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Implications for chloro- and pheopigment synthesis and preservation from combined compound-specific δ^{13}C, δ^{15}N, and Δ^{14}C analysis

S. Kusch[1,2], Y. Kashiyama[3,*], N. O. Ogawa[3], M. Altabet[4], M. Butzin[1,5], J. Friedrich[1], N. Ohkouchi[3], and G. Mollenhauer[1,2]

[1]Alfred Wegener Institute for Polar and Marine Research, Am Handelshafen 12, 27570 Bremerhaven, Germany
[2]Department of Geosciences, University of Bremen, Klagenfurter Strasse, 28359 Bremen, Germany
[3]Institute of Biogeosciences, Japan Agency for Marine-Earth Science and Technology, 2-15 Natsushima-cho, Yokosuka 237-0061, Japan
[4]School for Marine Science and Technology, University of Massachusetts Dartmouth, 706 S Rodney French Boulevard, New Bedford, Massachusetts 02744, USA
[5]MARUM Center for Marine Environmental Research, Leobener Strasse, 28359 Bremen, Germany
[*]now at: Department of Chemistry, University of Tsukuba, 1-1-1 Tennodai, Tsukuba, Ibaraki 305-8571, Japan

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Abstract. Chloropigments and their derivative pheopigments preserved in sediments can directly be linked to photosynthesis. Their carbon and nitrogen stable isotopic compositions have been shown to be a good recorder of recent and past surface ocean environmental conditions tracing the carbon and nitrogen sources and dominant assimilation processes of the phytoplanktonic community. In this study we report results from combined compound-specific radiocarbon and stable carbon and nitrogen isotope analysis to examine the time-scales of synthesis and fate of chlorophyll-*a* and its degradation products pheophytin-*a*, pyropheophytin-*a*, and 13^2,17^3-cyclopheophorbide-*a*-enol until burial in Black Sea core-top sediments. The pigments are mainly of marine phytoplanktonic origin as implied by their stable isotopic compositions. Pigment δ^{15}N values indicate nitrate as the major uptake substrate but ^{15}N-depletion towards the open marine setting indicates either contribution from N_2-fixation or direct uptake of ammonium from deeper waters. Radiocarbon concentrations translate into minimum and maximum pigment ages of approximately 40 to 1200 years. This implies that protective mechanisms against decomposition such as association with minerals, storage in deltaic anoxic environments, or eutrophication-induced hypoxia and light limitation are much more efficient than previously thought. Moreover, seasonal variations of nutrient source, growth period, and habitat and their associated isotopic variability are likely at least as strong as long-term trends. Combined triple isotope analysis of sedimentary chlorophyll and its primary derivatives is a powerful tool to delineate biogeochemical and diagenetic processes in the surface water and sediments, and to assess their precise time-scales.

Correspondence to: S. Kusch
(stephanie.kusch@awi.de)

1 Introduction

In both marine and terrestrial aerobic photoautotrophic organisms the light harvesting systems are based on the tetrapyrrole molecule chlorophyll-*a* being the major antenna pigment (e.g., reviewed in Falkowski, 2003). Accordingly, chlorophyll-*a* is ubiquitously found in marine surface waters and may ultimately become buried in marine sediments. However, chlorophyll-*a* undergoes several pre- and post-depositional diagenetic processes such as (autolytic) cell senescence, microbial or viral lysis, enzymatic or hydrolysis reactions, photo-oxidation and grazing (Owens and Falkowski, 1982; Carpenter et al., 1986; Repeta and Simpson, 1991; Spooner et al., 1995; Gossauer and Engel, 1996; Chen et al., 2003) altering its original chemical structure. In particular, chlorophyll-*a* of higher plants is decomposed enzymatically during senescence of the leaves (Matile et al., 1996), by bacterial breakdown and meso- and micro-faunal

grazing in soils (Hoyt, 1966; Chamberlain et al., 2006), and by oxidation and photo-degradation during transport (Sanger, 1988). Consequently, pigments found in marine sediments are proposed to be overwhelmingly derived from marine photoautotrophs.

Primary degradation products of chlorophyll-*a* include pheophytin-*a* and pyropheophytin-*a*, formed via demetallation and demethoxycarbonylation by either of the above-mentioned pathways (Chen et al., 2003), and 13^2,17^3-cyclopheophorbide-*a*-enol which is considered an exclusive grazing product (Goericke et al., 2000). These chloro- and pheopigments have been shown to be extremely labile whereas their macrocycles can be preserved over very long time-scales (Rho et al., 1973) allowing their stable carbon and nitrogen isotopic compositions to be used to characterize past surface water environments and photoautotrophic communities (Ohkouchi et al., 2005, 2006, 2008; Kashiyama et al., 2008a, b).

Various studies have demonstrated rapid decrease of chlorophyll-*a* and pheophytin-*a* concentrations over time periods on the order of hours to days or few weeks (Owens and Falkowski, 1982; SooHoo and Kiefer, 1982; Sun et al., 1993b; Goericke et al., 2000). Carpenter et al. (1986) found very low water-column half-lives of chlorophyll-*a* and pheopigments leading to photo-degradation within only 3 days. In general, degradation rates seem to be higher for sediments. Bianchi et al. (1993) report chlorophyll-*a* mean lives of up to 80 days for Hudson River sediments and Furlong and Carpenter (1988) calculate half-lives of pheopigments to be 30 to 50 days. This is attributed to protection of the pigments by aggregation with humic substances or protection by a sediment matrix. Overall, degradation rates were enhanced under oxic compared to anoxic conditions (Sun et al., 1993a, b) causing preferential preservation of pigments such as 13^2,17^3-cyclopheophorbide-*a*-enol in anoxic sediments (Ocampo et al., 1999). Nevertheless, the degradation rates reported to date are mainly based on relative concentration estimates prone to analytical biases and can, thus, not truly reflect natural preservation time-scales.

Here we present results from combined δ^{13}C, δ^{15}N, and Δ^{14}C analysis of chlorophyll-*a* and its primary degradation products pheophytin-*a*, pyropheophytin-*a*, and 13^2,17^3-cyclopheophorbide-*a*-enol from NW Black Sea core-top sediments. The sediments underlie contrasting environmental conditions ranging from river-influenced oxic bottom waters at the mouth of the Danube River towards suboxic bottom waters near the shelf break, and anoxic bottom waters in the open-marine Black Sea (Fig. 1). This redox gradient was chosen since it should strongly influence pigment preservation and diagenesis. We aim to investigate preferential preservation processes, to precisely trace the time-scales of early pigment diagenesis under different environmental conditions, and to assess potential effects on the stable isotope record.

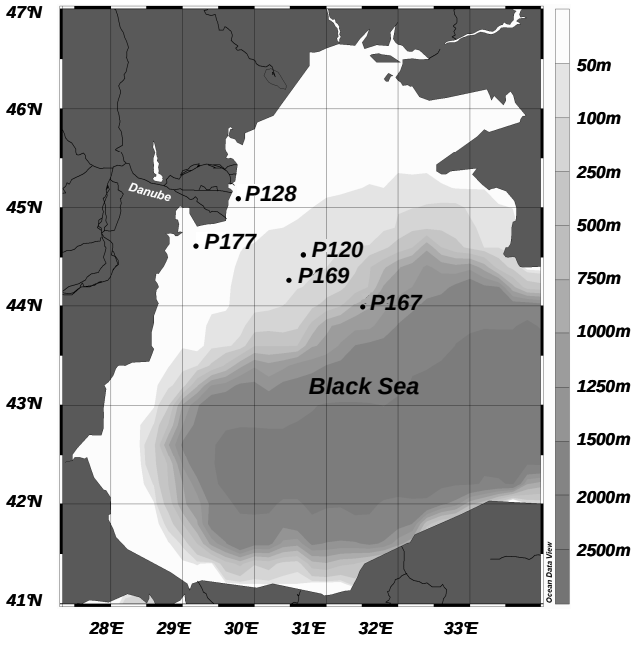

Fig. 1. Locations of sampling stations.

