LIMNOLOGY
and
OCEANOGRAPHY: METHODS

Limnol. Oceanogr.: Methods 3, 2005, 477–487
© 2005, by the American Society of Limnology and Oceanography, Inc.

Effects of sediment storage conditions on pigment analyses

Nina Reuss[1] and Daniel J. Conley[1,2]*
[1]National Environmental Research Institute, Frederiksborgvej 399, DK-4000 Roskilde, Denmark
[2]Department of Marine Ecology, University of Aarhus, Finlandsvej 14, DK-8200 Aarhus, Denmark

Abstract

Sediment pigments as an indicator of water column and benthic processes have become widely used during the last decades. However, the effect of different storage and handling conditions on the accuracy of sediment pigment analyses has not been adequately evaluated. This can potentially make comparisons between studies difficult and, at worst, call into question the validity of some studies. Even though sediment pigment studies often rely on relative changes of individual pigments with depth, the effect of storage and handling is a critical consideration in any investigation. Based on testing of a number of different protocols, this study recommends that all sediment samples should be frozen at –20°C or colder as soon as possible after sampling and stored at this temperature without additional treatment (e.g., freeze-drying) until just before analysis of pigments. Freezing at a lower temperature than –20°C or flushing with nitrogen gas did not improve preservation. Our results showed that, if dictated by other proxy analyses, raw sediment can be stored at 3°C for up to 6 months, whereas freeze-dried sediment should always be stored frozen. In addition, the use of an internal standard (for example, β-apo-8-carotenal) for normalizing pigment concentrations is highly recommended as it significantly reduces variability between runs.

It has been shown by numerous investigators that pigments degrade in the presence of light, heat, and oxygen (Jeffrey et al. 1997; Leavitt and Hodgson 2001; and references therein). In addition, extreme pHs, enzymes, and biological activity can all have substantial effect on the preservation of pigments after sampling (Leavitt and Hodgson 2001). Although analyses for sediment pigments have been carried out for decades, there has been no distinct consensus on the best protocols for the storage and handling of sediment samples for pigment analyses. Leavitt and Hodgson (2001) suggest that conditions that closely resemble those of the sediment (cool, dark, and anoxic) will reduce the loss of pigments. However, they also acknowledge that this is often not possible during collection and sampling of sediment cores in the field.

An extensive review and study of the effects of different storage conditions on water samples for pigment analyses, collected on filters, was conducted by Mantoura et al. (1997). Various methods of storage and transport of the filters were studied. Their general conclusions were that the filters should be stored as cold as possible (< –80°C), preferably at –196°C, and never stored freeze-dried. Proper recommendations for the storage and handling of sediments for pigment analysis have been put forward only recently by Leavitt and Hodgson (2001). They recommend avoiding direct light and storage of sediment in small airtight containers under inert gas in extreme cold, e.g., –80°C. However, to our knowledge, comparative studies of the effect of different storage and handling procedures on sedimentary pigment concentrations have not been published.

The aim of this study was to evaluate the effect of different storage and handling procedures often experienced between sampling and analysis. Samples from two contrasting sites in a eutrophic Danish estuary were investigated with one site located in anoxic bottom waters and the second site located in oxic bottom waters. This provided information on storage and handling effects on sediment deposited under different preservation conditions, potentially affecting further degradation after sampling. The different procedures evaluated included storage of raw and freeze-dried samples at various temperatures as well as a raw sample frozen and stored under inert gas as recommended by Leavitt and Hodgson (2001).

*E-mail address: Nina.Reuss@geol.lu.se. Lund University, Sölvegatan 12, SE-223 62 Lund, Sweden

Acknowledgments

We would like to thank Thomas Bianchi for comments and guidance throughout the planning and writing of this paper, and Morten Søndergaard, Peter Leavitt, and an anonymous reviewer for useful comments to improve the manuscript. Furthermore, we thank Jacob Carstensen for help with designing the study and statistical advice, Ingela Dahllöf for advice on the use of the internal standard, and Uffe Frisinette, skipper of R/V *Maria*, for help with site selection and sampling. This research was partially supported by European Union funding of the MOLTEN project (EVK3-CT-2000-00031) and the Danish National Science Foundation (21-02-0390).

Table 1. Storage conditions for Mariager Fjord samples from oxic and anoxic top and bottom*

Sediment	Storage conditions	Time
Control	—	Freeze-dry and run immediately
Raw sample	–80°C	6 months
Raw sample	–20°C under inert gas	6 months
Freeze-dry	–20°C	6 months
Freeze-dry	–20°C	1 y
Raw sample	–20°C	6 months
Raw sample	3°C	6 months
Raw sample	~22°C (room temperature)	1 month
Freeze-dry	~22°C (room temperature)	6 months
Freeze-dry	~22°C (room temperature)	1 y

*All samples stored raw were frozen and freeze-dried immediately before extraction and run on HPLC.

Materials and procedures

Study site—Mariager Fjord, Denmark, is a highly eutrophic estuary with anoxic bottom waters below 10 to 15 m because of strong stratification influenced by a shallow sill that reduces exchange of the highly saline bottom waters (Fallesen et al. 2000). Occasional intrusions of saline oxygen-rich bottom water from the coastal ocean can temporally oxygenate bottom waters for short periods. Nutrient concentrations and primary production in the estuary are some of the highest encountered in Danish estuaries (Andersen et al. 1998; Conley et al. 2000). The plankton community of the estuary is not diverse and is dominated by diatoms throughout most of the year, with occasional blooms of dinoflagellates and the ciliate *Mesodinium rubrum* (= *Myrionecta rubra*) dominating in the winter months (Andersen et al. 1998). The estuary was chosen for the exceptional good benthic preservation conditions at the deep anoxic site and the presence of a shallower oxic deposition site.

Sampling and storage—A deep anoxic site (56°39.777N, 09°58.409E, ~26.8 m) and a more shallow oxic site (56°40. 246N, 09°58.461E, ~8.6 m) in Mariager Fjord were sampled in late April 2003. Sediment cores were collected using a modified Haps sediment corer, effective in the very loose (water content = 90% to 98%) and highly organic sediment (LOI = 15% to 30%) of Mariager Fjord. A composite sample of the top (5-20 cm) and bottom (20-40 cm) sediments of each core were sampled, excluding the reactive surface layer of the sediment. The samples were kept cool and dark in tight plastic buckets and returned to the lab where they were homogenized and sub-sampled under subdued light within 10 h of sampling. Replicate sets of four sub-samples designated as anoxic top, anoxic bottom, oxic top, and oxic bottom of approximately 150 mL were placed in 250 mL bluecap bottles and freeze-dried or stored directly following the procedures described in Table 1. Freeze-dried samples were transferred to 50 mL vials before storage. The controls were frozen at –20°C overnight, freeze-dried, and five replicates were run immediately by high-performance liquid chromatography (HPLC) as described below. Triplicate samples were run for each of the storage treatments. All samples stored raw were frozen and freeze-dried immediately before pigment extraction.

