative to sedimentary pigment extraction for subsequent HPLC analyses were examined using sediment samples from a mudflat. We studied effects of both sample freeze-drying prior to extraction, and 2 extraction solvents (100% acetone and buffered methanol). We also examined the influence of extraction time on pigment recovery. Freeze-drying significantly improved pigment extraction from mud samples. Acetone yielded significantly better recoveries of pheopigments, but (in freeze-dried sediment) acetone and buffered methanol did not differ significantly in their extraction of chl *a* or carotenoids. Long-time extraction (up to 24 h) improved recovery of chl *a*, but not other pigments.

KEY WORDS: Sediments · Pigments · Extraction · HPLC

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Pigment studies provide useful information about oceanic plant communities. Measurements of chlorophyll *a* (chl *a*) in surface waters and superficial sediments provide estimates of plant biomass and productivity whereas carotenoid pigments are commonly used as taxonomic biomarkers to characterise planktonic and benthic microalgal communities (Riaux-Gobin et al. 1987, Plante-Cunney et al. 1993, Pinckney et al. 1994). Such studies are now often handled by the use of HPLC pigment analyses (Cariou-Le Gall & Blanchard 1995, Jeffrey et al. 1997, Brotas & Plante-Cunney 1998). HPLC analysis techniques allow separation of pigments prior to spectrophotometric and/or fluorimetric measurements, avoiding interference among pigments.

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Although HPLC techniques have been applied to the study of natural microphytobenthic communities (Riaux-Gobin et al. 1987, Plante-Cunney et al. 1993, Cariou-Le Gall & Blanchard 1995, Brotas & Plante-Cunney 1998, Paterson et al. 1998, Lucas & Holligan 1999, Buffan-Dubau & Carman 2000), studies of protocols adapted to sediment sample analysis are rare (Riaux-Gobin et al. 1987, Plante-Cunney et al. 1993, Brotas & Plante-Cunney 1996). The first step in chromatographic analysis consists of performing a qualitatively and quantitatively efficient extraction of pigments from samples into a compatible solvent. Pigment extraction efficiency can be influenced by many factors including the choice of the extraction solvent, based on its penetration power and solvation properties, the duration of extraction, and treatments applied to samples prior to extraction (Jeffrey et al. 1997). These points remain a matter of debate for sedimentary pigment analyses. Pure acetone and buffered methanol have been recommended as extraction solvents for phytoplankton pigment analyses (Wright et al. 1991, Jeffrey et al. 1997), and are both commonly used for microphytobenthos studies without explicit consideration of extraction efficiency (Riaux-Gobin et al. 1987, Plante-Cunney et al. 1993, Cariou-Le Gall & Blanchard 1995, Buffan-Dubau et al. 1996, Brotas & Plante-Cunney 1998, Paterson et al. 1998, Lucas & Holligan 1999). Durations of extractions reported in the literature vary from a few minutes (Brotas & Plante-Cunney 1998) to 24 h (Cariou-Le Gall & Blanchard 1995). While freeze-drying of sediment samples prior to extraction has been proposed, Riaux-Gobin et al. (1987) found that freeze-drying reduced efficiency of sedimentary pigment extraction whereas Plante-Cunney et al. (1993) observed that it improved reproducibility of results. Accordingly, the purpose of this work was to test these methodological issues. This note describes how choice of solvent, freeze-drying, and duration of extraction influenced our extraction efficiency.

Materials and methods. The study site, located near Cocodrie, Louisiana, USA (30° 15' N, 91° 21' W), is a mudflat surrounded by stands of *Spartina alterniflora* Loisel. Sediment samples were collected in June 1997 (extraction-duration experiment) and June 1999 (solvent and freeze-drying experiment). Sediment cores were hand collected using butyrate tubing (3.5 cm i.d.). Surficial sediment (top 1 cm) was collected, frozen on liquid nitrogen, and stored at -80°C . Further details of

