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# The purification of chlorins from marine particles and sediments for nitrogen and carbon isotopic analysis

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## Abstract

Methods are presented for the separation of chlorins from marine particles and sediments for nitrogen and carbon isotopic analysis. Carbon and nitrogen purities of 91 and 96%, respectively, were obtained for particulate chlorins, with recoveries of 88%. Sedimentary chlorins were obtained with carbon and nitrogen purities of 88 and 89%, respectively, and recoveries of 18%. The reproducibility of carbon and nitrogen isotopic analyses on particulate and sedimentary chlorins was 0.2%. The low recovery of chlorins from sediments is primarily a result of their complex distribution, requiring that only the one or two most abundant chlorins from a sample are purified. The procedure for particulate samples includes ultrasonic extraction with solvents, two phase separations, and reversed- and normal-phase high-performance liquid chromatographic separations (HPLC). The procedure for sedimentary chlorins includes ultrasonic extraction by solvent, solid-phase extraction on silica gel, reversed-phase HPLC, size-exclusion chromatography, and normal-phase HPLC. The purification procedures require ca. 4 h for particulate samples, and ca. 8 h for sediment samples when two samples are processed simultaneously. The application of these methods to surficial Black Sea sediments demonstrated that different chlorins have distinct nitrogen and carbon isotopic ratios. The  $\delta^{15}\text{N}$  differences are interpreted in terms of changes in the seasonal flux of material out of the euphotic zone, while the  $\delta^{13}\text{C}$  differences are thought to derive from the presence or absence of the chlorin phytol side-chain. © 2000 Elsevier Science Ltd. All rights reserved.

*Keywords:* Chlorins; Nitrogen; Isotopes; Sediments; Phytoplankton; Size exclusion chromatography; HPLC

## 1. Introduction

Earth and biological scientists often seek to determine the nitrogen and carbon isotopic ratios of primary producers in contemporary and historical environments. This effort can be confounded by wide distributions of isotopic values in an ecosystem and by the alteration of primary isotopic signatures during natural decomposition processes. Therefore, it is preferable to isolate molecular markers for primary producers, or “biomarkers,” from

natural samples for isotopic analysis. Chlorophyll is an ideal biomarker for phytoplankton, the primary producers in aquatic ecosystems, because it is produced solely by photoautotrophs and can be easily recovered from particulate and sedimentary material. Nevertheless, aside from methods for the purification of chlorophyll from higher plants (Bidigare et al., 1991) and phytoplankton cultures (Sachs et al., 1999) for stable N and C isotopic analyses, there are no published protocols for chlorophyll purification from sediments for isotopic analyses. This is a result of the difficulty in purifying these compounds from complex mixtures and matrices. Contributing to this difficulty are the physical and chemical properties of chlorin pigments (i.e. chlorophyll derivatives), which as a class are non-volatile, thermally unstable, light- and oxygen-sensitive, and chemically reactive.

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A previously published procedure for the purification of chlorophylls *a* (Chla) and *b* for N and C isotopic analysis, developed for terrestrial higher plants (Bidigare et al., 1991), relied upon precipitation of Chla with dioxane (Iriyama et al., 1974), followed by preparative reversed-phase HPLC (Watanabe et al., 1984). Since sedimentary chlorins are predominantly demetallated (e.g. magnesium-free), and particulate chlorins can be partially demetallated during collection, handling and storage, dioxane precipitation was considered unsuitable. In addition, dioxane precipitation of Chla can fail when pigment extracts are lipid-rich, as found with certain algal cultures (R. Bidigare, personal communication). Therefore our goal was to develop generally applicable protocols for the purification of chlorins from marine sedimentary and particulate samples for N and C isotopic analysis with the following criteria: (1) maintain isotopic integrity of the chlorins, (2) minimize sample size requirements, and (3) minimize time and labor per analysis. The methods (Fig. 1) are suitable for the wide diversity of chlorins commonly found in marine particles and sediments. Chlorin structures and abbreviations are shown in Fig. 2 and Table 1, respectively.

## 2. Methods

### 2.1. General laboratory procedures

In order to minimize exposure of chlorins to light and air, all manipulations were carried out under low-light conditions and samples were stored dry, in the dark, at  $-20^{\circ}\text{C}$  under a nitrogen atmosphere. Samples dissolved in solvent for more than a few minutes were kept in the dark under a nitrogen atmosphere. Exposure to polar solvents, such as methanol, was minimized to prevent alomerization and epimerization reactions from occurring (Hynninen, 1979; Otsuki et al., 1987; Zapata et al., 1987). All solvents were HPLC-grade. Glassware was cleaned with Micro (Cole-Parmer, Chicago, IL) and rinsed with tap water (3 $\times$ ), distilled water (3 $\times$ ), MeOH (3 $\times$ ), and acetone (3 $\times$ ). Pasteur pipets,  $\text{Na}_2\text{SO}_4$ , glass vials, glass fiber filters, sand, aluminum foil, and glass wool were combusted at  $450^{\circ}\text{C}$  for  $>8$  h. Teflon cap liners, cotton, and boiling chips (Hengar Granules, Hengar Co., Philadelphia, PA) were Soxhlet extracted in 7% MeOH/MeCl<sub>2</sub>.

### 2.2. The purification of particulate chlorophyll *a* for isotopic analysis

Detailed purification procedures for particulate chlorophyll are described in Sachs et al. (1999) and outlined in Fig. 1a. In brief, algal culture and marine particulate samples were collected by filtration through a 293 mm Gelman A/E filter. Culture samples were vacuum-filtered and marine particulate samples were pumped

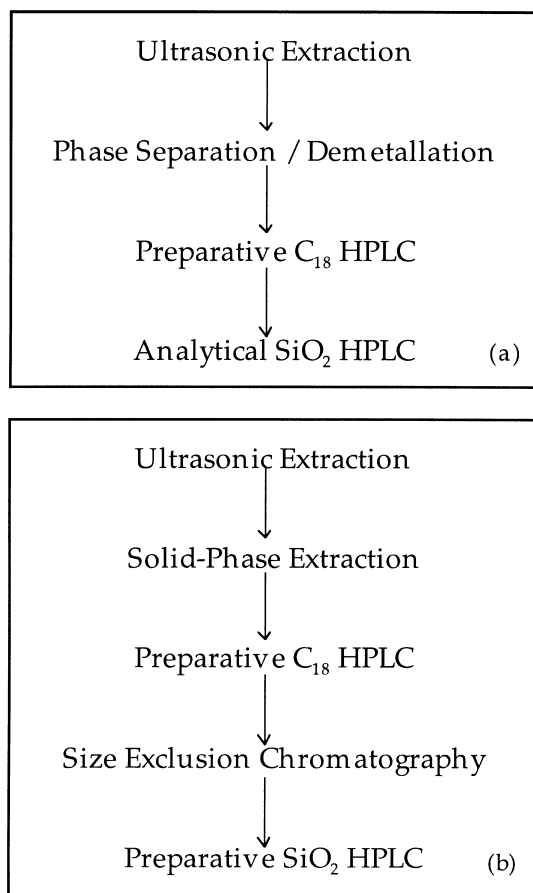


Fig. 1. Protocols for purifying chlorins from marine particles (a) and sediments (b).

from depth with garden hoses connected to a pneumatic pump (Lutz Pumps, Inc., Norcross, GA). Filters were immediately stored in liquid nitrogen, or at  $-20$  to  $-40^{\circ}\text{C}$  until liquid nitrogen storage could be obtained.