Pigment extraction and HPLC analysis—Freeze-drying has been shown to improve pigment extraction, and acetone to be an effective extraction solvent for sediment samples (Buffan-Dubau and Carman 2000), reducing the formation of degradation products. Extraction of freeze-dried sediment samples in acetone has been extensively used in studies of a range of marine sediment types (e.g., Lucas and Holligan 1999; Chen et al. 2001; Bianchi et al. 2002*a*; Hansen and Josefson 2003; Hodgson et al. 2003) and was chosen for the present study. Approximately 0.3 g at the anoxic site and 1 g at the oxic site of homogenized freeze-dried sediment was extracted in 5 mL cold HPLC grade 100% acetone in 20 mL glass vials with screw caps. The different amounts of sediment used were due to the large differences in pigment concentrations at the two sites. It is not expected that this difference in solid:solution ratios will effect the extraction of pigments from the sediments. The mixture was sonicated in an ice bath for 40 s and extracted in the freezer (–20°C) overnight. The extracts were filtered through a 0.5 µm, 25 mm filter (CAMEO 25 GF, Frisinette). Samples were diluted with Milli-Q water to a final concentration of 80% acetone to ensure good separation as suggested by Wright et al. (1991) and stored at 4°C in the dark (autosampler) until injection. β-apo-8-carotenal was added at the same time as the extraction solvent as an internal standard and the entire procedure was carried out under dim light.

Quantitative analyses of all pigments were conducted on a Shimadzu HPLC equipped with an on-line photodiode array detector (SPD-M10Avp) and fluorescence detector (RF-10Axl) with excitation set at 440 nm and emission at 660 nm (used for identification purposes only). Injection of 50 to 100 µL of the pigment-solvent complex was conducted using a Shimadzu SIL-10AF auto-sampler with the sample cooler set at 4°C. A reverse phase Supelcosil™ LC-18 column (5 µm particle size; 25 cm × 4.6 mm inner diameter) with a guard column was used for separation. The gradient program with a flow of 1.00 mL/min was a modification of Wright et al. (1991) as described by Chen et al. (2001). It starts isocratically with mobile phase A (80:20 methanol:0.5 M ammonium acetate, aq. pH 7.2 v/v); ascends to 100% B (90:10 acetonitrile: water v/v) in 4 min; then to 25% B and 75% C (100% ethyl acetate) over 34 min and remains isocratic for 1 min; the gradient returns to 100% B in 4 min, ascends to 100% A in 4 min, and finally runs isocratically with 100% A for 2 min.

Pigment identification and quantification—Chromatograms were integrated using Class VP 7.2 software. Identification of individual pigments was based on a combination of retention time and absorbance spectra compared with authentic pigment standards obtained from DHI Water & Environment, Denmark. Quantification of individual chlorophyll *a* (Chl *a*)

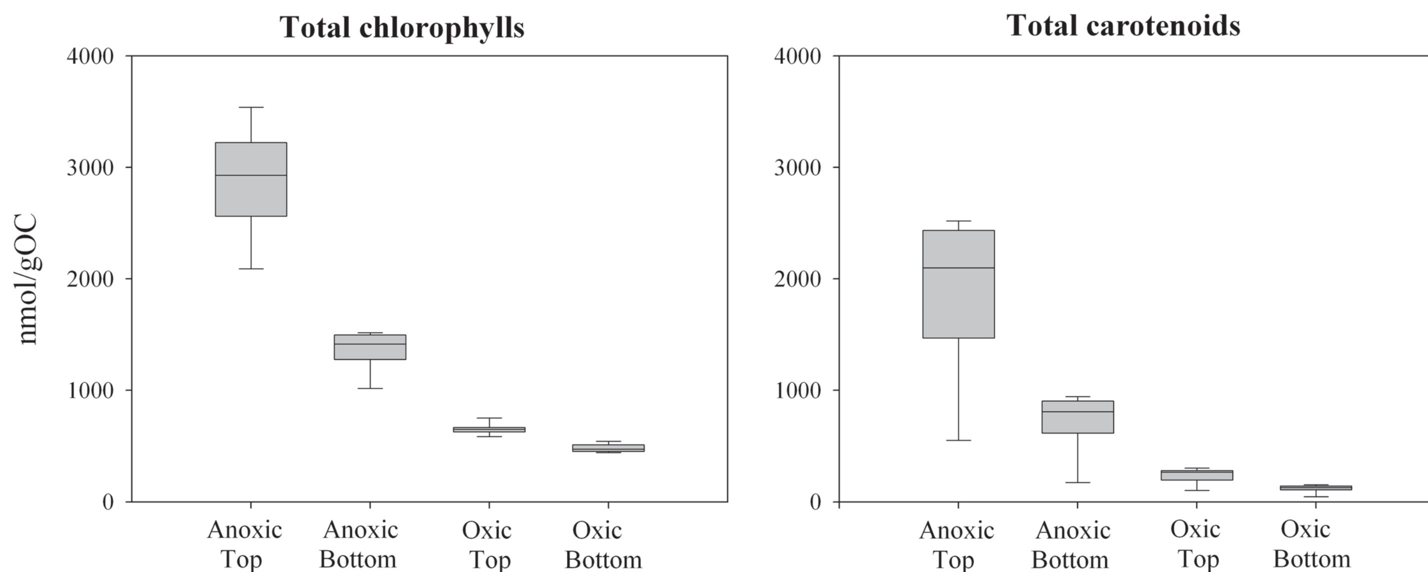

Fig. 1. Comparison of the four sediment samples based on total chlorophyll (Chl *a* and pheopigments *a*) and total carotenoids (4 major carotenoids: fucoxanthin, alloxanthin, diatoxanthin, β-carotene). Median (including control and all treatments), 10th, 25th, 75th, and 90th percentiles as vertical boxes with error bars. OC = organic carbon.

and pheopigments *a* was based on their absorbance at 666 nm, and for carotenoids and pheophytins *b* at 449 nm. Response factors (RF) for each pigment used for quantification were based on a single run of standards. The data are presented as nmol pigment per g organic carbon (nmol gOC^{-1}). Analyses of carbon content were carried out on a CHNS elemental analyzer (CE instruments EA1110). Organic carbon was determined as the difference between the total fraction of C, measured on freeze-dried samples, and the inorganic fraction, measured on combusted samples (2 h at 500°C). The organic carbon concentration ranged from 9.2 to 11.2 wt% in all samples, whereas inorganic carbon concentration were ~0.1 wt% at the anoxic site and ~2.9 wt% at the oxic site. In all samples normalization of concentrations were conducted relative to the internal standard, β-apo-8-carotenal.