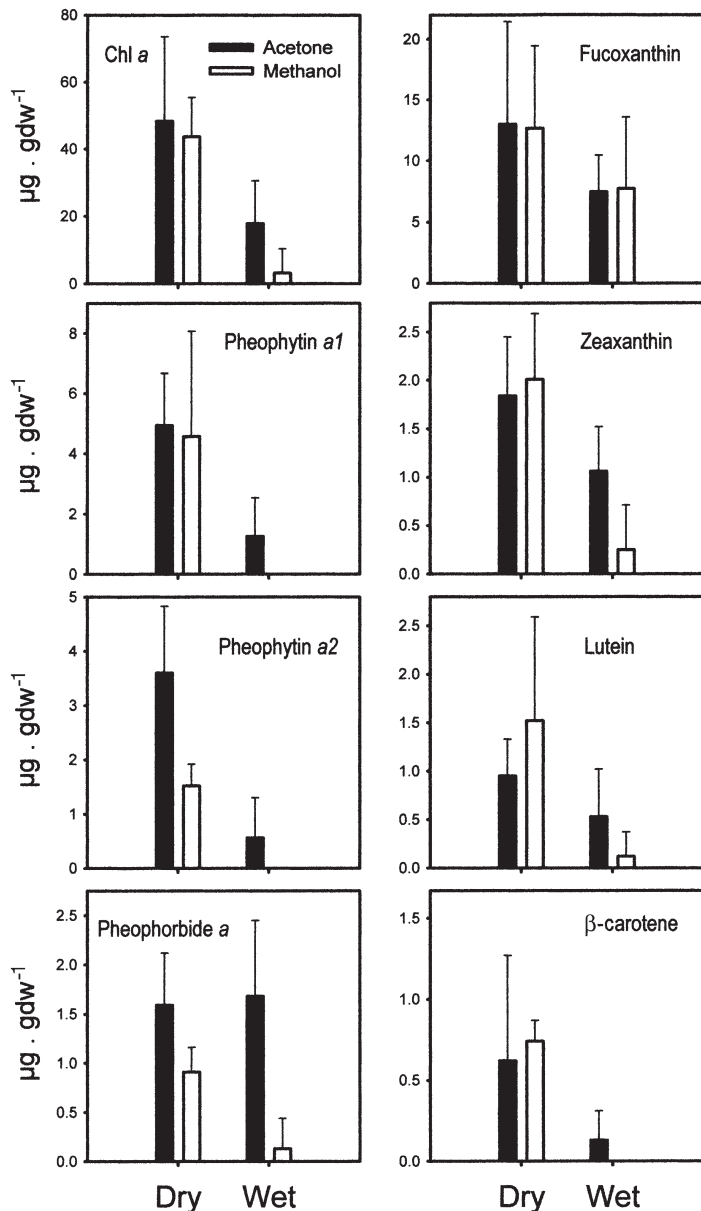


Fig. 1. Average pigment concentrations in superficial sediment (top 1 cm) determined from freeze-dried (Dry) and non-freeze-dried (Wet) samples extracted in 100% acetone and buffered methanol. Concentrations are normalized for sediment dry weight. Error bars are ± 1 SD ($n = 6$)

the study site, sample collection and preservation, as well as the liquid chromatograph and the HPLC analysis procedure (Wright et al. 1991) to determine benthic microalgal pigment concentrations, are described in Buffan-Dubau & Carman (2000). During analysis of the extraction-duration test, pheophorbide *a* was not properly detected with our system, thus we have no data on pheophorbide *a* concerning the extraction-duration test.

Extraction efficiencies of methanol versus acetone solvents, and the influence of freeze-drying were tested using sediment from 6 replicate cores. Sediment from each core was homogenized and divided into 4 subsamples (0.50 ± 0.13 g dry weight) and frozen at -80°C . Two subsamples from each core were freeze-dried. One of the freeze-dried and one of the non-freeze-dried samples from each core were extracted in acetone, and the remaining samples were extracted in methanol. Pigments were extracted by sonicating for 30 s in either 5 ml 100% cold acetone or 5 ml cold buffered methanol (2% 0.5 M ammonium acetate; Wright et al. 1991) and incubating overnight in the dark at -20°C . Extracts were then separated from sediment by centrifugation, filtered (Corning 0.2 μm), and stored in the dark at -80°C under nitrogen gas atmosphere. Immediately before HPLC analysis, extracts were diluted with water (Mallinckrodt, HPLC quality) to 70% acetone or 80% methanol to improve the sharpness of peaks (Wright et al. 1991). One hundred μl of each diluted extract was analysed by HPLC within 24 h following extraction.

Effects of varied extraction durations were tested using frozen superficial sediment (top 1 cm) partitioned in 3 subsamples (0.63 to 0.66 g dry weight) and freeze-dried. Pigments were extracted by sonicating each subsample for 30 s in 15 ml 100% cold acetone and incubating for 24 h in the dark at -20°C . During the 24 h incubation, extracts were sampled at 3, 6, 12, and 24 h, by collecting 1 ml aliquots, which were then analysed as described above.

Data were analysed using Sigmapstat 2.03 and Sys-Stat software. Three-way analysis of variance (ANOVA) (core, drying, and solvent) was used to analyze the influence of freeze-drying and acetone versus methanol. One-way ANOVA was used to determine the influence of extraction period on pigment recovery. When data did not meet the assumption of these statistical tests, transformation was applied (logarithmic for pigment concentrations and arcsine-square root for ratios). *A posteriori* comparisons were performed using Tukey's multiple-comparison test.