Immediately prior to extraction, filters were thawed at room temperature and subsamples were removed for whole-cell  $\delta^{15}\text{N}$  and  $\delta^{13}\text{C}$  determinations. Filters were ultrasonically extracted (3 $\times$ ) in 125 ml degassed acetone containing ca. 5 g  $\text{NaHCO}_3$ . Combined extracts were poured into a separatory funnel containing 125 ml water, and the chlorophyll was partitioned (3 $\times$ ) into 200 ml hexane. Chlorophyll in the hexane fraction was then quantified spectrophotometrically. The combined hexane fractions were back-extracted (1 $\times$ ) with 200 ml 15%  $\text{H}_2\text{O}$  in MeOH (v/v) to remove carotenoids, and chlorophyll was quantified. Demetallation of the chlorophyll, to form pheophytin, was accomplished by adding 200 ml 10% HCl (aq) to the hexane fraction and shaking for 1 min. The color of the hexane solution changed from emerald to pine green. The aqueous fraction was poured off and the hexane neutralized with 100 ml 2% (m/v)  $\text{NaHCO}_3$  (aq). The hexane phase was dried over

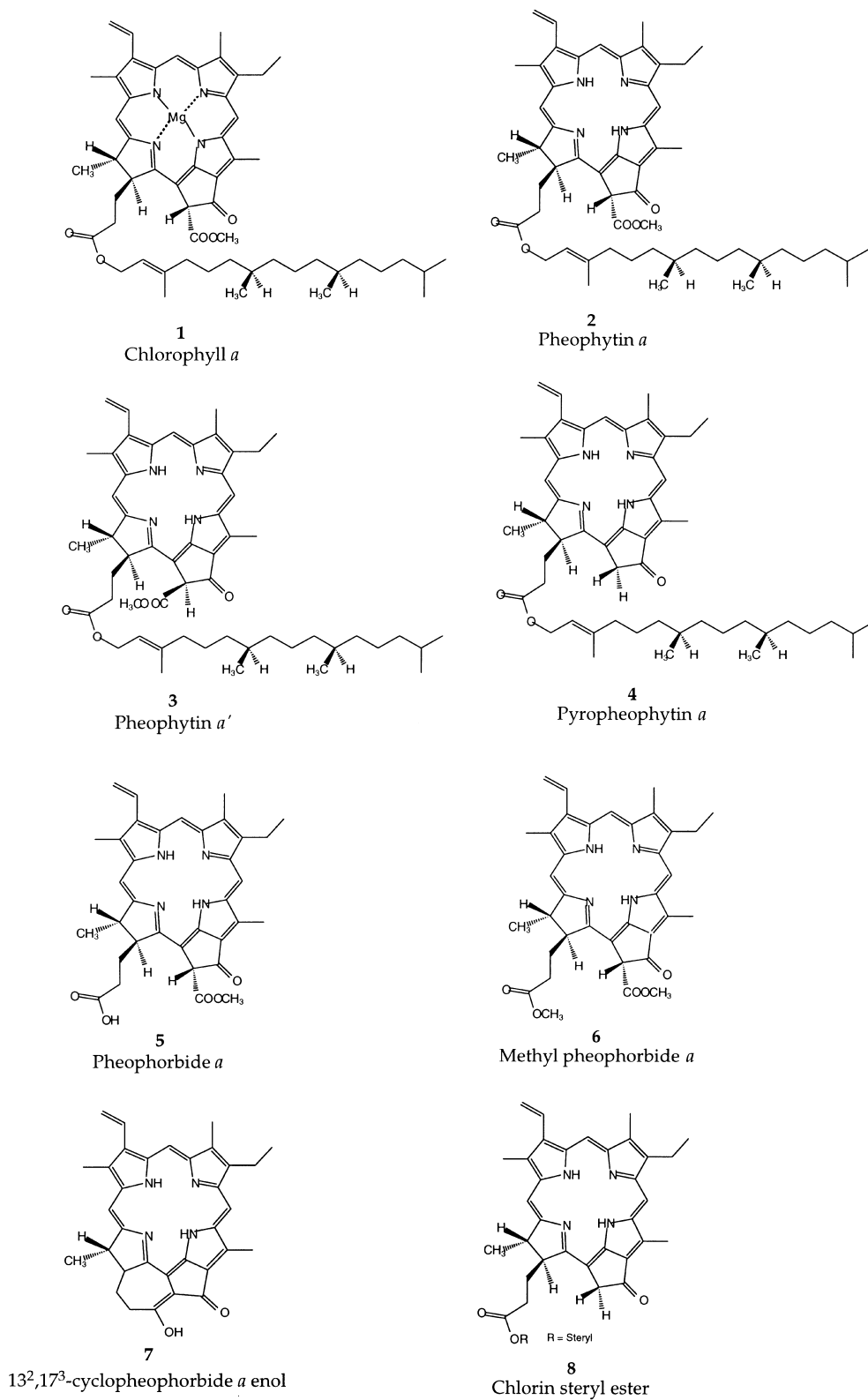


Fig. 2. Chlorin structures discussed in text.

Table 1  
Chlorin abbreviations

Chlorin	Abbrev.	Structure <sup>a</sup>
Chlorophyll <i>a</i>	Chl <sub>a</sub>	1
Pheophytin <i>a</i>	PTNa	2
Pheophytin <i>a'</i>	PTNa'	3
Pyropheophytin <i>a</i>	PPTNa	4
Pheophorbide <i>a</i>	PBDa	5
Pheophorbide <i>a'</i>	PBDa'	
Methyl pheophorbide <i>a</i>	MPBDa	6
Methyl pheophorbide <i>a'</i>	MPBDa'	
Pyropheophorbide <i>a</i>	PPBDa	
Methyl pyropheophorbide <i>a</i>	MPPBDa	
13 <sup>2</sup> ,17 <sup>3</sup> -cyclopheophorbide <i>a</i> enol	Chl686	7
Chlorin steryl esters	CSE	8

<sup>a</sup> Shown in Fig. 2.

Na<sub>2</sub>SO<sub>4</sub>, and rotary-evaporated to dryness. A final spectrophotometric quantification was performed before the dried extract was stored under nitrogen at -20°C.

Further purification of PTNa was achieved using preparative reversed-phase (C<sub>18</sub>) HPLC. Dried extracts were dissolved in a small amount of MeCl<sub>2</sub> and multiple injections of 50–200 µL (1–25 mg extract or 0.5–4 µmol PTNa) were made on to a preparative C<sub>18</sub> column (Kromasil Kr100-5-C18; 10 mm I.D.×250 mm, 5 µm particle size; Eka Nobel, Bohus, Sweden) fitted with a Kromasil 10 mm I.D.×50 mm guard column packed with 5 µm C<sub>18</sub>. An acetone (solvent A) and methanol (solvent B) gradient [(time — min, flow — ml/min %A): (0, 6, 95), (10, 6, 70), (15, 7, 65), (30, 6, 0)] resulted in retention times of ca. 20 and 21 min for PTNa and its epimer, PTNa', which were collected, quantified, rotary-evaporated to dryness and stored at -20°C.