Data manipulation and statistics—Normalization of the data was accomplished by scaling to the average value of the internal standard within each storage treatment across all four sediment samples. In the control runs, very low concentrations of the internal standard hampered the normalization. Instead, two outliers out of five samples from the anoxic top and anoxic bottom were identified by Dixons test on Chl *a* ($\alpha = 0.10$) and removed from the dataset. No outliers were identified in the oxic samples. The corrected dataset was statistically analyzed using SAS System 8.2.

Six common pigments were chosen for the statistical analysis of differences between the four sediment samples and storage treatments, including carotenoids with and without epoxygroups as well as Chl *a* and chlorophyll degradation products. The pigment concentrations varied significantly between the four samples (Kruskal-Wallis non-parametric test), and all further statistics were performed as one-way analysis of variance (ANOVA) to reveal differences of storage treatments within each sediment sample (anoxic top, anoxic bottom, oxic top, and oxic bottom). Variance homogeneity was assessed by means of Levine's test. In order to pass this test, all data were $\log_{10}$ transformed. Tukey's test was applied in all cases to compare individual treatments where the one-way ANOVA showed significant treatment effects. The effect of the use of the internal standard, β-apo-8-carotenal, was tested by comparing the coefficient of variation (CV = standard deviation/mean) for each pigment within a sediment sample before and after normalization with the internal standard.

Assessment

Differences between sediment samples—Large differences in the concentration of pigments in the four different sediment samples were apparent (Fig. 1). The highest concentrations of total chlorophyll pigments and total carotenoids were found in the anoxic top sample and the lowest concentrations found in the oxic bottom sample. This variation between sediment samples was expected based on the knowledge of differences in oxygen content at the sediment water interface at the two sites and previous investigation of down-core changes in pigment concentrations at the deep anoxic site (Reuss et al. 2005). Significantly different pigment concentrations were found between the four sediment samples ($\alpha = 0.01$, Kruskal-Wallis test). Therefore, all further analysis were conducted separately for each sediment sample to test for different effects of the storage treatments on sediment pigments, the main focus of this study.

Selection of pigments for further study—During analysis of pigments and their degradation products in sediments, more

than 30 identified and unidentified pigments were found in the samples. The total number of peaks within each sediment sample was largely the same for the different treatments after 6 months of storage. The highest number of peaks was found in the top samples and the lowest in the bottom samples (both in oxic and anoxic sediments). The control samples and sediment stored at room temperature for 1 month had the highest number of peaks in all runs, while samples stored for 1 y showed fewer peaks than in samples stored for 6 months. This indicates that storage of sediment samples does not add additional peaks from new degradation products as they were already present in sediment samples. In contrast, the observed reduction of peaks could be caused by lowered concentrations leaving fewer peaks above the limit of detection or from merging of the peaks. In order to focus on the effect of different storage treatments, we chose six common pigments with different labilities for further analysis.

β-carotene was chosen for further analysis because it was expected to be one of the most stable carotenoids (Leavitt and Hodgson 2001). Alloxanthin was also expected to show high stability as it contains no epoxy-group, which makes pigments more susceptible to degradation, and has been found in previous studies to preserve well in sediments (Hurley and Armstrong 1990). In contrast, fucoxanthin contains an epoxy group and was expected to show a high degree of degradation together with diatoxanthin. Diatoxanthin does not contain an epoxy group, but it can interconvert with diadinoxanthin as part of the xanthophyll cycle. Of the chlorophyll products Chl *a* is very labile compared with pheophytin *a*, which was expected to be very stable. It should be noted that for the oxic bottom sediment Chl *a*, fucoxanthin and, in some treatments, diatoxanthin are close to the limit of quantification and show poor absorbance spectra.

Effect of storage treatments—The effect of storage conditions on the concentration of pigments varied between sediment samples and for individual pigments (Fig. 2 and 3, Table 2). A general trend, however, was the particularly strong negative effect of storage of sediment as freeze-dried material at room temperature. The main reason for this effect was probably the relatively high temperature (room temperature, approximately 22-25°C) and enhanced oxygen availability after freeze drying since microbial activity should have been prevented by the freeze-drying process and photodegradation avoided by storage of the samples in darkness. The concentrations of individual pigments following freeze-drying and storage at room temperature were always significantly lower compared to the other storage treatments at the anoxic site (top and bottom) and most of the time at the oxic site (Table 2). This difference could be caused by a stronger effect of increased exposure to oxygen on samples from the anoxic site as opposed to samples from the oxic site. Organic material and pigments at the anoxic site are expected to consist of less refractory compounds than at the oxic site as they have not been exposed to the same degree of oxygen at the sediment-water interface causing extensive breakdown of pigments before permanent burial (Leavitt 1993; Sun et al. 1993; Bianchi et al. 2000).

Pigment concentrations in the control treatment (freeze-dried and run immediately) were, in general, not significantly different from most of the other treatments, excluding the storage of freeze-dried sediment at room temperature (both 6 months and 1 y) as described above, except for Chl *a* in the anoxic sediment and for diatoxanthin in all sediment types. This implies that there was generally no effect of storage as long as freeze-dried sediment is stored frozen at –20°C or raw sediment is stored refrigerated. Even storage of raw sediment at room temperature for 1 month showed good preservation of pigments. The relatively large volume of sample (~150 mL in 250 mL bottles) could allow for the raw samples to remain anoxic throughout most of the sample during storage, creating conditions conducive to the preservation of pigments. This may not be the case with smaller samples with a larger surface to volume ratio, even though storing small samples in equally small vials, as suggested by Leavitt and Hodgson (2001), can reduce the oxygen availability. The use of small storage vials for freeze-dried sediments in this study probably have had a similar effect.