Results and discussion. With the exceptions of pheophorbide *a* and fucoxanthin, pigment recoveries were significantly higher in freeze-dried than in non-freeze-dried sediments (Fig. 1, Table 1). Relative to the average of non-freeze-dried treatment, pre-freeze-drying of

sediment increased chl *a* recovery by a factor of 2.7 and 13.7 in acetone and methanol extracts, respectively (Table 2). A similar trend was observed in all other pigments with the exception of pheophorbide *a* (Table 2). Extraction of non-freeze-dried sediment in methanol was least efficient; it yielded no pheophytin (*a1* or *a2*) or β -carotene, and, with the exception of fucoxanthin, concentrations of all other pigments were low (Fig. 1). Water content of wet samples induced solvent dilution to unknown extents. It is likely that these uncontrolled dilutions reduced both solvation and penetration properties of the extraction solvent in wet samples.

The choice of extract solvent had a less dramatic influence on pigment recoveries than did freeze-drying (Fig. 1). Pheophytin *a2* and pheophorbide *a* were recovered in significantly greater concentrations in acetone than in methanol extracts (Table 1); acetone extracts contained approximately twice as much of these pigments relative to methanol extracts (Table 2). In freeze-dried sediments, methanol and acetone extracts yielded similar concentrations of chl *a*, pheophytin *a1*, fucoxanthin, and zeaxanthin (Tables 1 & 2). Lutein and β -carotene recoveries were greater (though not significantly) in methanol (Tables 1 & 2). Overall, total pigment concentration recovered in the acetone extraction (75.9 ± 37.5 SD $\mu\text{g g}^{-1}$) was similar to total pigments recovered in methanol (77.5 ± 20.7 $\mu\text{g g}^{-1}$). Jeffrey et al. (1997) compared the efficiency of different protocols that have been reported in the literature.

Among these protocols, efficiencies of sonication in 100% acetone and in buffered methanol were compared for extraction of total pigments from 3 phytoplanktonic microalgal cultures, which are particularly difficult to extract. Methanol treatment produced the best recoveries while acetone treatment underestimation was 13% for *Phaeodactylum tricornutum* (diatom), 20% for *Tetraselmis suecica*, and 63% for *Nannochloris atomus* (chlorophyte). Because protocols were tested on microalgal cultures, Jeffrey et al. (1997) considered pheopigments only as markers for chl *a* degradation induced by extraction procedures. In our analysis of freeze-dried natural sediment, total pigments other than pheopigments (chl *a*, fucoxanthin, lutein, zeaxanthin, and β -carotene) were recovered in similar quantities from acetone (64.8 ± 34.6 $\mu\text{g g}^{-1}$) and methanol (56.3 ± 22.9 $\mu\text{g g}^{-1}$) extracts. Thus, our results differ from those of Jeffrey et al. (1997), possibly as a consequence of differences in the nature of the analyzed samples (pure cultures vs a complex mixture of algae in a sediment matrix).

In freeze-dried sediments, total pheopigment (pheophytins *a* and pheophorbides *a*) recovery, however, was greater in acetone (10.1 ± 3.3 $\mu\text{g g}^{-1}$) than in methanol (7.0 ± 4.0 $\mu\text{g g}^{-1}$). Chl *a* recovery was also slightly higher in acetone (48.3 ± 25.2 $\mu\text{g g}^{-1}$) than in methanol (43.6 ± 11.7 $\mu\text{g g}^{-1}$). This indicates that increases in pheopigment concentrations obtained with acetic treatment did not originate from the

Table 1. Results of 3-way ANOVA (water content, solvent, and core) on the extraction efficiency of pigments. Neither the 'core' factor, nor any interaction terms associated with 'core' were significant ($p > 0.05$). Thus significant influences of water content and solvent (and their interaction) are shown. ns = not significant, Ac = acetone, MeOH = methanol.

Data are illustrated in Fig. 1

Pigment	Water	Solvent	Interaction
Chl <i>a</i>	Dry > Wet $p < 0.001$	ns	ns
Pheophytin <i>a1</i>	Dry > Wet $p < 0.001$	ns	ns
Pheophytin <i>a2</i>	Dry > Wet $p < 0.001$	Ac > MeOH $p < 0.001$	$p = 0.021$
Pheophorbide <i>a</i>	ns	Ac > MeOH $p < 0.001$	ns
Fucoxanthin	ns	ns	ns
Zeaxanthin	Dry > Wet $p < 0.001$	ns	$p = 0.045$
Lutein	Dry > Wet $p = 0.002$	ns	ns
β -carotene	Dry > Wet $p < 0.001$	ns	ns