Final purification of PTNa was achieved using normal-phase (SiO<sub>2</sub>) HPLC. Extracts were redissolved in a small amount of 10% acetone/hexane and multiple injections of 50–200 µl (0.5–2 mg PTNa) were made on to an analytical silica column (Alltech Spherisorb; 4.6 mm I.D.×100 mm, 3 µm particle size; Alltech Associates, Inc, Deerfield, IL). Isocratic elution with 4% acetone/hexane at 2 ml/min resulted in retention times of 11 and 13 min for PTNa' and PTNa, respectively, which were collected, quantified, dried under a N<sub>2</sub> stream and stored at -20°C. PTNa recoveries for the entire procedure were 88±18%, or about 90–95% for each step, while nitrogen and carbon purities averaged 96±3.4% and 91±4.3%, respectively. Two samples prepared in tandem can be processed in 8 h.

### 2.3. Purification of sedimentary chlorins for isotopic analysis

A flow diagram outlining the procedures for chlorin purification from sediment samples appears in Fig. 1b.

Frozen sediments (50–125 g wet wt or 20–50 g dry wt) were thawed at room temperature, transferred to 800 ml centrifuge tubes, and successively sonic-extracted with an immersion probe (Vibra Cell; 70% duty cycle, output control 8; Sonics and Materials, Inc., Danbury, CT) for 9 min each in 700 ml 100% MeOH, 50% MeOH/MeCl<sub>2</sub>, 25% MeOH/MeCl<sub>2</sub>, and 100% MeCl<sub>2</sub>. The centrifuge tube was immersed in an ice water bath to mitigate pigment decomposition and solvent evaporation. Following extraction, samples were centrifuged (Model 2K; 10 min, 1200 r.p.m; Needham Heights, MA), and the supernatants were combined, quantified and rotary-evaporated to dryness.

Solid-phase extraction (SPE) was accomplished by redissolving the extract, containing ca. 2–7 µmol chlorin, in 10 ml 7% MeOH/MeCl<sub>2</sub> and applying it to a column of flash SiO<sub>2</sub> (Matrex Silica Si; 5 cm×10 cm, 60 Å Pore Diameter; Amicon Corp., Danvers, MA). The column was prepared by pouring a slurry of 75 g unactivated silica in MeCl<sub>2</sub> into a 5×45.7 cm flash chromatography column (Ace Glass, Inc., Vineland, NJ) containing a plug of glass wool and a thin (5 mm) layer of sand at the bottom. Prior to sample application, the column was eluted with 600–1000 ml 7% MeOH/MeCl<sub>2</sub> under N<sub>2</sub> pressure of 2–5 p.s.i, and a thin layer (5 mm) of sand was applied to the top of the silica by slowly pouring it into 200–400 ml of the solvent. The sample was eluted from the column with 600 ml 7% MeOH/MeCl<sub>2</sub>. The first ca. 100 ml to elute were orange, containing β-carotene and other carotenoids. The next ca. 200 ml to elute were dark green to brown/black and contained most sedimentary chlorins in the wide polarity range from chlorin steryl esters (non-polar) to pheophorbide *a* (free chlorin acid). A hand-held UV lamp was useful for monitoring the chromatography, since the chlorin fraction was an intense pink/orange color under UV light. This fraction was collected, rotary-evaporated to dryness, and stored at -20°C. Typically some pink/orange fluorescence, indicative of chlorins, remained at the origin. However, total chlorin recoveries (assuming an extinction coefficient (ε<sub>666</sub>) of 5×10<sup>4</sup> for the red band (King and Repeta, 1994a) and a molecular mass of 550 or 850 amu, depending on the sample) averaged > 100%, making it unlikely that the material remaining on the column accounted for a significant fraction of the total. Yields greater than 100% can arise from the baseline correction of visible spectra of highly impure samples. The purity of the chlorin fraction after SPE was 4.0±1.2% for 7 Mediterranean and 4 Black Sea sediments. This compares to a purity of <1% for the whole extract prior to SPE. In addition, the procedure removes salt, circumventing the need for cumbersome phase separations which resulted in severe emulsions and loss of sample, and much of the colored material that interferes with visible absorption spectrophotometry (Fig. 3a).

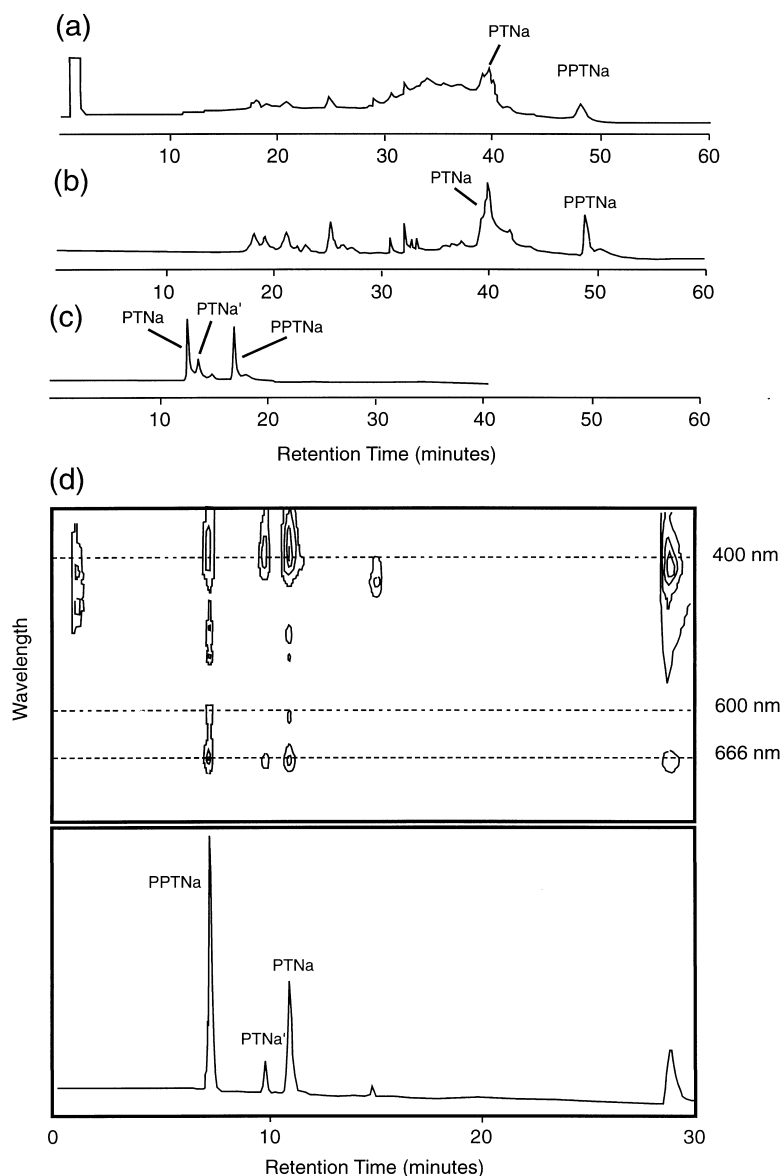


Fig. 3. Analytical  $C_{18}$  HPLC chromatograms of a surficial Black Sea sediment after (a) solvent extraction, (b) solid-phase extraction, and (c) size-exclusion chromatography. Note the different time scales in (a) and (b) and the use of a different gradient in (c). An analytical  $SiO_2$  HPLC chromatogram (d) was generated during the final purification step. Note the elution order is reversed on the normal-phase column used to generate (d).