Effect on individual pigments—The variability of Chl *a* and its major degradation product, pheophytin *a*, was high in the anoxic top sediment sample and showed low Chl *a* concentration and high pheophytin *a* concentration in the control sample (Fig. 2). There was no apparent external factor, such as problems in processing the samples, that could explain this variability. A possible explanation for the large variability is that some of the pigment was sequestered within the sediment matrix and rendered unextractable by the method used here. A laboratory study of pigment degradation in sediment under simulated natural conditions (Sun et al. 1993) showed an initial increase in extractable Chl *a* in the first few days of the experiment and a subsequent stabilization under anoxic conditions. They also found better extraction after freezing of the sediment, previously defined as a release of a 'bound' pool of Chl *a* (Sun et al. 1991). This pool was suggested to be associated with a variety of detrital matrices protecting the pigments from extraction and probably also degradation (Furlong and Carpenter 1988). Storage of the sediment for an extensive period, as in this study, may have broken some of these bonds in the sediment matrix enhancing the extraction efficiency with time. Enhanced pigment extraction after extensive freezing has also been found for several pigments including Chl *a* for water samples collected on filters and stored at –196°C and –90°C, resulting in more than 100% recovery after 60 d (Mantoura et al. 1997).

Concentrations of diatoxanthin in control samples were often one of the lowest, whereas the highest concentrations at the oxic site (top and bottom) were found in samples stored freeze-dried at –20°C for 1 y (Fig. 3). This implies that this pigment is produced from other pigments during storage.

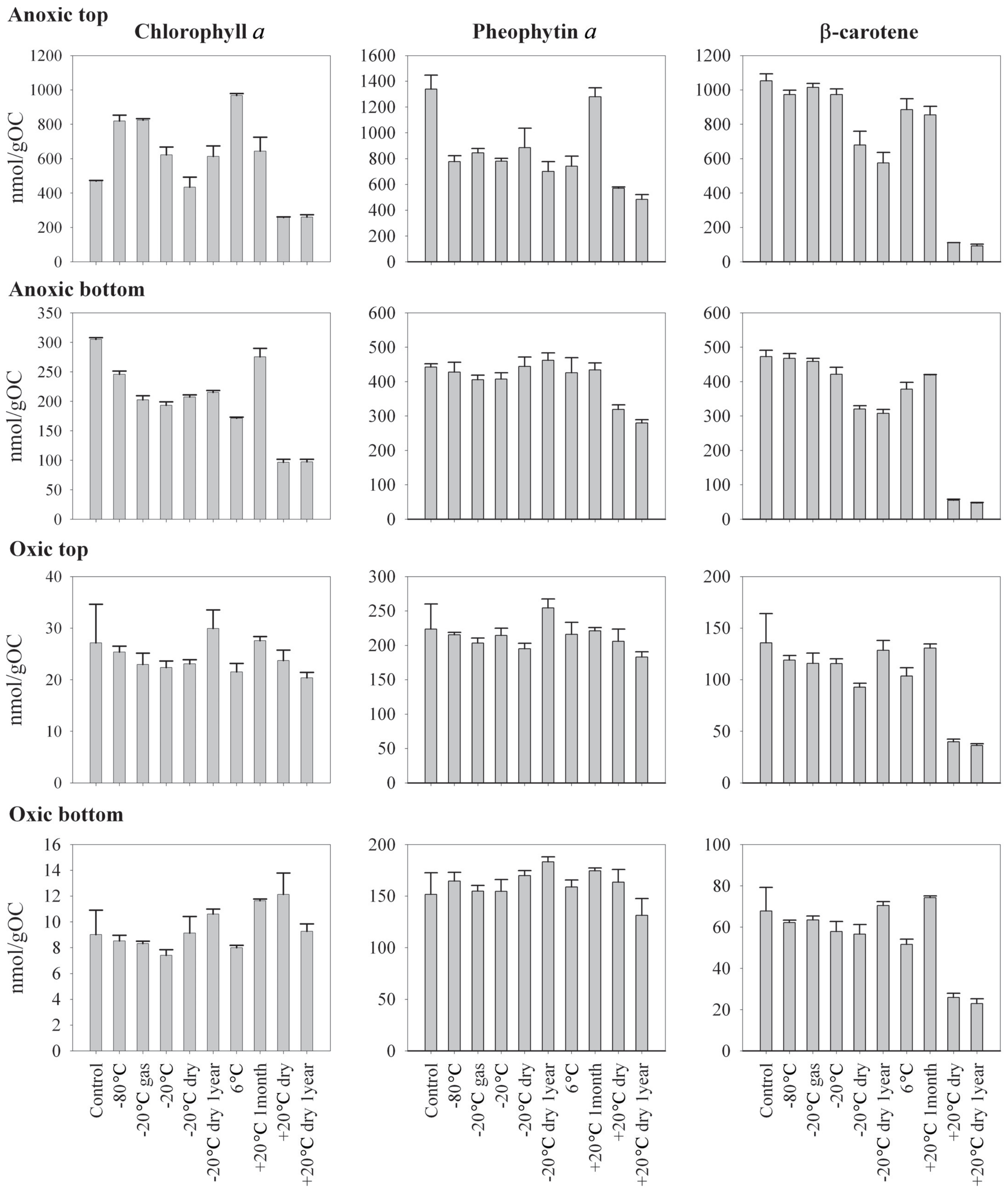

Fig. 2. Indicators of total biomass: Chl *a*, pheophytin *a*, and β-carotene. Average ±1 SD. All treatments were conducted over a 6-month period unless otherwise noted. OC = organic carbon.

Table 2. Statistics on differences of treatments within each sediment type*

Sediment type and pigment	ANOVA† Treat	Tukey's test on treatment‡ Control	−80°C	−20°C gas	−20°C	−20°C dry	−20°C dry, 1 y	3°C	22°C, 1 month	22°C dry	22°C dry, 1 y
Anoxic top											
Chl *a*	***	C	A	A	B	C	B	A	B	D	D
Pheophytin *a*	***	A	B	B	B	B	BC	B	A	CD	D
β-carotene	***	A	AB	AB	AB	C	C	AB	B	D	D
Fucoxanthin	***	A	A	AB	ABC	BC	C	A	AB	D	D
Diatoxanthin	***	D	AB	A	ABC	C	CB	A	D	E	E
Alloxanthin	***	A	B	B	B	B	B	B	B	C	D
Anoxic bottom											
Chl *a*	***	A	DE	C	E	DE	D	F	B	G	G
Pheophytin *a*	***	A	A	A	A	A	A	A	A	B	B
β-carotene	***	A	AB	AB	BC	DE	D	C	BC	E	F
Fucoxanthin	***	A	A	A	A	A	A	A	A	B	B
Diatoxanthin	***	D	AB	BC	BC	C	A	BC	D	E	E
Alloxanthin	***	A	AB	AB	AB	AB	BC	AB	C	D	E
Oxic top											
Chl *a*	—										
Pheophytin *a*	**	AB	AB	AB	AB	B	A	AB	AB	AB	B
β-carotene	***	AB	AB	AB	AB	B	A	AB	A	C	C
Fucoxanthin	***	AB	A	A	A	AB	A	A	A	BC	C
Diatoxanthin	***	E	AB	B	AB	BC	A	AB	DE	CD	CD
Alloxanthin	***	A	A	A	A	A	A	A	A	B	B
Oxic bottom											
Chl *a*	***	ABCD	BCD	DC	D	ABCD	ABC	DC	AB	A	ABC
Pheophytin *a*	***	AB	A	AB	AB	A	A	AB	A	AB	B
β-carotene	***	ABC	ABC	ABC	ABC	BC	AB	C	A	D	D
Fucoxanthin	***	AB	AB	AB	AB	AB	AB	AB	A	B	C
Diatoxanthin	—										
Alloxanthin	***	B	AB	AB	AB	AB	AB	AB	A	C	D