2 Materials and methods

2.1 Sampling

Core-top sediments from stations P128, P177, P120, P169, and P167 from the NW Black Sea (Fig. 1) were taken using a multicorer on RV *Poseidon Cruise P363* in March 2008. The samples were sliced into 1 cm slices and stored frozen at −20 °C in glass jars until analysis. The uppermost 0 to 2 cm or 0 to 3 cm slices were recombined for analysis to yield enough chloropigments for radiocarbon measurements. If available, co-occurring bivalve shells were isolated from each sample and radiocarbon dated. For TOC radiocarbon analyses, subsamples of 100 to 500 mg of the freeze-dried samples were separated. Seawater samples at the respective stations were taken using a CTD rosette equipped with 30 L Niskin bottles and poisoned using $HgCl_2$ to prevent bacterial activity.

2.2 Pigment extraction and purification

Pigments were extracted and purified as described in Tyler et al. (2010). Briefly, the freeze-dried sediment was ground and homogenized. Dehydrated acetone was added (2× sediment volume) and samples were ultrasonicated for 15 min cooled in ice water using an ultrasonic bath. Afterwards samples were centrifuged at 777 × G for 5 min. The acetone was removed and the extraction was repeated three times. After volume reduction under a cold stream of N_2, the acetone-fraction was transferred into a hexane-MilliQ bilayer (1:3), homogenized using vortex and centrifuged

at 427 × G for 1 min. The hexane-layer was transferred and the extraction procedure was repeated until the hexane-fraction was colorless. The hexane-fraction was then reduced in volume under cold N_2 and rinsed through a column of pre-combusted sodium sulphate. Afterwards dehydrated dimethylformamide (DMF) was added and solvents were homogenized by vortex before storing at −30 °C overnight. For HPLC-analysis the DMF layer was separated and analysed.

The HPLC-analysis was performed using an Agilent 1200 Series HPLC/DAD System. For isolation of the chloro- and pheopigments from the extract, a semi-preparative Agilent Zorbax Eclipse XDB C18 column (9.4 × 250 mm; 5 µm) with a XDB C18 guard column (4.6 × 12.5 mm; 5 µm) were used. The chloro- and pheopigments were eluted isocratically with 75% acetonitrile/pyridine (100:0.5) and 25% ethyl acetate/pyridine (100:0.5) for 5 min, followed by a linear gradient of ethyl acetate/pyridine to 50% in 50 min. The flow rate was set to 4.2 ml min^{-1} and the oven temperature to 30 °C. Detection of the chloro- and pheopigments was achieved by a photodiode array detector.

To achieve high single pigment purities, a second purification step was applied using an analytical Agilent Zorbax Eclipse PAH column (4.6 × 250 mm; 5 µm) equipped with a PAH guard column (4.6 × 12.5 mm; 5 µm). Isocratic elution of the pigments was achieved using 80% acetonitrile/pyridine (100:0.5) and 20% 2-butanone/pyridine (100:0.5) for 5 min, followed by two linear gradients of 2-butanone/pyridine (100:0.5), first to 60% in 25 min and second to 100% in 10 min. The flow rate was 1 ml min^{-1}. The oven temperature was 10 °C for chlorophyll-*a* and pheophytin-*a* and 30 °C for pyropheophytin-*a* and 13^2,17^3-cyclopheophorbide-*a*-enol.

HPLC processing blanks for each pigment methodology were determined by collection of the respective effluent volume. The solvent was dried using N_2 and the blank size (Supplement Table S1) and ^{13}C (−29.6‰), ^{15}N (5.1‰), and ^{14}C (−1000‰) isotopic compositions were determined. All $\Delta^{14}C$ concentrations reported are corrected for blank carbon addition using isotope mass balance.

2.3 Isotope measurements

The stable carbon and nitrogen isotopic compositions of chloro- and pheopigments were measured on a modified FlashEA1112 Automatic Elemental Analyser connected to a Thermo Finnigan Delta plus XP isotope ratio mass spectrometer (IRMS) via a ConFlo III Interface (Ogawa et al., 2010). Seawater nitrate, chemically converted to N_2O (McIlvin and Altabet, 2005), was measured for its $\delta^{15}N$ composition using an automated purge-and-trap system connected to a GV Instruments IsoPrime system. The stable carbon and nitrogen isotopic compositions of bulk sediment was analysed using a CE Elemental Analyser coupled with a Finnigan Delta plus IRMS via a ConFlo II Interface. The isotopic compositions are expressed as conventional $\delta^{13}C$ relative to Vienna PeeDee Belemnite (VPDB) and $\delta^{15}N$ relative to atmospheric N_2.

For radiocarbon measurements, chloro- and pheopigments, and bivalve shells were converted into CO_2. Cleaned bivalves were acid-hydrolyzed. Pigment samples were transferred into pre-combusted quartz tubes and 150 µg pre-combusted copper oxide (CuO) was added as oxygen source. The samples were evacuated and flame-sealed (H_2 jet burner) under vacuum as described in Mollenhauer and Rethemeyer (2009). Afterwards, the samples were combusted at 900 °C for 8 h. The resulting CO_2 gas was stripped of water and quantified under vacuum.

TOC was submitted as unprocessed samples and AMS measurements were performed using standard methods including acidification, combustion, and graphitization (McNichol et al., 1994). AMS measurements of chloropigment isolates were performed following the protocol for small samples (Pearson et al., 1998). Where sample sizes were sufficient, dual measurements were obtained. Radiocarbon ages are reported as $\Delta^{14}C$ (Stuiver and Polach, 1977). Additionally, calibrated radiocarbon ages are given for pigments for determination of their "true" age and calculation of degradation time-scales. Calibration of pre-bomb samples was achieved using the calibration software CALIB 6.0 (Stuiver et al., 2010) applying a reservoir correction of 400 years (Siani et al., 2000). The "true" age of bomb-^{14}C containing pigments was determined using a modeled surface water $DI^{14}C$ curve.

2.4 Black Sea surface water DIC model

The history of Black Sea surface water ^{14}C concentrations after AD 1950 is poorly constrained by observations. For this reason the temporal evolution of surface water $\Delta^{14}C$ was estimated by means of a heuristic one-dimensional model to assess the "true" ages of bomb-^{14}C containing pigments. Following Butzin and Roether (2004), the input of ^{14}C via air-sea exchange and river runoff is balanced by radioactive decay, downward mixing, and by an additional first-order apparent loss or gain term which parameterizes up- or downwelling and net horizontal exchange. The temporal evolution of surface water $\Delta^{14}C$ is then given by

$$\frac{\partial {}^{14}R_{\mathrm{wat}}}{\partial t} = K\frac{\partial {}^{214}R_{\mathrm{wat}}}{\partial z^2} - (\lambda+\mu){}^{14}R_{\mathrm{wat}} \quad (1)$$

where ${}^{14}R_{\mathrm{wat}}$ is the scaled surface water $^{14}C/^{12}C$ ratio at a given time t (following Toggweiler et al., 1989), K is an apparent vertical diffusivity, z is water depth, λ is the decay rate of ^{14}C ($= 1.2096 \times 10^{-4}\,\mathrm{yr}^{-1}$) (Godwin, 1962). The free parameters K (an apparent vertical diffusivity) and μ (the proportionality or time constant of the apparent source (sink) term) are used to fit the model to the observations. The model input are ^{14}C fluxes representing air-sea exchange (${}^{14}F_{\mathrm{air}}$)