Solid-phase extraction was followed by preparative reversed-phase HPLC (Waters 600E pump and 900 photodiode array detector), accomplished by redissolving the extract in 200–1000  $\mu$ L 10% MeOH/ $MeCl_2$  and injecting 50–200  $\mu$ L aliquots ( $\leq 10$  mg extract and 2  $\mu$ mol chlorin) on to a preparative  $C_{18}$  column (Kromasil Kr 100-5-C18; 10 mm I.D.  $\times$  250 mm, 5  $\mu$ m particle size; Eka Nobel, Bohus, Sweden), fitted with a 10 mm I.D.  $\times$  50 mm guard column (Kromasil 5  $\mu$ m  $C_{18}$  packing). An aqueous methanol (10%  $H_2O/MeOH$ ; solvent

A) and acetone (solvent B) gradient was employed as follows: (time — min, flow — ml/min, %A): (0, 6, 75), (15, 6, 0), followed by 8 min isocratic elution. The chromatogram was monitored at 666 nm and prominent chlorin peaks (Table 2) were collected with a fraction collector (Gilson FC 203; Gilson Medical Electronics, Middleton, WI) into 18 ml glass vials. Care was taken to collect the entire chlorin peak in order to prevent isotopic alteration of a sample resulting from across-peak isotopic heterogeneity (Bidigare et al., 1991). Recoveries

Table 2  
Retention times for sedimentary chlorins on preparative reversed-phase (C<sub>18</sub>) HPLC

Chlorin	<i>t<sub>R</sub></i> (min)
PPBDa	6.5
MPPBDa	9
PTNa	15
PPTNa	17
CSEs	18–20

varied widely with the distribution of chlorins in a sample, but averaged  $24 \pm 9\%$ . Samples with simple chlorin distributions had higher recoveries than those containing a wide diversity of chlorins due to the impracticality of collecting and purifying numerous chlorins from a single sample. The purity of individual chlorins ranged from 12 to 78%, averaging  $43 \pm 22\%$ .

Size exclusion chromatography (SEC) was next performed by redissolving the extracts in 200  $\mu\text{L}$  MeCl<sub>2</sub> and injecting 20–80  $\mu\text{L}$  aliquots (containing  $\leq 0.5$  mg of sample and 0.5  $\mu\text{mol}$  chlorin) on to a SEC column having an exclusion limit of  $1.5 \times 10^3$  Daltons (Shodex GPC K-801; 8 mm I.D.  $\times$  300 mm; Showa Denko K.K., Tokyo, Japan) to which was attached a Shodex K-G guard column (8 mm I.D.  $\times$  100 mm). The sample was eluted with degassed MeCl<sub>2</sub> at 1.0 ml/min (Waters 600E pump) and monitored at 666 nm (Waters 990 photodiode array detector). Chlorin peaks were collected into 4 ml vials, quantified spectrophotometrically, dried under a stream of N<sub>2</sub>, and stored at  $-20^\circ\text{C}$ . Retention times for common chlorin classes were: CSEs, 5.3–5.6 min; PTNs and carotenoids, 5.8–7.0 min; pheophorbides, 7.0–8.4 min (Fig. 6). Chlorin recoveries and purities averaged  $89 \pm 12\%$  and  $78 \pm 13\%$ , respectively.

Preparative normal-phase HPLC was accomplished by redissolving the sample in 200–500  $\mu\text{L}$  of 10–30% acetone/hexane (depending on the polarity of the chlorin) and injecting 100  $\mu\text{L}$  (containing ca. 1 mg sample and 0.5  $\mu\text{mol}$  chlorin) on to a SiO<sub>2</sub> column (Kromasil KR100-5-Sil; 10 mm I.D.  $\times$  250 mm, 5  $\mu\text{m}$  SiO<sub>2</sub> particles) fitted with a Kromasil guard column (10 mm I.D.  $\times$  50 mm, 5  $\mu\text{m}$  SiO<sub>2</sub> particles). Isocratic elution at 6 ml/min (Waters 600E pump) was employed with an acetone/hexane mixture that varied depending on the chlorin being purified (Table 3). The chromatogram was monitored at 666 nm (Waters 990 photodiode array detector), and chlorin peaks were collected into 18 ml glass vials, spectrophotometrically quantified, rotary-evaporated to dryness, and stored at  $-20^\circ\text{C}$ . As with preparative C<sub>18</sub> HPLC, care was taken to collect the entire chlorin peak in order to negate the effect of any across-peak isotopic heterogeneity (Bidigare et al., 1991). Recoveries and purities averaged 67 and 88%, respectively.

Table 3  
Solvent mixtures for isocratic elution of sedimentary chlorins on preparative SiO<sub>2</sub> HPLC. Values are ratios of acetone to hexane, and the approximate retention times for the chlorins are shown

Chlorin	Acet/hex	<i>t<sub>R</sub></i> (min)
PPTNa	5/95	9.5
PTNa'	5/95	12
PTNa	5/95	13
MPPBDa	20/80	7
PPBDa	30/70	6.5

#### 2.4. Chlorin identification

Individual chlorins were identified based on chromatographic retention times and visible absorption spectra of authentic standards synthesized in our laboratory from spirulina (Sachs, 1997) using procedures optimized from standard protocols (Falk, 1964; Fuhrhop & Smith, 1975; King, 1993; Scheer, 1991; Svec, 1978).

#### 2.5. High-performance liquid chromatography

A standard analytical reversed-phase HPLC method was used to "fingerprint" all samples. A Waters 600E Multisolute Delivery System was used in conjunction with a Waters 990 Photodiode Array Detector and Software (Waters Corporation, Milford, MA). The injector was a Rheodyne 7125 Syringe Loading Sample Injector (Rheodyne, Inc., Cotati, CA), fitted with a 200  $\mu\text{L}$  sample loop (1000  $\mu\text{L}$  for preparative work). A 3  $\mu\text{m}$  C<sub>18</sub> column (Adsorbosphere HS C18 3  $\mu\text{m}$ ; 4.6 mm I.D.  $\times$  150 mm, 3  $\mu\text{m}$  particle size; Alltech Associates, Inc, Deerfield, IL) was employed, and a solvent gradient from 100% A to 100% B in 30 min, followed by a 30 min isocratic elution of solvent B, was used. Solvent A was 20% 0.5 N ammonium acetate (aq) in MeOH. Solvent B was 20% acetone in MeOH. The flow rate was maintained at 1.5 ml/min. The gradient was optimized from previously published methods (Mantoura and Llewellyn, 1983; Zapata et al., 1987; Wright et al., 1991) to resolve the entire suite of pigments found in marine phytoplankton. It was used in this work because the retention times of most algal pigments eluting during this gradient are well-known and documented. It was not optimized for the analysis of sedimentary chlorins, some of which (i.e. CSEs) do not elute under these conditions.