*All treatments were carried out over 6 months unless otherwise stated.
†***Significant differences at $\alpha = 0.01$; **$\alpha = 0.05$; —, nonhomogeneity of variance.
‡In Tukey's test, different letters indicate that there is statistically significant difference between treatments within the sediment sample, and the same letters indicate that there is not at $\alpha = 0.05$.

However, higher concentrations after 1 y could also be an artifact due to merging of peaks in the chromatogram after extensive storage time. The merging of peaks after 1-y storage time was observed for the entire chromatogram that was characterized by fewer large wide peaks. This may also explain why the other pigments show only minor decreases or no decrease with extended storage time. It is therefore recommended not to store samples for periods longer than ~6 months to avoid artifacts of merging peaks in samples.

The two most stable carotenoids, β-carotene and alloxanthin, always showed a significant reduction in concentration in samples stored freeze-dried at room temperature in relation to all other treatments (Fig. 2 and 3). The more labile pigments such as fucoxanthin and Chl *a* showed less extensive or nonsignificant reduction using this storage type relative to the other treatments especially at the oxic site. This could indicate that the more labile pigments have been degraded already in the top of the sediment or in the water column prior to deposition and less material is left for degradation in the deep sediment. In addition, selective loss of β-carotene in freeze-dried samples has previously been found for water samples (Mantoura et al. 1997).

The pigments chosen for further analysis in this study did not only represent different stabilities, but also total algal abundance (Chl *a*, pheophytin *a*, and β-carotene) and the dominating phytoplankton community of diatoms and dinoflagellates (fucoxanthin and diatoxanthin) and the autotrophic ciliate *Mesodinium rubrum* with cryptophytes as endosymbionts (alloxanthin). However, several other pigments were observed in the sediment, including lutein, pheophytin *b*, and canthaxanthin. Canthaxanthin was only of minor importance in the anoxic sediment, whereas both canthaxanthin

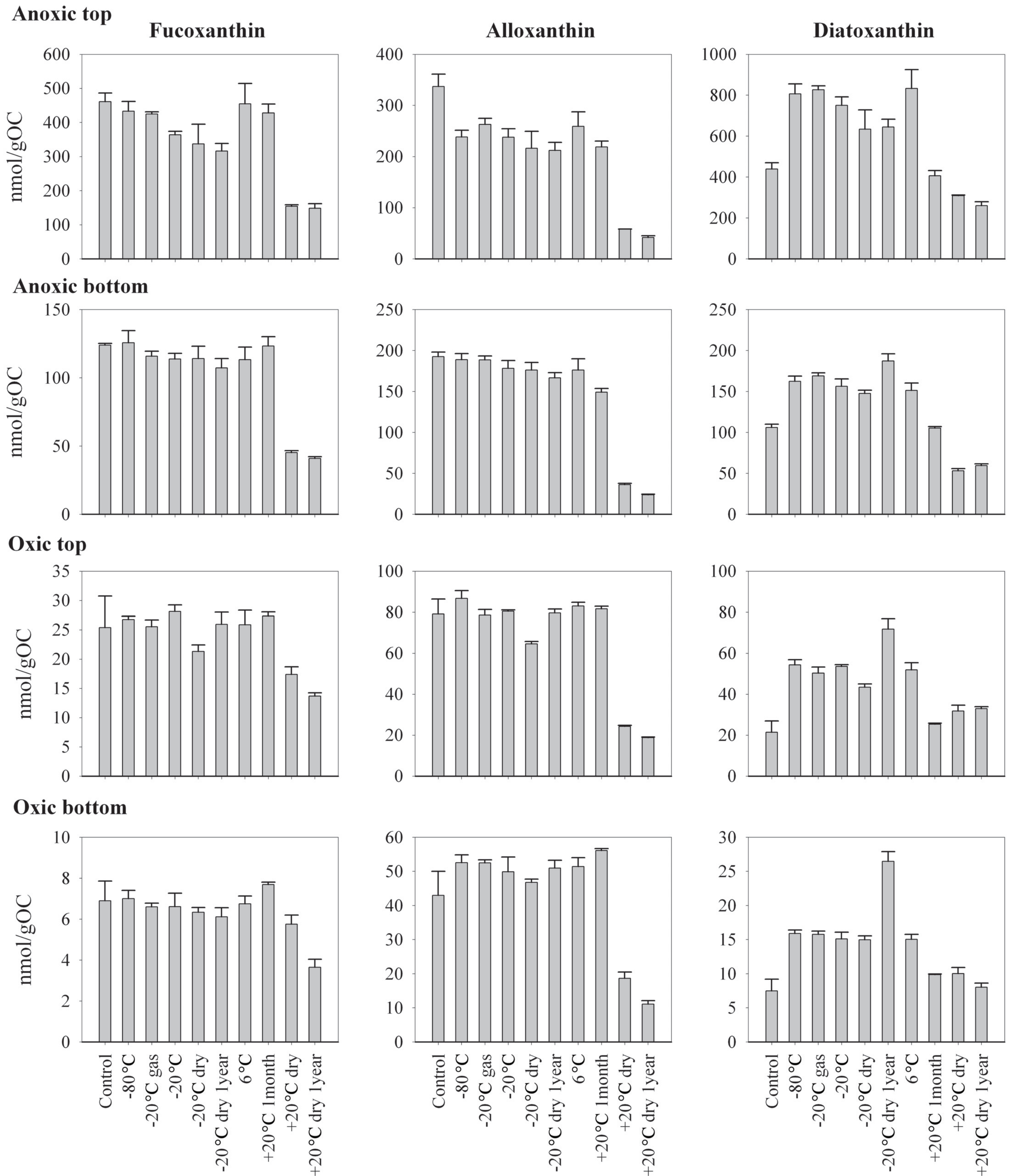

Fig. 3. Primary carotenoid biomarkers: fucoxanthin, alloxanthin, and diatoxanthin. Average ±1 SD. All treatments were conducted over a 6-month period unless otherwise noted. OC = organic carbon.