and continental river runoff ($^{14}F_{riv}$). Air-sea exchange is estimated as

$$^{14}F_{air} = \Phi\ pCO_2/\Sigma CO_2\ (^{14}R_{atm} - {}^{14}R_{wat}) \quad (2)$$

where Φ is the average invasion rate of CO_2 ($=20\,mol\,m^{-2}\,yr^{-1}/330\,ppm$) (Broecker et al., 1985), pCO_2 is the partial pressure of atmospheric CO_2 (Enting et al., 1994), ΣCO_2 is the average concentration of dissolved inorganic carbon in surface water ($=2\,mol\,m^{-3}$), and $^{14}R_{atm}$ is the atmospheric $^{14}C/^{12}C$ ratio in the Northern Hemisphere (Enting et al., 1994; Levin et al., 2008). The ^{14}C delivery due to river runoff is crudely estimated as

$$^{14}F_{riv} = V_{run}/A\,^{14}R_{riv} \quad (3)$$

where V_{run} is the runoff to the Black Sea ($=350\,km^3\,yr^{-1}$) (e.g., Tolmazin, 1985), A is the surface area of the Black Sea ($=420\,000\,km^2$), and $^{14}R_{riv}$ is the fluvial $^{14}C/^{12}C$ ratio. As observations of $^{14}R_{riv}$ were not available, $^{14}R_{riv} = {}^{14}R_{atm}$ was assumed, i.e., atmospheric ^{14}C deposited in catchment areas enters the Black Sea instantaneously and without dilution. While this is clearly an overestimation, the contribution of $^{14}F_{riv}$ amounts only about 1‰ of the atmospheric input flux. Equation (1) is numerically integrated from year 1810 to 2010 and evaluated at 20 m depth since highest chlorophyll-*a* concentrations have been found between 10 m and 30 m water depth (Krupatkina et al., 1991; Repeta and Simpson, 1991; Chu et al., 2005). The solution is fitted to scattered observations (Ostlund and Dryssen, 1986; Jones and Gagnon, 1994; Siani et al., 2000; Fontugne et al., 2009; this study) using K and μ as fit coefficients. Values of K and μ are about $7 \times 10^3\,m^2\,yr^{-1}$ ($\sim 2\,cm^2\,s^{-1}$) and $\sim 1 \times 10^{-3}\,yr^{-1}$, respectively.

The model results indicate a rapid increase in surface water $\Delta^{14}C$ during the 1960's and a slow decrease since about 1970, respectively (Supplement Table S2; see also Fig. 4). The model curve reflects the spike of atmospheric nuclear weapons testing in the early 1960's and the comparatively small ^{14}C input during more recent times.

3 Results

3.1 Stable carbon and nitrogen isotopic compositions

Bulk sedimentary $\delta^{13}C$ values increase from -26.1 ± 0.1‰ (P128) to -23.2 ± 0.1‰ (P167) with distance seawards, which is in agreement with an increasing contribution of marine production at the offshore locations (Table 1, Fig. 2). In contrast, $\delta^{13}C$ values of chloro- and pheopigments range from -24.5 ± 0.1‰ to -26.7 ± 0.1‰ without a distinct spatial pattern. Pigment isotopic values within samples are heterogeneous covering a ~2‰ range except for P128, where all pigments have virtually identical $\delta^{13}C$ values. The $\delta^{13}C$ values are in the range previously reported

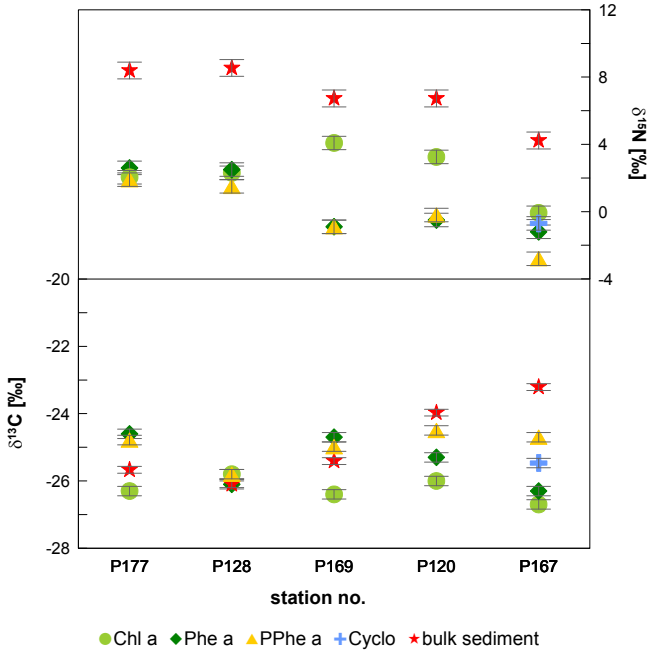

Fig. 2. Stable nitrogen and carbon isotopic compositions ($\delta^{13}C$ and $\delta^{15}N$) of purified chloro- and pheopigments Error bars give 1σ-analytical uncertainty. Chl-*a*: chlorophyll-*a*, Phe-*a*: pheophytin-*a*, PPhe-*a*: pyropheophytin-*a*, Cyclo: 13^2,17^3-cyclopheophorbide-*a*-enol.

for marine phytoplankton (Sachs et al., 1999). The pigment $\delta^{15}N$-values range from 4.0 ± 0.4‰ to -2.8 ± 0.4‰. Generally, chlorophyll-*a* is the most ^{15}N-enriched whereas pyropheophytin-*a* is the most ^{15}N-depleted pigment within each sample. Along-transect data reveal a gradual isotopic depletion (~4‰) with increasing distance from the river mouth. Only chlorophyll-*a* $\delta^{15}N$ values at stations P120 and P169 deviate from this trend. The general trend is, moreover, consistent with a steady decrease of bulk sedimentary $\delta^{15}N$ values (ranging from 8.5 ± 0.5‰ to 4.2 ± 0.5‰) with distance offshore, which is in agreement with previously reported data (Reschke et al., 2002). Seawater nitrate (NO_3^-) $\delta^{15}N$ values from 0 m to 92 m water depth range from 4.9 ± 0.2‰ to 8.4 ± 0.2‰ (Table 2).

3.2 Radiocarbon composition

TOC radiocarbon concentrations (Table 1, Fig. 3) are lowest at stations P128 (-166.6 ± 2.1‰) and P177 (-161.0 ± 3.4‰) at the mouth of the Danube River. At these stations contribution of pre-aged (reworked) terrigenous organic matter is likely (Kusch et al., 2010). With further distance offshore $\Delta^{14}C$ values increase to -64.2 ± 3.2‰ (P120) and -51.2 ± 3.2‰ (P169) and show bomb-^{14}C contribution (44.5 ± 2.5‰) at anoxic core location P167.