#### 2.6. Isotope analyses

Purified chlorins were transferred to smooth-walled tin capsules (8  $\times$  6 mm; Elemental Microanalysis, Manchester, MA) in 200  $\mu\text{l}$  of acetone, dried under a 60 W light bulb, folded with forceps and stored in a desiccator until

isotopic analysis. Smooth-walled, as opposed to pressed, capsules were used to minimize solvent “creep”. Filters from particulate samples were dried at 60°C, placed into tin boats (5×9 mm; Elemental Microanalysis), folded with forceps and stored in a desiccator until isotopic analysis. Sediment samples were prepared using a modified “cup and saucer” technique (Cowie and Hedges, 1991) in which 10–100 mg of dry sediment, or 50–200 mg of wet sediment, was placed into a pressed silver cup (8×5 mm; Elemental Microanalysis) and placed on to an unfolded silver cup (to retain overflow). After drying the sample in an oven (50°C, > 12 h) one drop of 2 N HCl was added to the cup with a pipette, and the sample was dried for > 4 h before being acidified again with 1 drop of 2 N HCl. This procedure was repeated until effervescence ceased, at which time the sample was dried (50°C for > 24 h), folded with forceps (both cups) and stored in a desiccator until analysis. Nitrogen and carbon isotope ratios were measured on a Finnigan MAT delta S isotope ratio mass spectrometer (Finnigan Corporation, 355 River Oaks Parkway, San Jose, CA 95134) connected to a CHN analyzer (Heraeus Rapid Elemental Analyzer) by an automated trapping box for the sequential cryogenic purification of CO<sub>2</sub> and N<sub>2</sub> (Stable isotope Laboratory at the Marine Biological Laboratory, Woods Hole, MA; Fry et al., 1992), thus permitting rapid δ<sup>15</sup>N and δ<sup>13</sup>C analyses on the same sample. Standard delta notation is used for reporting stable isotopic ratios of nitrogen and carbon: δ<sup>n</sup>χ ≡  $\left[ \frac{(^n\chi / (^{n-1}\chi))}{(^n\chi / (^{n-1}\chi))_{\text{std}}} - 1 \right] \times 1000\text{‰}$  where <sup>n</sup>χ = <sup>15</sup>N or <sup>13</sup>C, and the isotopic standards for C and N are Peedee Belemnite limestone (δ<sup>13</sup>C = 0‰; Craig, 1953) and atmospheric N<sub>2</sub> (δ<sup>15</sup>N = 0‰; Hoering, 1955). Nitrogen isotopic blank corrections were not required since the N blank was typically < 0.1 nmol N. However, carbon isotopic blank-corrections (–25.1‰) were required for small chlorin samples purified by SiO<sub>2</sub> HPLC when the carbon blank was 13–30% of the sample carbon. The measurement precision for δ<sup>15</sup>N and δ<sup>13</sup>C analyses of purified chlorins from particulate and sedimentary samples was 0.16‰ for nitrogen and 0.12‰ for carbon, the pooled standard deviations of replicate analyses.

### 2.7. Elemental analyses

Elemental (CHN) analyses were performed on an EA 1108 elemental analyzer with Eager 200 data acquisition software (Fisons Instruments, Inc., Beverly, MA). Particulate and sediment samples were prepared as described above for isotope analyses. Organic samples (i.e. whole or partially purified extracts and chlorins) were prepared by placing 50–1000 μg of sample, dissolved in < 200 μl acetone or MeCl<sub>2</sub>, into a smooth wall tin capsule (8×6 mm; Elemental Microanalysis, Manchester, MA), and evaporating the solvent by heating from

above with a 60 W lamp. Dried samples were folded with forceps and stored in a desiccator until analysis. Mass determinations were performed with a Sartorius Micro balance (Sartorius AG, Gottingen, Sweden). All values were blank-corrected for C and N (1) associated with the sample capsules (typically < 0.1 nmol N, and 0.2–7% of sample carbon), (2) added to organic samples during sample transfer and drying (typically < 0.1 nmol N, and 0.3–10% of sample carbon), and (3) associated with chromatography (< 0.1 nmol N, and 13–30% of sample carbon).

### 2.8. UV/vis spectrophotometry

Visible spectra of chlorins in the wavelength range from 350–750 nm were taken on a Hewlett–Packard HP8452A Diode Array Spectrophotometer (Rockville, Maryland) or a Varian Techtron DMS-200 Spectrophotometer (Varian Techtron Limited, Springvale Road, Mulgrave, Victoria 3170, Australia). The instrument was referenced against the appropriate solvent contained in a 1-cm quartz cuvette. Reference spectra were obtained from the literature (Svec, 1978; King, 1993) and from standards synthesized from spirulina (Sachs, 1997) using optimized procedures (Falk, 1964; Fuhrhop and Smith, 1975; Svec, 1978; Scheer, 1991; King, 1993).

## 3. Results

The procedure for nitrogen and carbon isotopic determination in particulate and sedimentary chlorins was designed for all commonly occurring chlorins. It was used successfully to measure the N and C isotopic ratios of particulate Chla and Chla', in addition to sedimentary PTNa, PTNa', PPTNa, PPBDa, and MPPBDa. Aside from having broad applicability and high recoveries, the technique produced highly pure products while maintaining isotopic integrity. This section describes results of experiments to determine elemental and isotopic purity of the chlorins thus prepared.

### 3.1. Chlorin purities

The increasing purity of particulate Chla (e.g. PTNa) following individual steps of the purification protocol (Fig. 4) was assessed by comparing spectrophotometrically determined PTNa abundances in six batch cultures of marine phytoplankton to total N and C determined by elemental analysis (Table 4). Culture details are described elsewhere (Sachs et al., 1999). Spectrophotometric quantification of PTNa was performed at 666 nm with an extinction coefficient of 1.01 × 10<sup>5</sup> and a Soret/red (S/R) band absorbance ratio of 2.22 (King, 1993). Purities are lower limits since S/R ratios of purified PTNa in this

work were normally between 2.40 and 2.45. Nitrogen purity of PTNa was  $56 \pm 24\%$ ,  $90 \pm 10.4\%$ , and  $96 \pm 3\%$ , respectively, after solvent extraction and phase separation, preparative C<sub>18</sub> HPLC, and SiO<sub>2</sub> HPLC (Fig. 4). Carbon purities of PTNa were  $10 \pm 8\%$ ,  $51 \pm 38\%$ , and  $91 \pm 4\%$  after the three steps (Fig. 4).