Table 3. Mean and coefficient of variation (CV in %) for individual pigments for each site corrected and uncorrected by internal standard

Type†	Chl *a*	Pheophytin *a*	β-carotene	Fucoxanthin	Alloxanthin	Diatoxanthin
Corrected‡						
Anoxic top	605.0 (6.3)**	785.5 (7.3) n.s.	684.6 (6.1)*	340.2 (7.4) n.s.	194.0 (7.2) n.s.	607.8 (6.7) n.s.
Anoxic bottom	189.4 (3.2)**	400.9 (5.3) n.s.	319.6 (3.4)**	99.9 (5.3)**	142.7 (4.4)**	132.4 (3.9)**
Oxic top	24.1 (6.6)*	212.1 (4.7) n.s.	98.1 (5.5)—	23.5 (4.7)*	66.4 (5.0) n.s.	46.2 (4.9) n.s.
Oxic bottom	9.5 (6.0)**	161.8 (5.3) n.s.	54.0 (5.3)**	6.3 (6.1) n.s.	43.3 (5.1)**	14.6 (4.9)**
Uncorrected						
Anoxic top	634.3 (14.3)	806.3 (8.5)	723.8 (12.5)	353.2 (8.4)	204.2 (10.8)	639.1 (10.6)
Anoxic bottom	179.7 (13.6)	379.4 (9.1)	303.2 (13.0)	94.2 (9.4)	134.6 (10.6)	125.8 (11.1)
Oxic top	25.5 (13.5)	223.0 (8.5)	103.1 (12.3)	24.7 (9.6)	69.6 (9.6)	48.3 (10.1)
Oxic bottom	9.1 (14.3)	153.5 (9.9)	51.2 (12.7)	5.9 (8.7)	40.9 (10.5)	13.7 (12.2)

†All treatments combined excluding 'control' where the internal standard was not used.
‡**Significant differences between the CV of uncorrected and corrected data at $\alpha = 0.05$; *$\alpha = 0.1$; n.s = not significant; —, not determined due to non-homogeneity of variance.

and a pigment provisionally identified as *cis*-canthaxanthin (Henriksen 2004) constituted some of the more important peaks in the oxic sediment. This indicates a larger contribution of pigments from either blue-green or green algae, possibly from the macroalgae *Ulva lactuca* that can be found in high concentrations in adjacent shallow areas of Mariager Fjord.

Use of internal standard—The effect of using an internal standard to normalize pigment concentrations that have gone through an extensive extraction procedure was tested in addition to the storage study. ANOVA was performed on the $\log_{10}$ transformed coefficient of variation (CV) for each pigment before and after normalizing the concentrations within each sediment sample including all treatments except the control, where the internal standard was not used. There was always a reduction in the coefficient of variation following normalization, however, this reduction was not always significant (Table 3) probably due to large variation between the treatments. For the pigments Chl *a* and β-carotene the reduction was always significant, while other pigments showed a less significant reduction in the CV, e.g., pheophytin *a* and fucoxanthin. However, even though the reduction in CV was not always significant, it was always lower after normalization to the internal standard, increasing the probability of seeing statistically significant differences between treatments. It is, therefore, highly recommended to always include an internal standard in the analysis of pigments that require several extraction steps. This recommendation has been put forward for other analyses, such as DNA-analysis (Petersen and Dahllöf 2005), and is a common practice for quantitative chromatography in analytical chemistry. Mantoura and Repeta (1997) also highly recommend the use of an internal standard added immediately before extraction for the study of pigments in water samples as it reduces handling errors, while Leavitt and Hodgson (2001) recommend the use of an internal standard (e.g., Sudan II) applied with the injection solution to the evaporated extract to reduce injection uncertainties.

Discussion

Storage practice—This study was inspired by work on effects of storage and handling of filers for pigment analysis of water column samples and the fact that for sediment samples, there is no comparable study of the effects of different storage and handling on pigment preservation. Few studies mention the effects of storage and handling. For example, Lucas and Holligan (1999) found that freeze-dried sediment stored at –70°C for 2 to 9 months had no significant effect on pigment concentrations, whereas Klein and Riaux-Gobin (1991) found that sediment samples stored frozen (< –20°C) for 5 to 7 months did not influence chlorophyll concentrations. The most common practice for storage of sediment for pigment analysis since the 1970s has been freezing of sediment immediately after collection until extraction on wet or freeze-dried samples. However, it has also been common before the 1990s to store samples for pigment analysis as freeze-dried sediment in the dark at room temperature. This practice, rarely reported in papers, is known from hearsay between laboratory assistants and scientists at institutes conducting pigment analysis. In more recent studies, the common practice is freezing of the sediment at –20°C or in liquid nitrogen just after sampling and transfer to –70°C or –80°C on return to the laboratory until analysis (e.g., Buffan-Dubau and Carman 2000; Chen et al. 2003; Hodgson et al. 2003). A significant contribution from the present study is therefore the comparison of the effects of storage of sediments at –20°C and –80°C for pigment preservation. We did not find significant differences in pigment concentrations between storage at these temperatures and acknowledge that differences due to storage may not be a major issue comparing early and new studies. However, developments in extraction procedures and the separation and identification of pigments may still have significant effects and should be considered when comparing concentrations between studies.

The results from the present sediment study differ from the results of the storage experiment of water samples col-

lected on filters where large differences in pigment preservation were observed for storage at different temperatures (Mantoura et al. 1997). For long term storage (328 d) at –196°C, they found almost 100% recovery of carotenoids, whereas the recoveries were approximately 80% for storage at –20°C and only 25% for storage of freeze-dried filters at room temperature. Material collected on filters is more susceptible to degradation compared to material in sediments that are protected within the sediment matrix. In addition, organic material in sediments has been exposed to degradation in the water column and surface sediment before being incorporated into the deep sediment and is expected to consist of more refractory compounds than organic material from the water column.

The sediment matrix effect of protecting the pigments from degradation could also have had an effect on the extraction efficiency during this study. If extended freezing or storage in general increases the extraction efficiency by gradual release of the pigments from the sediment matrix, this effect counteracts degradation of pigments during storage and results in apparently full or more than 100% recovery with time. Release of a bound pool of Chl after freezing has been reported by Sun et al (1993). In addition, this release of bound pigments was also suggested as the mechanism for increased pigment concentrations over a 60-d storage experiment of filters (Mantoura et al. 1997). The absence of a decline in most pigments after 6 months of storage in our study could be a consequence of this initial or gradual release of pigment combined with subsequent degradation. Another possible explanation is the merging of peaks, as suggested for diatoxanthin, which were observed for the samples stored for 1 year.