Table 2. Ratios of average pigment concentrations (data from Fig. 1) in all combinations of 2 solvents (acetone [Ac] and methanol [MeOH]), and for freeze-dried versus non-freeze-dried sediments. *No pigment recovered in non-freeze-dried methanol extractions

Pigment	Ratio of concentrations			
	Dry:Wet		Ac:MeOH	
Chl <i>a</i>	Ac	2.7	Dry	1.1
	MeOH	13.7	Wet	5.6
Pheophytin <i>a1</i>	Ac	3.9	Dry	1.1
	MeOH	*	Wet	*
Pheophytin <i>a2</i>	Ac	6.5	Dry	2.4
	MeOH	*	Wet	*
Pheophorbide <i>a</i>	Ac	0.95	Dry	1.8
	MeOH	6.8	Wet	12.5
Fucoxanthin	Ac	1.7	Dry	1.0
	MeOH	1.6	Wet	1.0
Zeaxanthin	Ac	1.7	Dry	0.9
	MeOH	8.2	Wet	4.3
Lutein	Ac	1.8	Dry	0.6
	MeOH	12.6	Wet	4.4
β -carotene	Ac	4.7	Dry	0.8
	MeOH	*	Wet	*

Table 3. Sediment pigment concentrations (mean \pm 1 SD, n = 3) obtained over a range of extraction periods in acetone (3, 6, 12, and 24 h), and 1-way ANOVAs of pigment concentrations versus times of incubation (*a posteriori* test is Tukey's)

Pigment	Concentration ($\mu\text{g g}^{-1}$ dw)				ANOVA p
	3 h	6 h	12 h	24 h	
Chl <i>a</i>	43.0 \pm 3.7	41.1 \pm 3.3	44.0 \pm 3.4	51.6 \pm 1.0	0.01 (24 h > 6 h, 24 h > 3 h)
Fucoxanthin	33.2 \pm 2.6	31.8 \pm 5.5	33.2 \pm 1.6	34.9 \pm 0.5	0.69
Diadinoxanthin	8.8 \pm 0.7	8.7 \pm 1.4	8.6 \pm 0.4	9.3 \pm 0.4	0.68
Lutein	1.4 \pm 0.1	1.3 \pm 0.3	1.8 \pm 0.9	1.6 \pm 0.2	0.60
Zeaxanthin	0.7 \pm 0.03	0.6 \pm 0.1	0.9 \pm 0.4	0.7 \pm 0.05	0.58
β -carotene	1.1 \pm 0.1	1.0 \pm 0.2	1.1 \pm 0.1	1.2 \pm 0.04	0.61
Pheophytin a1	6.8 \pm 0.4	6.2 \pm 1.0	6.2 \pm 30.4	6.9 \pm 0.4	0.37
Pheophytin a2	4.3 \pm 0.4	4.0 \pm 0.5	4.3 \pm 0.8	4.2 \pm 0.2	0.82

degradation of chl *a* induced by the extraction procedure, but rather were the result of better extractability of *in situ* pheopigments from sediment samples. The determination of sedimentary pheopigment composition is generally needed for ecological studies of benthic communities; e.g. chl *a*/pheopigments *a* ratio is an indicator of the physiological state of the microphytobentos and pheophorbides *a* are considered as markers for *in situ* grazing activities (Bianchi et al. 1988, 1991). More generally, the study of benthic pheopigment composition is considered as a useful way for investigating the trophic dynamics of natural systems (Bianchi et al. 1991, Brotas & Plante-Cunney 1998). Our results showed that the use of methanol as an extraction solvent may underestimate pheopigment concentrations as well as chl *a* concentrations in benthic field samples. Thus, we do not recommend extraction of sedimentary pigments in methanol prior to HPLC analyses.

The length of extraction significantly influenced recovery of chl *a* only (Table 3). Recovery of chl *a* after 24 h of extraction was 18% greater than after 6 h of extraction (Table 3). We do not know why the duration of extraction enhanced chl *a* recovery rate. However, it is known that the type of algal cells present influences extraction efficiency (Jeffrey et al. 1997). Some benthic diatoms are among the algae known to be difficult to extract. We know that the microphytobenthic communities in this study were dominated by diatoms (Buffan-Dubau & Carman 2000). It is therefore likely that our samples contained a proportion of such difficult-to-extract diatoms, and influenced the results concerning extraction time for chl *a*.

In conclusion, our results indicate that the most reliable extraction method for muddy samples should include freeze-drying of samples prior to extraction in 100% acetone. When sonication is applied, long-duration (>3 h) extractions of samples in the solvent are not required to reliably determine concentrations of major sedimentary pigments other than chl *a*.

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