As with particulate samples, the increasing purity of sedimentary chlorins (Fig. 5) was assessed by performing spectrophotometry and elemental analyses after each purification step. Chlorins from a surficial (0–10 cm) Black Sea sediment (2129 m water depth; Box Core 2, Stn. 2, R/V Knorr 134-9, 14–28 May, 1988) consisted principally of PPTNa, with lesser amounts of PTNa and PTNa'. The nitrogen purity of PPTNa increased from 21 to 89% during the 5-step procedure, while that of carbon increased from 6 to 88% (Fig. 5). The final purities are considered lower limits, as discussed above, since the S/R ratio used in spectrophotometric quantitations is believed to be 10% too low. PTNa and PTNa' from the same sediment sample had purities (based on C/N ratio) of 84 and 87%, respectively. These results

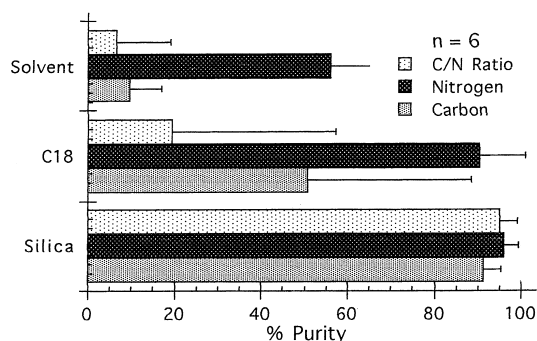


Fig. 4. Purity (%) of PTNa following each step of the procedure for the purification of particulate chlorins for isotopic analysis. Purities based on the elemental abundances of carbon and nitrogen, as well as the measured versus theoretical (13.75) C/N ratio. All values are averages from 6 phytoplankton cultures, and standard deviations are plotted.

compared favorably with the average purity of PPBDa and MPPBDa (88%) obtained by gravimetry from three eastern Mediterranean sapropels and a Black Sea sapropel (Sachs, 1997).

## 4. Discussion

### 4.1. Chlorin purification protocol

The protocols for purifying chlorins from particles and sediments for N and C isotopic analysis consist of sequential adsorption and size exclusion chromatography steps to separate impurities from individual chlorins in complex mixtures. The procedure yields products that are at least 88% pure, with a precision of better than 0.2‰ for both isotopes. Overall recoveries of pure chlorins were 88 and 18% from particulate and sedimentary samples, respectively. Recoveries of purified chlorins from sediments were low because typically only the most abundant one or two chlorins was purified. For example, Fig. 3b shows the distribution of chlorins in a surficial Black Sea sediment extract after solid-phase extraction. Only the two largest peaks, PTNa and PPTNa ( $t_R = 40.3$  and  $49.1$  min, respectively),

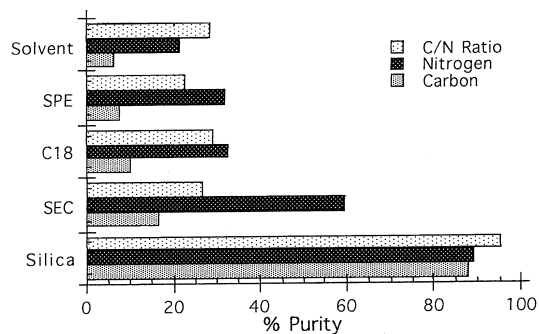


Fig. 5. Purity (%) of a Black Sea surficial sedimentary chlorin (PPTNa) after successive purification steps.

Table 4

Purity of algal PTNa, in percent, following each step of the purification protocol: 1 = phase separation; 2 = preparative C<sub>18</sub>HPLC; 3 = analytical SiO<sub>2</sub> HPLC

Culture <sup>a</sup>	1st N Purity	1st C Purity	2nd N Purity	2nd C Purity	3rd N Purity	3rd C Purity
TW4	33	11	99	99	99	95
PHA4	62	18	98	83	95	96
PAV4	28	1	71	4	97	95
AMP4	62	7	95	41	97	87
DUN4	55	2	89	14	97	90
TW3			91	63	89	86
EH3	96	19				

<sup>a</sup> Culture abbreviations are as follows: TW = *Thalassiosira weissflogii*, PHA = *Phaeodactylum tricorutum*, PAV = *Pavlova lutheri*, AMP = *Amphidinium carterae*, DUN = *Dumaliella tertiolecta*, EH = *Emiliania huxleyi*.



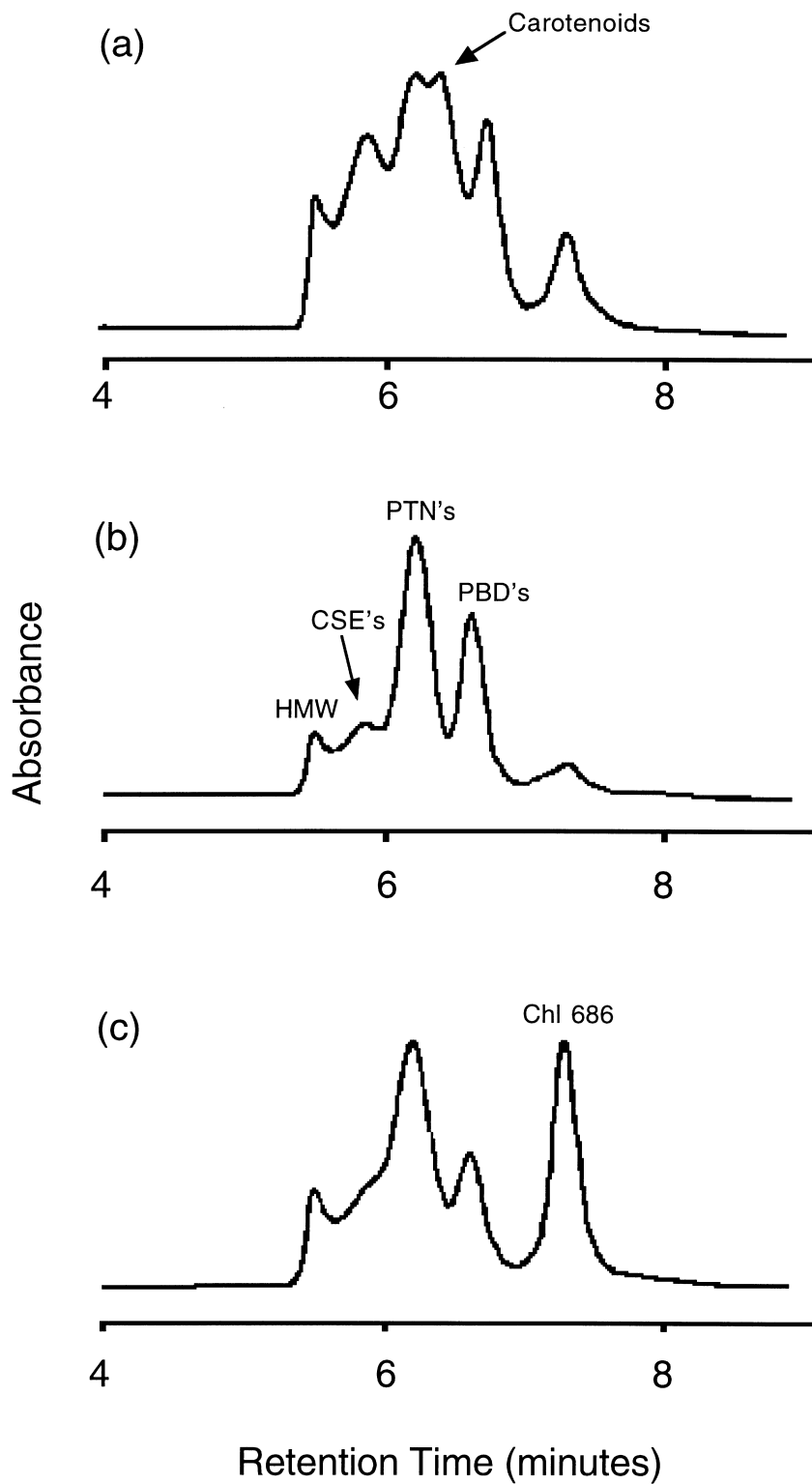


Fig. 6. Size-exclusion chromatograms of a surface Peru margin sediment extract at (a) 440 nm, (b) 666 nm, and (c) 686 nm. The abbreviation HMW refers to high molecular weight chlorins.

accounting for about 50% of the total chlorin in the sample, were collected and purified. Thus, overall recoveries of pure chlorins had to be less than 50%.