Comparison of pigment degradation rates in sediments—Studies of the decomposition of bulk organic carbon have shown that pigments are some of the most labile compounds in the sediment. In a study of marine organic matter from the central equatorial Pacific, Wakeham et al. (1997) were able to assign overall reactivity of different biochemical classes and found pigments (chlorophylls) to be the most labile, followed by lipids, amino acids, and finally carbohydrates (from polysaccharides) as the most refractory group. Degradation of organic matter in the sediment is affected by several factors such as microbial decay as well as bioturbation and oxygen conditions at the sediment water interface (Berner 1980; Canfield 1994). Chlorophyll degradation pathways include allomerization (oxidation), demetallation (loss of the Mg), and dephytylation (loss of phytyl chain), with the five-ring phorbin being relatively stable (Porra et al. 1997). Carotenoids are found mostly in the *trans*-form and can be converted to the *cis*-form during degradation and subsequently broken down to colorless compounds by destruction of the long chain of alternating double bonds constituting the chromophore of the carotenoids (Leavitt 1993; Leavitt and Hodgson 2001). General models of early diagenesis of organic matter in the sediment are often based on the G-model (Berner 1980), assuming first-order kinetics indicated by exponential decreases in concentrations down-core. This type of kinetics has been shown also to apply to sediment pigments deposited in oxygenated marine sediments (Furlong and Carpenter 1988; Sun et al. 1991; Sun et al. 1994), while good preservation and lack of systematic alteration down-core has been observed in anoxic sediments (Villanueva and Hastings 2000). However, most pigment degradation takes place in the water column by photooxidation, senescence, grazing, and microbial decay and secondly at the sediment water interface (Furlong and Carpenter 1988; Hurley and Armstrong 1990; Bianchi et al. 2002b; Chen et al. 2003). This is reflected by degradation rates in the range of days in the water column, years in surface sediments and millennia in deep sediments (Leavitt 1993). The depositional environment therefore plays a significant role in the total amount and further degradation of pigments in the sediment as shown by the results from the present study. The study sites represented two contrasting depositional environments: one with permanently anoxic conditions and one with mostly oxygenated bottom waters, and revealed differences in both pigment concentrations and effects of storage after sampling. Based on pigment concentrations, the two sites are good representatives of other coastal sites with good preservation, typically with anoxic bottom waters and laminated sediments, and shallow or open coastal sites with oxic bottom waters (Reuss et al. 2005; Bianchi et al. 2002*a*; Chen et al. 2001).

Multi-proxy studies—Multi-proxy studies have been shown to be a reliable way of describing paleoecological changes within a system (Engstrom et al. 1985; Hall et al. 1999; Battarbee 2000; Kauppila et al. 2005). However, certain constraints on storage conditions may apply in multi-proxy studies and individual analysis can require different storage conditions or treatment of the sediment. For example, the determination of germinable diatoms requires that the sediment not be frozen (Hansen and Josefson 2003), whereas the analysis of general geochemical biomarkers such as carbon, nitrogen, biogenic silica, and metals, as well as various isotopes require dried or preferably freeze-dried sediment. Transport of sediment samples over longer distances may necessitate freeze-drying due to lower cost and problems keeping raw samples cold. Results from the present study suggest that storage of raw samples at 3°C up to 6 months will not significantly affect pigment preservation assuming a relatively large sample size or comparable small storage vials to minimize oxygen availability. On the other hand, freeze-dried sediment should always be stored frozen because storage at room temperature over an extended period significantly decreases the pigment concentration in these samples.

Comments and recommendations

Results from the present study support the newest recommendations from Leavitt and Hodgson (2001) in that sediment samples for pigment analysis should be frozen at –20°C or colder as soon as possible after collection and stored at this

temperature preferably no longer than 6 months. All further treatment of the samples such as freeze-drying should be postponed until just before extraction and analysis of the pigments. However, in this study no significant improvement of preservation was observed for more rigorous measures such as extreme cold and flushing with an inert gas. If the analysis of other proxies require non-freezing of the sediment (e.g., seeding experiments) samples should as a minimum be refrigerated, while freeze-dried material (e.g., needed for dating) should always be stored frozen. All comparisons of pigment concentrations between sites, regardless of old or new studies, should be evaluated with caution due to differences in preservation and sedimentation conditions inherent of the site, and the effect of storage evaluated as a potential loss factor for sediment pigments.

The use of an internal standard for normalizing pigment concentrations in the sediment is strongly recommended as it significantly reduced the variation between replicate samples in the present study. Prior recommendations of the use of internal standard in quantitative chromatography methods, however, are primarily concerned with the uncertainties of injection. We recommend that the internal standard be deployed together with the extraction solvent (dissolved in the solvent or added at the same time as the solvent) as recommended for water samples (Mantoura and Repeta 1997) to account for inaccuracies that can also arise during the extraction and handling procedures required for pigment analysis.

References

Andersen, F., and others. 1998. Mariager Fjord. Udvikling og status 1997. Århus Amts Trykkerri. (in Danish).

Battarbee, R. W. 2000. Palaeolimnological approaches to climate change, with special regard to the biological record. Quaternary Sci. Rev. 19:107-124.

Berner, R. A. 1980. Early diagenesis. A theoretical approach. Princeton Univ. Press.

Bianchi, T. S., and others. 2002*a*. Do sediments from coastal sites accurately reflect time trends in water column phytoplankton? A test from Himmerfjärden Bay (Baltic Sea proper). Limnol. Oceanogr. 47:1537-1544.

———, B. Johansson, and R. Elmgren. 2000. Breakdown of phytoplankton pigments in Baltic sediments: effects of anoxia and loss of deposit-feeding macrofauna. J. Exp. Mar. Biol. Ecol. 251:161-183.

———, C. Rolff, B. Widbom, and R. Elmgren 2002*b*. Phytoplankton pigments in Baltic Sea seston and sediments: seasonal variability, fluxes, and transformations. Est. Coast. Shelf Sci. 55:369-383.

Buffan-Dubau, E., and K. R. Carman 2000. Extraction of benthic microalgal pigments for HPLC analyses. Mar. Ecol. Prog. Ser. 204:293-297.

Canfield, D. E. 1994. Factors influencing organic carbon preservation in marine sediments. Chem. Geol. 114:315-329.

Chen, N., T. S. Bianchi, and J. M. Bland 2003. Implications for the role of pre- versus post-depositional decay of chlorophyll-a in the lower Mississippi River and Louisiana shelf. Mar. Chem. 81:37-55.