Table 1. Blank-corrected radiocarbon (Δ^{14}C) and stable isotopic compositions (δ^{13}C and δ^{15}N) of purified chloro- and pheopigments, bivalve shells, total organic carbon (TOC), and bulk sediment. Where sample sizes were sufficient, samples were split prior to ^{14}C analysis. Errors are given as 1σ analytical uncertainties. n.d. = not determined.

sample	δ^{13}C [‰][a]	δ^{15}N [‰][a]	Δ^{14}C [‰][a]	cal year AD[b]	ID No.[c]
P128 (45°04.69′ N, 29°46.64′ E; 17 m b.s.l.) 0–2 cm					
chlorophyll-*a*	−25.8 ± 0.1	2.1 ± 0.4	n.d.		
pheophytin-*a*	−26.1 ± 0.1	2.5 ± 0.4	−127.9 ± 11.9	1230 ± 105	70 736
pheophytin-*a*	−26.1 ± 0.1	2.5 ± 0.4	−109.3 ± 11.6	1380 ± 70	70 737
pyropheophytin-*a*	−25.8 ± 0.1	1.5 ± 0.4	32.9 ± 12.8	1963 ± 1/2009 ± 6	70 738
Scarpharca sp.	−1.1 ± 0.1		56.1 ± 3.3		70 752
TOC 0–1 cm[d]	−25.7 ± 0.1		−166.6 ± 2.1		66 558
bulk sediment	−26.1 ± 0.1	8.5 ± 0.5			
P177 (44°35.76′ N, 29°11.43′ E; 22 m b.s.l.) 0–3 cm					
chlorophyll-*a*	−26.3 ± 0.1	2.0 ± 0.4	−183.2 ± 15.3	690 ± 160	71 683
pheophytin-*a*	−24.6 ± 0.1	2.6 ± 0.4	−6.6 ± 18.1	1962 ± 2	70 746
pyropheophytin-*a*	−24.8 ± 0.1	1.9 ± 0.4	−55.8 ± 12.9	1950 ± 1	70 747
Abra fragilis	−0.6 ± 0.1		54.0 ± 3.3		70 754
TOC 0–1 cm[d]	−25.2 ± 0.1		−161.0 ± 3.4		66 564
TOC 2–3 cm	−24.7 ± 0.1		−103.4 ± 4.2		71 682
bulk sediment	−25.7 ± 0.1	8.4 ± 0.5			–
P120 (44°30.66′ N, 30°40.93′ E; 91 m b.s.l.) 0–3 cm					
chlorophyll-*a*	−26.0 ± 0.1	3.1 ± 0.4	−36.7 ± 15.5	1959 ± 2	71 685
pheophytin-*a*	−25.3 ± 0.1	−0.5 ± 0.4	−50.6 ± 7.6	1956 ± 2	70 748
pyropheophytin-*a*	−24.5 ± 0.1	−0.2 ± 0.4	6.2 ± 12.4	1962 ± 1	70 749
TOC 0–1 cm[d]	−23.7 ± 0.1		−64.2 ± 3.2		70 734
TOC 2–3 cm	−23.6 ± 0.1		−209.7 ± 3.2		71 681
bulk sediment	−24.0 ± 0.1	6.7 ± 0.5			
P169 (44°15.48′ N, 30°29.19′ E, 96 m b.s.l.) 0–3 cm					
chlorophyll-*a*	−26.4 ± 0.1	4.0 ± 0.4	181.1 ± 17.1	n.d.	71 684
pheophytin-*a*	−24.7 ± 0.1	−0.9 ± 0.4	21.7 ± 14.3	1963 ± 1	70 750
pyropheophytin-*a*	−25.0 ± 0.1	−0.9 ± 0.4	n.d.		
Modiolus sp.	0.9 ± 0.1		9.9 ± 3.8		70 753
TOC 0–1 cm[d]	−23.4 ± 0.1		−51.2 ± 3.2		70 735
bulk sediment	−25.4 ± 0.1	6.7 ± 0.5			
P167 (43°58.88′ N, 31°30.83′ E; 1336 m b.s.l.) 0–2 cm					
chlorophyll-*a*	−26.7 ± 0.1	−0.1 ± 0.4	−34.1 ± 16.5	1959 ± 3	70 743
chlorophyll-*a*	−26.7 ± 0.1	−0.1 ± 0.4	19.9 ± 13.2	1963 ± 1	70 751
pheophytin-*a*	−26.3 ± 0.1	−1.2 ± 0.4	13.6 ± 12.2	1963 ± 1	70 740
pheophytin-*a*	−26.3 ± 0.1	−1.2 ± 0.4	1.7 ± 9.2	1962 ± 1	70 741
pyropheophytin-*a*	−24.7 ± 0.1	−2.8 ± 0.4	105.5 ± 18.0	1969 ± 3/1981 ± 6	70 742
13^2,17^3-cyclopheophorbide-*a*-enol	−25.5 ± 0.1	−0.7 ± 0.4	48.3 ± 12.2	1964 ± 1/2002 ± 6	70 744
13^2,17^3-cyclopheophorbide-*a*-enol	−25.5 ± 0.1	−0.7 ± 0.4	−7.1 ± 8.3	1962 ± 1	70 745
TOC 0–1 cm[d]	−24.6 ± 0.1		44.5 ± 2.5		66 557
bulk sediment	−23.2 ± 0.1	4.2 ± 0.5			

[a] Blank-corrected.
[b] Calendar year AD calibrated using CALIB 6.0 for years <AD 1950 and modelled surface DI^{14}C values for years ≥AD 1950.
[c] NOSAMS ID number.
[d] Data from Kusch et al. (2010).

Table 2. Seawater chemical properties and nitrate nitrogen isotopic composition. n.d. = not determined.

sample	water depth [m]	temperature [°C]	salinity	O_2 concentration [µmol l^{-1}]	NO_3^- concentration [µmol l^{-1}]	$\delta^{15}N\ NO_3^-$ [‰]
P128	1.5	7.5	15.3	368	8.4	8.2 ± 0.2
						7.2 ± 0.2
	15	6.3	18.0	307	0.3	4.9 ± 0.2
P177	6	6.9	16.8	359	2.3	8.1 ± 0.2
	12	6.8	16.8	360	2.3	8.0 ± 0.2
	22	6.2	17.1	330	3.0	8.4 ± 0.2
						7.0 ± 0.2
P120	5	8.1	18.0	324	0.2	n.d.
	40	7.9	18.0	320	0.3	n.d.
	68	7.3	18.2	303	0.5	n.d.
	88	7.4	18.3	292	0.5	n.d.
P169	5	8.1	17.7	333	0.2	n.d.
	75	7.5	18.3	281	1.1	4.9 ± 0.2
	92	8.1	19.1	142	1.9	5.0 ± 0.2
P167	2	8.3	17.2	341	0.2	n.d.
	25	8.1	18.1	322	0.0*	7.2 ± 0.2
	52	7.2	18.5	321	0.2	n.d.
	70	8.1	19.3	113	1.1	7.7 ± 0.2
	150	8.5	21.1	4	0.0	n.d.
	500	8.9	22.0	0	0.0	n.d.

* This measurement is considered prone to an analytical artifact.

All bivalve shell $\Delta^{14}C$ values are bomb-influenced and range from 56.1 ± 3.3‰ at the shallowest nearshore station (P128, *Scarpharca* sp.) to 9.9 ± 3.8‰ further offshore (P169, *Modiolus* sp.), which is consistent with utilization of a more ^{14}C-depleted dissolved inorganic carbon (DIC) pool with increasing water depth.

Chloro- and pheopigment $\Delta^{14}C$ values (Table 1, Fig. 3) vary over a range of ∼290‰, overall showing increasing $\Delta^{14}C$ values with distance from the river mouth. For chloro- and pheopigments dual ^{14}C measurements were performed where sample sizes were sufficient. With respect to the uncertainties associated with sample preparation, combustion backgrounds, and analytical errors of the AMS measurement, we consider differences of the reported ^{14}C concentrations insignificant if they are within 2σ-analytical uncertainties.

Lowest $\Delta^{14}C$ values are found for pheophytin-*a* at station P128 (−127.9 ± 11.9‰ and −109.3 ± 11.6‰, i.e., agree within 1σ-analytical uncertainty) and chlorophyll-*a* at station P177 (−183.2 ± 15.3‰) at the mouth of the Danube River. However, in both samples the other pigments are significantly less depleted in ^{14}C (>120‰). Pyropheophytin-*a* at station P128 contains bomb-^{14}C (32.9 ± 12.8). Likewise, $\Delta^{14}C$ values of pheophytin-*a* (−6.6 ± 18.1‰) and pyropheophytin-*a* (−55.8 ± 12.9‰) at station P177 are notably increased relative to chlorophyll-*a* at this station.