Solid-phase extraction proved to be a rapid, effective technique for removing significant quantities of non-chlorin colored material from sedimentary lipid extracts. Fig. 3a shows a chromatogram from a surficial Black Sea sediment lipid extract. The absorbance of 666 nm light by “baseline” material all but camouflaged individual chlorin peaks. Following SPE the individual chlorins emerged (Fig. 3b). Removal of the colored baseline material by SPE thus increased nitrogen and carbon purities of PPTNa by 48 and 20%, to 32 and 7.2%, respectively (Fig. 5).

Size exclusion chromatography, which separates compounds on a styrene-divinyl benzene gel, based on the effective length of their longest dimension, was a rapid and, effective technique for removing impurities with similar polarities from sedimentary chlorins. Previous researchers have successfully used SEC to separate carotenoids from chlorins (Repeta & Gagosian, 1984, 1987; King & Repeta, 1994b) and metalloporphyrins from crude oil (Fish & Komlenic, 1984). Size-exclusion chromatograms of a surface Peru margin sediment extract are shown in Fig. 6. Good separation between pheophytins and pheophorbides was observed (Fig. 6b), with carotenoids eluting between them (Fig. 6a). The chlorin peak preceding the pheophytins comprises the chlorin steryl esters (King and Repeta, 1991) and the peak preceding them is high molecular weight chlorin material (King and Repeta, 1994a). The chromatogram in Fig. 3c demonstrates the purity of pheophytins (PTNa, PTNa', PPTNa) after SEC. By comparison to the chromatogram after SPE (Fig. 3b) it is clear that much of the chlorin contamination has been removed. Indeed, nitrogen and carbon purities of PPTNa from a Black Sea sediment increased by 27 and 65%, to 60 and 16%, respectively (Fig. 5) after SEC.

Reverting back to adsorption HPLC following SEC proved effective at separating most remaining contaminants from individual sedimentary chlorins (Fig. 3d) as purities then averaged 88% (Fig. 5). Recoveries from SiO<sub>2</sub> HPLC were relatively low, averaging 67 ± 7.6%, a result of the separation, from the chlorin peak of interest, of structural isomers and allomers that had coeluted during preparative C<sub>18</sub> HPLC and SEC. The entire purification procedure for sedimentary chlorins can be accomplished in one day if two samples are processed simultaneously.

#### 4.2. Minimum purification required for chlorin $\delta^{15}\text{N}$ and $\delta^{13}\text{C}$

It has been suggested (Macko, 1981) that sediment and particulate lipid extracts may contain primarily chlorin nitrogen, and therefore may be used as surrogates

for chlorophyll  $\delta^{15}\text{N}$  analyses. This appears not to be the case for particles or sediments. For instance, the nitrogen and carbon purity of Chla (as PTNa) in solvent extracts from 6 phytoplankton cultures averaged 56 and 10%, respectively (Fig. 4), while sedimentary chlorin purities in surficial Black Sea sediments were 21 and 6%, based on nitrogen and carbon respectively (Fig. 5). The purity of sedimentary chlorins was possibly lower still in 7 Eastern Mediterranean sapropel and 4 subsurface Black Sea sediment samples, averaging < 1% by weight after solvent extraction (Sachs, 1997). Since the impurities had significantly different N and C isotopic ratios than the chlorins, whole lipid extract  $\delta^{15}\text{N}$  and  $\delta^{13}\text{C}$  values were not suitable isotopic proxies for chlorins. For instance, in surficial Black Sea sediments, purified chlorins had  $\delta^{15}\text{N}$  and  $\delta^{13}\text{C}$  values ranging from -3.4 to -4.9‰ and -24.9 to -25.0‰, respectively, while the sedimentary lipid extract had a  $\delta^{15}\text{N}$  value of -2.55‰ and a  $\delta^{13}\text{C}$  value of -27.4‰ (Table 5).

The amount of purification required to obtain material with an isotopic value similar to fully purified sedimentary chlorins differed for nitrogen and carbon. A pure chlorin  $\delta^{15}\text{N}$  signature was approached in Black Sea surface sediments after preparative C<sub>18</sub> HPLC was performed, as PPTNa (-4.4‰) and PTNa (-4.6‰) were both 0.1‰ from the weighted average  $\delta^{15}\text{N}$  of fully purified chlorins (-4.5‰; Fig. 5). For carbon, all 5 purification steps were required in order to obtain  $\delta^{13}\text{C}$  values similar to fully purified chlorins. For instance, the  $\delta^{13}\text{C}$  values of the same C<sub>18</sub> HPLC-purified PPTNa (-26.7‰) and PTNa (-25.7‰) were 2.6 and 1.6‰ depleted in <sup>13</sup>C, respectively, compared to the weighted average  $\delta^{13}\text{C}$  of fully purified chlorins (-24.1‰; Fig. 5). Thus, the non-chlorin component of the lipid extract was depleted in <sup>13</sup>C and enriched in <sup>15</sup>N relative to the chlorins, and  $\delta^{15}\text{N}$  values decreased while  $\delta^{13}\text{C}$  values increased with increasing sedimentary chlorin purity.

Table 5

Isotopic ratios and elemental purities of Black Sea surficial sedimentary chlorins after sequential purification steps. Isotope ratios are in ‰ and purities are in percentage

Sample	$\delta^{15}\text{N}$	N Purity	$\delta^{13}\text{C}$	C Purity
Whole sediment	1.2		-23.3	
Lipid extract	-2.6	21	-27.4	6
SPE Chlorins	-3.6	32	-26.7	7
C <sub>18</sub> HPLC PPTNa	-4.4	33	-26.7	10
C <sub>18</sub> HPLCPTNa	-4.6	39	-25.7	18
SEC PTNa + PPTNa		60		16
SiO <sub>2</sub> chlorins <sup>a</sup>	-4.5	89	-24.1	88

<sup>a</sup>  $\delta^{15}\text{N}$  value is a weighted average for PPTNa, PTNa, PTNa', and Chl686.  $\delta^{13}\text{C}$  value is a similar weighted average, excluding PTNa', for which no  $\delta^{13}\text{C}$  measurement was available. The N and C purities are for PPTNa.