Chen, N. H., T. S. Bianchi, B. A. McKee, and J. M. Bland 2001. Historical trends of hypoxia on the Louisiana shelf: application of pigments as biomarkers. Org. Geochem. 32:543-561.

Conley, D. J., H. Kaas, F. Moehlenberg, B. Rasmussen, and J. Windolf. 2000. Characteristics of Danish Estuaries. Estuaries 23:820-837.

Engstrom, D. R., E. B. Swain, and J. C. Kingston. 1985. A palaeolimnological record of human disturbance from Harvey's Lake, Vermont: geochemistry, pigments and diatoms. Freshwat. Biol. 15:261-288.

Fallesen, G., F. Andersen, and B. Larsen. 2000. Life, death and revival of the hypertrophic Mariager Fjord, Denmark. J. Mar. Sys. 25:313-321.

Furlong, E. T., and R. Carpenter. 1988. Pigment preservation and remineralization in oxic coastal marine sediments. Geochim. Cosmochim. Acta 52:87-99.

Hall, R. I., P. R. Leavitt, R. Quinlan, A. S. Dixit, and J. P. Smol. 1999. Effects of agriculture, urbanization, and climate on water quality in the northern Great Plains. Limnol. Oceanogr. 44:739-756.

Hansen, J. L. S., and A. B. Josefson 2003. Accumulation of algal pigments and live planktonic datoms in aphotic sediments druing the spring bloom in the transition zone of the North and Baltic Seas. Mar. Ecol. Prog. Ser. 248:41-54.

Henriksen, P. 2004. Estimating nodularin content of cyanobacterial blooms from abundance of *Nodularia spumigena* and its characteristic pigments— a case study from the Baltic entrance area. Harmful Algae 4:167-178 [doi:10.1016/j.hal.2004.02.003].

Hodgson, D. A., and others. 2003. Colonization, succession, and extinction of marine floras during a glacial cycle: A case study from the Windmill Islands (east Antarctica) using biomarkers. Paleoceanogr. 18 (3):1076 [doi:10.1029/2002PA000775].

Hurley, J. P., and D. E. Armstrong. 1990. Fluxes and transformations of aquatic pigments in Lake Mendota, Wisconsin. Limnol. Oceanogr. 35:384-398.

Jeffrey, S. W., R. F. C. Mantoura, and S. W. Wright. 1997. Phytoplankton pigments in oceanography. UNESCO Publishing.

Kauppila, P., K. Weckstrom, S. Vaalgamaa, H. Pitkanen, A. Korhola, N. Reuss, and S. Drew. 2005. Tracing pollution and recovery from sediments of an urban estuary, the northern Baltic Sea: are we far from ecological reference conditions? Mar. Ecol. Prog. Ser. 290:35-53.

Klein, B., and C. Riaux-Gobin. 1991. Algal pigment diversity in coastal sediments from Kerguelen (sum-Antarctic Islands) reflecting local dominance of green algae, euglenoids and diatoms. Polar Biol. 11, 439-448.

Leavitt, P. R. 1993. A review of factors that regulate carotenoid and chlorophyll deposition and fossil pigment abundance. J. Paleolimnol. 9:109-127.

——— and D. A. Hodgson. 2001. Sedimentary pigments, p. 295-325. *In* J. P. Smol, H. J. B. Birks, and W. M. Last [eds.], Tracking environmental change using lake sediments, Volume 3: Terrestrial, algal, and siliceous indicators. Kluwer Academic Publishers.

Lucas, C. H., and P. M. Holligan. 1999. Nature and ecological implications of algal pigment diversity on the Molenplaat tidal flat (Westerschelde estuary, SW Netherlands). Mar. Ecol. Prog. Ser. 180:51-64.

Mantoura, R. F. C., and D. Repeta. 1997. Calibration methods for HPLC, p. 407-428. *In* S. W. Jeffrey, R. F. C. Mantoura, and S. W. Wright [eds.], Phytoplankton pigments in oceanography. UNESCO Publishing.

———, S. W. Wright, S. W. Jeffrey, R. G. Barlow, and D. E. Cummings. 1997. Filtration and storage of pigments from microalgae, p. 283-306. *In* S. W. Jeffrey, R. F. C. Mantoura, and S. W. Wright [eds.], Phytoplankton pigments in oceanography. UNESCO Publishing.

Petersen, D. G., and I. Dahllöf. 2005. Improvements for comparative analysis of changes in diversity of microbial communities using internal standards in PCR-DGGE. FEMS Microbiol. Ecol. 53(3):339-348 [doi:10.1016/j.femsec.2005.01.001].

Porra R. J., E. E. Pfündel, and N. Engel, 1997. Metabolism and function of photosynthetic pigments p. 85-126. *In* S. W. Jeffrey, R. F. C. Mantoura, and S. W. Wright [eds.], Phytoplankton pigments in oceanography. UNESCO Publishing.

Reuss, N., D. J. Conley, and T. S. Bianchi. 2005. Preservation conditions and the use of sediment pigments as a tool for recent ecological reconstruction in four Northern European estuaries. Mar. Chem. 95:283-302 [doi:10.1016/j.marchem.2004.10.002].

Sun, M. Y., R. C. Aller, and C. Lee. 1991. Early diagenesis of chlorophyll-*a* in Long-Island Sound sediments—a measure of carbon flux and particle reworking. J. Mar. Res. 49:379-401.

———, R. C. Aller, and C. Lee. 1994. Spatial and temporal distributions of sedimentary chloropigments as indicators of benthic processes in Long Island Sound. J. Mar. Res. 52:149-176.

———, C. Lee, and R. C. Aller. 1993. Laboratory studies of oxic and anoxic degradation of chlorophyll-a in Long Island Sound sediments. Geochim. Cosmochim. Acta 57:147-157.

Villanueva, J., and D. W. Hastings. 2000. A century-scale record of the preservation of chlorophyll and its transformation products in anoxic sediments. Geochim. Cosmochim. Acta 64:2281-2294.

Wakeham, S. G., C. Lee, J. I. Hedges, P. J. Hernes, and M. L. Peterson. 1997. Molecular indicators of diagenitic status in marine organic matter. Geochim. Cosmochim. Acta 61:5363-5369.

Wright, S. W., S. W. Jeffrey, R. F. C. Mantoura, C. A. Llewellyn, T. Bjørnland, D. Repeta, and N. Welschmeyer. 1991. Improved HPLC method for the analysis of chlorophylls and carotenoids from marine phytoplankton. Mar. Ecol. Prog. Ser. 77:183-196.

Submitted 12 November 2004
Revised 16 July 2005
Accepted 31 August 2005