At intermediate station P120 pheophytin-*a* (−50.6 ± 7.6‰) and chlorophyll-*a* (−36.7 ± 15.5‰) agree within 1σ-analytical uncertainty. Pyropheophytin-*a* from the same sample, however, shows bomb-^{14}C contribution (32.9 ± 12.8‰). Chlorophyll-*a* at station P169, in similar water depth as P120, shows the most enriched $\Delta^{14}C$ value of the data set (181.1 ± 17.1‰). Co-occurring pyropheophytin-*a* (21.7 ± 14.3‰) is significantly less enriched in ^{14}C.

At anoxic station P167 all pigments investigated show incorporation of bomb-^{14}C relative to the pre-bomb surface water DIC ^{14}C concentration of the Black Sea (Fig. 3). Overall, $\Delta^{14}C$ values show a range of ∼140‰. Except for pyropheophytin-*a*, which shows the highest bomb-^{14}C incorporation (105.5 ± 18.0‰) at this station, dual measurements could be performed on all isolated pigments from this sample. Dual measurements of pheophytin-*a* (13.6 ± 12.2‰ and 1.7 ± 9.2‰) are within 1σ-analytical uncertainty. Chlorophyll-*a* from this station yields $\Delta^{14}C$ values of 19.9 ± 13.2‰ and −34.1 ± 16.5‰, agreeing within 2σ-analytical uncertainty. In contrast, at this station $\Delta^{14}C$ values of 13^2,17^3-cyclopheophorbide-*a*-enol (48.3 ± 12.2‰ and −7.1 ± 8.3‰) are significantly different (>2σ-uncertainty).

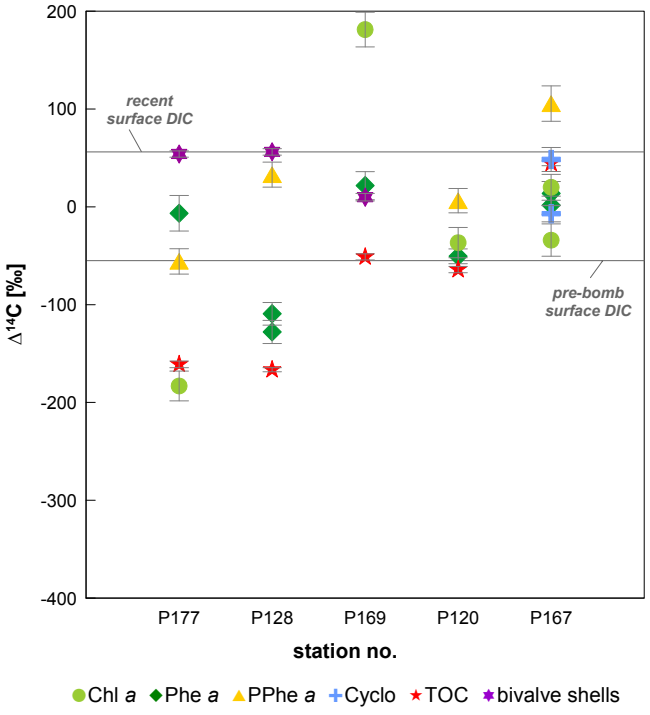

Fig. 3. Radiocarbon concentrations ($\Delta^{14}C$) of purified chloro- and pheopigments. Error bars denote 1σ analytical uncertainties. Chl-*a*: chlorophyll-*a*, Phe-*a*: pheophytin-*a*, PPhe-*a*: pyropheophytin-*a*, Cyclo: 13^2,17^3-cyclopheophorbide-*a*-enol.

4 Discussion

4.1 Chloro- and pheopigment origin and biogeochemical C and N cycle of the prevailing photoautotrophs

The stable carbon and nitrogen isotopic compositions of primary pigments reflect those of the substrates, the assimilation pathway, and the growth conditions of the photoautotrophic community (Sachs et al., 1999; Ohkouchi et al., 2005, 2006, 2008; Kashiyama et al., 2008a, b). Empirically it has been shown that chlorophyll-*a* is enriched in ^{13}C and depleted in ^{15}N relative to total cellular carbon and nitrogen by 1.8 ± 0.8‰ and 4.8 ± 1.4‰, respectively (Sachs et al., 1999; Ohkouchi et al., 2006, 2008). Therefore, after accounting for fractionation, our data imply that mean $\delta^{13}C_{cell}$ values of the phytoplanktonic community (-26.3 ± 0.8‰ to -28.5 ± 0.8‰) are in agreement with previous estimates of surface water particulate organic matter from the NW Black Sea shelf area (Banaru et al., 2007) and values reported for marine phytoplanktonic chloropigments (Sachs et al., 1999). Although similar values have been reported for terrestrial plants (Kennicutt et al., 1992), we consider their contribution generally to be minor due to the manifold degradation processes occurring on land and during transport (e.g., Hoyt, 1966; Sanger, 1988; Matile et al., 1996; Chamberlain et al., 2006).

It is obvious that the $\delta^{13}C$ values of all pigments except those at station P128 show a ~2‰ scatter, which would not be expected if the pheopigments were entirely produced by decomposition of one homogenous chlorophyll-*a* pool. It has to be considered that in natural systems isotope values always represent mixtures of a continuum of (potentially superimposed) isotope values present in a certain biomarker sample. Therefore, measured pigment isotope values result from the fractional contributions of all pigment molecules present in a particular biomarker fraction, which might be produced at different times within the interval during which the sediments investigated were deposited. The $\delta^{13}C$ scatter might, therefore, be due to growth season effects (Sachs et al., 1999) if the pigments derived from different phytoplankton blooms (including different species) occurring at different seasons associated with a seawater DIC ^{13}C isotopic variability. Indeed, in the Black Sea the phytoplankton growth seasons are coupled with spatially and temporarily varying pigment fluxes (King, 1995), and seasonal variations in DIC $\delta^{13}C$ could, e.g., be driven by decreasing carbon isotopic fractionation during times of high productivity and low surface water $CO_{2(aq)}$ concentrations (Gruber et al., 2002) or admixture of seasonally varying riverine DIC $\delta^{13}C$ (Kanduć et al., 2007). In case of 13^2,17^3-cyclopheophorbide-*a*-enol (only detectable at anoxic station P167) an additional isotopic effect could be the cleavage of the phytol side chain, which is normally more ^{13}C-depleted than the tetrapyrrole macro cycle (Sachs et al., 1999; Ohkouchi et al., 2008). For this latter effect Ohkouchi et al. (2008), for example, report an average ^{13}C-enrichment of 1.8‰ for chlorophyllide-*a* compared to chlorophyll-*a*. However, such a process cannot explain the observed ~2‰ ^{13}C-enrichment of pyropheophytin-*a* relative to chlorophyll-*a* and pheophytin-*a* at station P167. Therefore, only seasonal isotopic changes in the C source (DIC) may explain the observed variability for all investigated pigments.

Fractionation-corrected $\delta^{15}N_{cell}$ values range from approximately 8.8 ± 1.5‰ to 2.0 ± 1.5‰ in very good agreement with bulk sedimentary $\delta^{15}N$ (8.5 ± 0.5‰ to 4.2 ± 0.5‰). Nitrogen isotopic variations within each sample and especially the noticeable ^{15}N-enrichment of chlorophyll-*a* at stations P120 and P169 could be caused by seasonal variations of the nitrogenous nutrient sources similar to above assumptions about seasonality. Chlorophyll-*a* produced at the end of a phytoplanktonic bloom limited by nitrogen would result in ^{15}N enrichment of the substrate as the source becomes depleted and, thus, pigments would reflect such enriched $\delta^{15}N$ values (Altabet and Francois, 1994).