The amount of purification required for suitable particulate Chla isotopic ratios can only be estimated, since isotopic determinations were not made on the partially purified chlorin. However, since the N purity of Chla from six algal cultures averaged 90.4% after the first chromatography step (C<sub>18</sub> HPLC; Fig. 4), and Chla is 5.1‰ depleted in <sup>15</sup>N relative to total cellular nitrogen (Sachs et al., 1999), then the partially purified Chla would be enriched in <sup>15</sup>N by ca. 0.5‰ assuming the 9.6% of non-Chla N has an isotopic signature similar to total N. Results from 4 Mediterranean Sea suspended particulate samples support this estimate, and indicate that  $\delta^{15}\text{N}$  values for partially purified (i.e. C<sub>18</sub> HPLC) Chla, being  $94 \pm 11\%$  pure (Sachs, 1997), would be ca. 0.3‰ enriched in <sup>15</sup>N relative to fully purified Chla. If such tolerances are sufficient, then only the first chromatographic purification is required for  $\delta^{15}\text{N}$  determinations of particulate Chla. If, however, the final 6–10% of contaminant N has a markedly different  $\delta^{15}\text{N}$  value than total cellular N, these may be underestimates of the effect of contaminant N on C<sub>18</sub> HPLC-purified Chla.

For  $\delta^{13}\text{C}$  determinations of particulate Chla it is recommended that both chromatographic purifications (e.g. C<sub>18</sub> and SiO<sub>2</sub> HPLC) be performed. The Chla from cultured algae averaged only 51% purity for C after C<sub>18</sub> HPLC, and improved to >90% purity after SiO<sub>2</sub> HPLC (Fig. 4). Although little systematic difference between Chla and cellular  $\delta^{13}\text{C}$  was observed in batch cultures of marine phytoplankton (Sachs et al., 1999), it is widely accepted that lipids are depleted in <sup>13</sup>C relative to total algal carbon (Galimov and Shirinsky, 1975; DeNiro and Epstein, 1977). This suggests that contaminants in partially purified Chla from particulate lipid extracts may have significantly different  $\delta^{13}\text{C}$  values. In natural systems, where detritus and non-planktonic carbon can comprise large fractions of particulate C, there may be differences between the  $\delta^{13}\text{C}$  value of Chla and particles.

#### 4.3. Isotopic differences between sedimentary chlorins

Chlorins purified from surficial Black Sea sediment had different N and C isotopic ratios (Table 6) that may represent primary signatures imparted to Chla during different growing seasons, or that may result from natural decompositional processes. For instance, PPTNa was 0.9‰ depleted in <sup>15</sup>N relative to PTNa (Table 6). This difference can be explained in terms of changes in the seasonal flux of organic matter out of the euphotic zone. Most of the material flux to Black Sea sediments occurs during two annual phytoplankton blooms (Hay et al., 1990). The large dinoflagellate and diatom bloom that occurs in the spring (Hay et al., 1990) is associated with a maximum in the flux of PPTNa out of the euphotic zone (King, 1993), while the smaller coccolithophorid-dominated bloom that occurs in the fall

Table 6

N and C isotopic ratios in purified chlorins from surficial Black Sea sediments. Values are in ‰

Chlorin	$\delta^{15}\text{N}$	$\delta^{13}\text{C}$
PPTNa	−4.8	−24.9
PTNa	−3.9	−25.0
PTNa'	−3.4	
Chl686	−4.9	−23.1

(Hay et al., 1990) is associated with a maximum in the PTNa flux (King, 1993). The observation that minima in sinking particulate  $\delta^{15}\text{N}$  values coincide with maxima in mass fluxes to sediment traps in both the North Atlantic (Altabet et al., 1971; Altabet and Deuser, 1985) and the Arabian Sea (Schafer and Ittekkot, 1993) implies that the difference in Black Sea sedimentary PPTNa and PTNa  $\delta^{15}\text{N}$  values may result from deposition of these components during the spring (diatom and dinoflagellate) and fall (coccolithophorid) blooms, respectively. The large mass and PPTNa fluxes associated with the spring bloom would thus be expected to have lower  $\delta^{15}\text{N}$  values than the PTNa and smaller mass flux associated with the fall bloom. The dynamics of the two blooms thus appear to be discernible in sedimentary chlorin N isotopic ratios, suggesting the potential for reconstructing historical seasonal fluxes (and hence, paleoproductivity) in the Black Sea.

Also observed in surficial Black Sea sediments was a 0.5‰ <sup>15</sup>N depletion in PTNa relative to the diastereomer, or epimer, PTNa' (Table 6). Chlorin epimers readily arise non-enzymatically (Hynninen, 1979) during pigment handling and natural decomposition processes (Mantoura and Llewellyn, 1983). In another instance where the  $\delta^{15}\text{N}$  of the two epimers was measured — a suspended particulate sample from the deep chlorophyll maximum in the Mediterranean Sea — PTNa was depleted by 2.0‰ relative to PTNa' (Sachs, 1997). In the Black Sea sediment, the epimer accounted for 23% of the combined PTNa + a', while in the Mediterranean Sea particulate sample it accounted for 28% of total PTNa + a'. However, a strict mass balance was not possible since the amount of each compound transformed to other chlorins or to colorless products is not known. Since the C-13<sup>2</sup> diastereoisomerization of PTNa, to form 13<sup>2</sup>(S)-PTNa', is thought to result in significant additional steric strain on the chlorin macrocycle due to the cis arrangement of the C-17 propionic ester group and the carbomethoxy group at C-13<sup>2</sup> (Fig. 2) (Hynninen and Sievers, 1981), it is possible that the relief of that strain through configurational changes in the chlorin macrocycle results in nitrogen isotopic fractionation.

Lastly, a carbon isotopic depletion of 1.9‰ was observed in Chl686 (−23.1‰) relative to PTNa (−25.0‰) and PPTNa (−24.9‰) from surficial Black Sea sediment (Table 6). This difference is likely attributable to the

absence of the phytol side chain on Chl686 (Ocampo et al., in press). The phytol ester group in chlorophyll, a lipid, is expected to be depleted in  $^{13}\text{C}$  relative to protein (Galimov and Shirinsky, 1975; DeNiro and Epstein, 1977), and experimentally determined isotopic depletions of phytol are between 1.6 and 5.1‰ (Bogacheva et al., 1979). In addition, since this novel chlorin had approximately the same  $\delta^{15}\text{N}$  (−4.9‰) as PPTNa (−4.8‰), it is suggested that Chl686 is derived from PPTNa.

## 5. Conclusion

Nitrogen and carbon isotopic ratios in most common chlorins can be determined in marine particulate and sediment samples with a precision of ca. 0.2‰ for both isotopes. The entire procedure can be accomplished in about 4 h for particulate samples and 8 h for sediment samples. The chlorins thus produced from particulate samples had nitrogen purities of 96% and carbon purities of 91%. Sedimentary chlorin N and C purities were 89 and 88%, respectively. The chlorin recovery from particulate samples was 88%, while that for sediment samples was 18%. The low recovery from sediments, relative to particles, is attributed to the complex distribution of chlorins in sediments and to the additional purification steps required. Using these techniques, different chlorins in a surficial Black Sea sediment had different isotopic ratios. The  $\delta^{15}\text{N}$  variation is attributed to coincidental changes in the seasonal flux of material and certain chlorins out of the euphotic zone. The  $\delta^{13}\text{C}$  differences are likely a result of the presence or absence of the phytol side-chain in different chlorins. The measurement of stable isotopic ratios in biomarker compounds can significantly augment the environmental information obtained from bulk isotopic analyses, while circumventing any isotopic alteration caused during natural decomposition processes.

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