Since chlorophyll-*a* $\delta^{15}N_{cell}$ values (8.8 ± 1.5‰ to 4.7 ± 1.5‰) at all stations show good agreement with the respective seawater nitrate $\delta^{15}N$ values (Table 2), nitrate must be the major substrate assimilated by the phytoplanktonic community (presuming a high NO_3^- uptake/availability ratio). It has to be considered that seawater nitrate $\delta^{15}N$ values

reflecting a seasonal signal during the cruise (March, 2008) are compared with sedimentary pigment δ^{15}N values integrating over several years ($\sim$1 to 8 years depending on the sedimentation rates). The slight seawater δ^{15}N enrichment as compared to other open ocean settings (Liu and Kaplan, 1989; Brandes et al., 1998; Sigman et al., 2000, 2005) is coincident with low nitrate concentrations (Table 2) suggesting that the isotopic enrichment is caused by nitrate depletion by blooming phytoplankton. Additionally, the enrichment of seawater nitrate δ^{15}N values can also derive from input of ^{15}N-enriched agricultural nitrate from the Danube River as implied by the high nitrate concentrations at the mouth of the Danube River (P128 and P177, Table 2). Nevertheless, the good spatial agreement of seawater nitrate δ^{15}N values of river mouth and offshore study sites indicates an isotopically homogenous nitrate pool. Therefore, an overall trend of pigment ^{15}N-depletion with distance offshore indicates that distinct groups of phytoplankton using different nitrogen assimilation processes occur in the surface waters of the study sites. Enriched δ^{15}N values at the mouth of the Danube River imply predominant NO_3^- assimilation, whereas more depleted values towards the central basin may result from contribution of pigments derived from diazotrophs with typical stable nitrogen isotopic composition in the range of -3‰ to 0‰ (Minagawa and Wada, 1986; Montoya et al., 2002). Such contribution from N_2-fixing biomass is consistent with the nutrient availability observed during the sampling campaign P363 in March 2008. In front of the Danube delta observed nitrogenous nutrient supply was high enough (N:P > 80) to maintain nitrate assimilation; here phosphorus is the limiting nutrient in agreement with previous observations (Cauwet et al., 2002). In contrast, further offshore towards station P167 nitrogen became limiting over phosphorus (N:P < 10). Since the anoxic water body of the Black Sea is devoid of nitrate, the only source of nitrate is the suboxic layer where nitrate δ^{15}N values are highly enriched (e.g., Murray et al., 2005; Fuchsman et al., 2008). Such ^{15}N-enrichment would also be reflected in enriched pigment δ^{15}N values if nitrate supplied from the suboxic zone was assimilated by the photoautotrophs. In fact, the observed effect is opposite (^{15}N-depletion). The permanent pycnocline of the Black Sea may prevent effective upward mixing of nitrate from deeper, suboxic waters. Thus, in the central basin of the Black Sea N_2-fixation by cyanobacteria (Uysal, 2000, 2006) might account for a significant fraction of total nitrogen assimilation in surface waters. Various lineages of the cyanobacterium *Synechococcus* have previously been reported to be capable of N_2-fixation (e.g., Huang et al., 1988; Ikemoto and Mitsui, 1994). Indeed, in the Black Sea *Synechococcus* sp. cell abundances were reported to increase from the mouth of the Danube River towards the central basin (Uysal, 2006) likely providing increasing contributions of a diazotrophic nitrogen source with distance offshore as implied by the pigment δ^{15}N data. Although in situ evidence for N_2-fixation is yet missing, it was proposed to be an input source necessary to balance the Black Sea N cycle (e.g., McCarthy et al., 2007; Fuchsman et al., 2008).

Alternatively, the observed ^{15}N-depletion of the pigments at the offshore stations may be explained by contribution of pigments derived from phytoplankton assimilating ammonium (NH_4^+) directly supplied from deeper waters. Previously reported ammonium δ^{15}N values range from approximately 6 to 8‰ in the suboxic zone and from around 3 to 4‰ in the upper anoxic water column of the Black Sea (Velinsky et al., 1991). Considering the isotopic fractionation of ammonium during uptake by phytoplankton (Pennock et al., 1996) and the isotopic fractionation during pigment synthesis, the expected pigment δ^{15}N values can be estimated to range from roughly -5 to -29‰. Unfortunately, little is known about the supply of ammonium from deeper suboxic or anoxic waters, but Kuypers et al. (2003) suggest that likely all ammonium diffusing up into the suboxic zone may be oxidized by anaerobic ammonium oxidation. Based on our pigment isotope data and the poor information about the potential role of N_2-fixation and direct assimilation of ammonium in the euphotic zone of the Black Sea, we cannot ascribe a higher probability to either of the two processes.

4.2 Time-scales of chloro- and pheopigment synthesis and assessment of controlling processes

Chlorophyll-*a* is generally expected to rapidly decompose after cell death. A sediment trap study in the Black Sea showed that the flux of chlorophyll-*a* to a water depth of 100 m is only 0.2% of the production in 0 to 55 m water depth (Repeta and Simpson, 1991). Due to this instability of primary pigments, the Δ^{14}C concentrations of pigments deposited in surface sediments would be expected to mirror recent surface water DIC ^{14}C concentrations. Instead, ^{14}C concentrations of chlorophyll-*a* (-183.2 ± 15.3‰) and pheophytin-*a* (-127.9 ± 11.9‰ and -109.3 ± 11.6‰) at stations P177 and P128, respectively, are much lower than DIC Δ^{14}C values represented by co-occurring bivalves from 17 m (56.1 ± 3.3‰) to 96 m water depth (9.9 ± 3.8‰) and previously reported values (Jones and Gagnon, 1994; Fontugne et al., 2009), translating into ages of 570 to 1260 cal yrs BP (Table 1). Potential explanations for the low Δ^{14}C concentrations include utilization of different DIC pools, protective mechanisms associated with sedimentary matrices or faecal pellets, and contribution of pre-aged terrestrial pigments.

Although the Danube River catchment is geologically characterized by large areas of carbonaceous rocks, we regard the old pigment ages at stations P177 and P128 unlikely an artefact of riverine hardwater DIC utilization by marine phytoplankton for the following reasons. At both stations corresponding bivalve shells, which were produced in shallow water depths (P128 at 17 m; P177 at 22 m) in agreement with subsurface chlorophyll-*a* maximum depths (Chu et al., 2005), show bomb-^{14}C concentrations, thus, unambiguously reflecting a modern DIC pool. Potential ^{14}C

isotopic differences related to different DIC species ($CO_{2(aq)}$, HCO_3^-, and CO_3^{2-}) utilized by phytoplankton and bivalves are, in general, considerably less pronounced (Zeebe and Wolf-Gladrow, 2001) compared to the >150‰ difference between the bivalves and the old pigments. An additional reason for excluding a hardwater effect is the pigments' ^{14}C isotopic difference at each river mouth. Phytoplanktonic utilization of hardwater DIC would result in depleted ^{14}C concentrations of all analysed pigments, but pheophytin-*a* and pyropheophytin-*a* at P177, and pyropheophytin-*a* at P128 reflect post-bomb ^{14}C concentrations, i.e., are not influenced by ^{14}C-depleted DIC. Moreover, the riverine nutrient input favours large marine phytoplanktonic blooms at the river mouth producing high amounts of marine biomass (Cociasu et al., 1996). Thus, ^{14}C concentrations of pigments synthesized by riverine freshwater organisms, which might indeed be influenced by Danube hardwater DIC, should likely be obscured.

Aggregation with humic substances or protection by sediment matrices in general has previously been proposed to improve the preservation potential of pigments (Bianchi et al., 1993). Adsorption might occur both as surface monolayer and inside mineral pores large enough for pigments protecting them from photo-oxidation and hydrolytic enzymes (Mayer, 1994). Adsorption appears likely because of very high organic carbon/surface area ratios found for sediment water interface samples of the Black Sea (Mayer, 1994). The sediment discharge from the Danube River (Jaoshvili, 2002) should provide minerals appropriate for this process to act effectively either during water column transit or immediately after initial sedimentation. A similar protective mechanism might be the packaging of pigments into dense and rapidly sinking faecal pellets after grazing by herbivores (Taguchi et al., 1993), which are quickly exported from the photic zone protecting pigments from photo-oxidation. Such a process, however, would only account for the preservation of digestive pigments like pheophytin-*a*, pyropheophytin-*a*, and 13^2,17^3-cyclopheophorbide-*a*-enol but not for the original chlorophyll-*a*. Chloro- and pheopigments protected by either of the above ways may afterwards be carried into younger sediments through bioturbation. However, such process should not discriminate against pheophytin-*a*, which is significantly younger than chlorophyll-*a* at both stations, and therefore we consider this process not the sole control on the observed ^{14}C-depleted chlorophyll-*a* and pheophytin-*a*.

Contribution of terrestrial biomass as indicated by low TOC and leaf-wax lipid (Kusch et al., 2010) radiocarbon concentrations, could account for older chloro- and pheopigments, if intact leaf fragments or antenna proteins of their photosystems, respectively, have been sequestered on land before delivery to the Black Sea. Such sequestration might occur in the Danube Delta, which is a periodically flooded wetland area characterized by interconnected lakes and ponds. A flood event, as recurrently occurring (e.g., Bakker, 2007; Jugara Tiron et al., 2009), or dredging processes may release plant leaves previously preserved in anoxic lake sediments preventing degradation. Contribution from pre-aged intact leaf fragments could also explain why only chlorophyll-*a* and pheophytin-*a* are noticeably depleted in ^{14}C whereas pyropheophytin-*a* is enriched in most samples, and why only those stations immediately in front of the river mouth are affected. Both chlorophyll-*a* and pheophytin-*a* are primary pigments in the photosystem II (although the latter in small amounts) and, thus, would be the only pigments present in preserved intact leaves or leaf fragments. However, we did only find negligible amounts of chlorophyll-*b*, which is abundantly present in the photosystems of terrestrial plants. Furthermore, the southward deflection of Danube River sediments inhibits large scale dispersal of terrigenous organic matter across the NW Black Sea shelf (Panin and Jipa, 2002) and restricts the deposition of terrestrial organic matter (including pigments) to the river mouth. Thus, we consider the contribution of pre-aged intact chlorophyll-protein complexes within leaves or leaf fragments the most likely explanation for the ^{14}C-depleted chlorophyll-*a* and pheophytin-*a* at stations P177 and P128, respectively.

Except for chlorophyll-*a* and pheophytin-*a* at stations P177 and P128, respectively, all investigated pigments contain bomb-^{14}C, i.e., they are enriched relative to the pre-bomb surface water DIC ^{14}C concentration of −54.8‰ (Jones and Gagnon, 1994). Thus, they were synthesized after AD 1950 (Stuiver and Polach, 1977). Accordingly, pigment ages of bomb-^{14}C containing pigments can only be estimated by comparison with the surface water DIC ^{14}C record from AD 1950 until the sampling year AD 2008. Since measured bomb surface water DIC ^{14}C values are only available for AD 1988 (Jones and Gagnon, 1994), we use model DIC ^{14}C results for AD 1810 to AD 2010 (Fig. 4; Supplement Table S2) adapted to match the few existing data available for this time interval (Ostlund and Dryssen, 1986; Jones and Gagnon, 1994; Siani et al., 2000; this study).

Estimated average calendar years of pigment synthesis derived from comparison with the DIC ^{14}C record range from AD 1956 to AD 1969 (Table 1), because post-bomb surface DIC ^{14}C concentrations were not equilibrated to values <30‰ (Fig. 4; Supplement Table S2) until AD 2008. Only for pyropheophytin-*a* at station P128 (AD 1963 or AD 2009), and pyropheophytin-*a* (AD 1969 or 1981) and 13^2,17^3-cyclopheophorbide-*a*-enol at station P167 (AD 1964 or AD 2002) two possible average synthesis years can be derived. However, in all cases the earlier possible date is regarded more likely because of its similarity to estimated years of pigment synthesis at the other stations (AD 1956 to AD 1964) and, in the case of 13^2,17^3-cyclopheophorbide-*a*-enol, with the date estimated for a second aliquot (AD 1962) analysed separately.

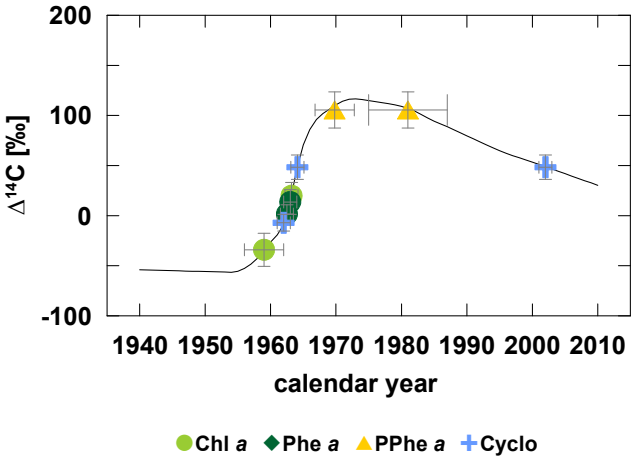

Fig. 4. Modelled Black Sea surface water DIC Δ^{14}C history. As example, potential calendar years of pigment synthesis derived from correlation with Δ^{14}C concentrations are given for station P167. Error bars denote 1σ analytical uncertainties. Chl-*a*: chlorophyll-*a*, Phe-*a*: pheophytin-*a*, PPhe-*a*: pyropheophytin-*a*, Cyclo: 13^2,17^3-cyclopheophorbide-*a*-enol.

The modelled surface DIC ^{14}C concentrations do not reach values high enough to correspond to that of the most ^{14}C-enriched pigment, chlorophyll-*a* at station P169 (181.12 ± 17.1‰). This might be caused by either uncertainties of the model related to the scarcity of input data, since the chlorophyll-*a* Δ^{14}C value is in the range previously reported for Northern Hemisphere oceanic surface water DIC (e.g., Druffel, 1987), or contribution of terrestrial chlorophyll-*a* such as from a recent leaf fragment, reflecting atmospheric bomb-^{14}C concentrations.

Overall, bomb-^{14}C containing pigments are older than expected. The time period estimated for their synthesis (AD 1956 to AD 1969) is approximately coincident with the time interval of serious eutrophication (AD 1970 to AD 1990) on the NW Black Sea shelf caused by a dramatic increase of anthropogenic nutrients discharged by the Danube River (e.g. Aubrey et al., 1996; Oguz, 2005). This enhanced nutrient input caused an up to tenfold increase of the phytoplankton photosynthetic activity and, thus, pigment abundance, which might have enhanced pigment preservation. Besides higher biomass quantity, pigment preservation could also have been enhanced through reduced light penetration in the uppermost water column (Aubrey et al., 1996; Oguz, 2005), which likely limited photo-oxidation of pigments during that time. Moreover, eutrophication-triggered recurrent hypoxia of shallow shelf bottom waters (Aubrey et al., 1996) could have further enhanced pigment preservation by decreasing both oxidation and grazing. Therefore, the preservation efficiency could have been considerably enhanced during this eutrophication time period obscuring more recent pigment ^{14}C concentrations. Therefore, we regard the Black Sea eutrophication phase a likely time period of synthesis of the bomb-^{14}C containing pigments. The temporal deviation of the estimated years of pigment synthesis (AD 1956 to AD 1969) and the major eutrophication phase (AD 1970 to AD 1990) may be caused by the uncertainties associated with the radiocarbon analysis and the uncertainties of the surface water DIC ^{14}C model related to the scarcity of input data during the initial incorporation of bomb-^{14}C into the surface water DIC pool. Furthermore, the temporal deviation may be caused by some undetermined additional blank C introduced during HPLC processing or wet chemical sample preparation. Accordingly, eutrophication-induced light-limitation and hypoxia are likely further controls on the preservation time-scales of primary pigments.

4.3 Chloro-and pheopigment preservation time-scales and implications for the stable carbon and nitrogen isotopic compositions

If the decomposition of chlorophyll-*a* to its derivative pigments in the sediment occurred on time-scales of several years to decades, the ^{14}C concentrations of the latter within the same sedimentary layer as the precursor should decrease with increasing degradative loss of functional groups, i.e. a decrease in ^{14}C concentrations would be expected from chlorophyll-*a* to pheophytin-*a* and pyropheophytin-*a* due to increasing time needed for the decompositional process. An evaluation of the precise time-scales of pre- and post-depositional degradation processes is only possible for station P167, the only core location situated well below the oxycline (Table 2) where bioturbation can be excluded to cause pigment age differences. Likewise, no age artefact from low sediment accumulation is expected since the sedimentation rate at this station is ~0.9 mm year^{-1} (extrapolated from Gulin et al., 2002). Except for pyropheophytin-*a* all pigment ^{14}C concentrations agree within 2σ-analytical uncertainty at station P167. Thus, their decompositional conversion from their respective parent chlorophyll-*a* occurs within a few years. Although the ^{14}C concentrations of pyropheophytin-*a* are significantly different from those of the other pigments at station P167, the calendar age difference is also only a few years (Table 1) caused by the steep gradient of bomb-^{14}C incorporation into surface water DIC during the 1960s (Fig. 4).

In contrast to what would be expected, pyropheophytin-*a* is younger than chlorophyll-*a* and pheophytin-*a*. This pattern is also evident at stations P128, P177, and P120. These younger ages are coupled with a ^{15}N-depletion of pyropheophytin-*a* compared to chlorophyll-*a* and pheophytin-*a* at the respective stations, which might be caused by seasonal nutrient source variations coupled with nitrogen isotopic variability. Seasonally varying isotopic compositions of nutrients (δ^{15}N) and DIC (δ^{13}C and Δ^{14}C) could potentially result in differences of the isotopic compositions of the individual pigments extracted from the sediments, if each of them is predominantly derived from a different phytoplankton bloom occurring at different seasons. For

instance, it has been reported that the spring bloom results in a pyropheophytin-*a* mass flux maximum, while the autumn bloom results in a pheophytin-*a* flux maximum (King, 1995). This explanation might apply in particular for the differences between ^{14}C concentrations of pyropheophytin-*a* and the other pigments at the more offshore locations P120 and P167. Additional variability in the ^{14}C concentration of DIC (Broecker and Peng, 1980) utilized by certain plankton groups and, as a result, preserved in their respective pigments, might arise from seasonal variations of the average dwelling depths of blooming phytoplankton groups. Seasonal variations in subsurface chlorophyll-*a* maximum depths have been reported for the Black Sea (Chu et al., 2005), but seasonally resolved DIC ^{14}C data are not available.

The stable carbon and nitrogen isotopic compositions of the individual pigments reflect the conditions at the time of synthesis. At station P128 the oldest pigment (pheophytin-*a*) is approximately 700 years older than the youngest pigment (pyropheophytin-*a*). Our data suggest that during this time period the pigment nitrogen isotopic composition and, thus, the nutrient assimilation pathway, and source nitrate isotopic composition has not changed substantially if pigments are derived from phytoplankton. Likewise, no major changes of the nutrient source and assimilation pathway are obvious at station P177 where the oldest (chlorophyll-*a*) and the youngest (pheophytin-*a*) measured pigments are even ∼1200 years apart. However, if chlorophyll-*a* and pheophytin-*a* at these two river mouth stations are derived from terrestrial plants, such temporal assessment is invalid. All pigments probably derive from phytoplankton at stations P120 and P169 where chlorophyll-*a* δ^{15}N values differ considerably from the δ^{15}N values of the other pigments (likely driven by ^{15}N isotopic variability due to seasonal changes and/or nutrient assimilation pathway, as discussed in Sect. 4.1) while their ^{14}C concentration differences are insignificant. Accordingly, in the NW Black Sea shelf area, the pigments' stable carbon and nitrogen isotopic compositions seem to be more strongly influenced by seasonal variations of the isotopic compositions of a nutrient source and spatial differences of the nutrient assimilation pathway than by potential temporal and perhaps anthropogenic changes in nutrient supply during the last millennium.

5 Conclusions

This study reports the first results from combined compound-specific radiocarbon and stable carbon and nitrogen isotopic analysis of chloro- and pheopigments. The combined δ^{13}C and δ^{15}N isotope analysis of chlorophyll-*a* and its degradation products pheophytin-*a*, pyropheophytin-*a*, and 13^2,17^3-cyclopheophorbide-*a*-enol from NW Black Sea surface sediments provides evidence for their origin from a mainly nitrate assimilating phytoplanktonic community. Additionally, towards the central ocean basin the pigment δ^{15}N values imply contributions from either N_2-fixation or uptake of ammonium supplied from deeper waters. The chloro- and pheopigment ^{14}C concentrations are much lower than expected translating into minimum and maximum pigment ages of approximately 40 to 1200 years. This is most likely the result of protection against degradation by processes such as association with minerals or eutrophication-induced hypoxia and light limitation. Nevertheless, the isotopic heterogeneity is also caused by seasonally varying isotopic compositions of nutrient source, habitat, and growth period which appear to be at least as strong as the long-term trends.

Overall, the combined triple isotopic analysis of sedimentary chlorophyll and its primary degradation products can delineate biogeochemical and diagenetic processes in surface waters and sediments and simultaneously assess the timescales of these processes.

Supplementary material related to this article is available online at: http://www.biogeosciences.net/7/4105/2010/bg-7-4105-2010-supplement.pdf.

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References

Altabet, M. A. and Francois, R.: Sedimentary Nitrogen Isotopic Ratio as a Recorder for Surface Ocean Nitrate Utilization, Global Biogeochem. Cy., 8, 103–116, doi:10.1029/93GB03396, 1994.

Aubrey, D., Moncheva, S., Demirov, E., Diaconu, V., and Dimitrov, A.: Environmental changes in the western Black Sea related to anthropogenic and natural conditions, J. Marine Syst., 7, 411–425, 1996.

Bakker, M. H. N.: Transboundary river floods: vulnerability of continents, international river basins and countries, Ph.D. thesis, Oregon State University, 261 pp., available at: http://hdl.handle.net/1957/3821, 2007.

Banaru, D., Harmelin-Vivien, M., Gomoiu, M.-T., and Onciu, T.-M.: Influence of the Danube River inputs on C and N stable isotope ratios of the Romanian coastal waters and sediment (Black Sea), Mar. Pollut. Bull., 54, 1385–1394, 2007.

Bianchi, T. S., Dibb, J. E., and Findlay, S.: Early Diagenesis of Plant Pigments in Hudson River Sediments, Estuar. Coast. Shelf S., 36, 517–527, 1993.

Brandes, J. A., Devol, A. H., Yoshinari, T., Jayakumar, D. A., and Naqvi, S. W. A.: Isotopic composition of nitrate in the central Arabian Sea and eastern tropical North Pacific: A tracer for mixing and nitrogen cycles, Limnol. Oceanogr., 43, 1680–1689, 1998.

Broecker, W. S. and Peng, T. H.: Seasonal variability in the $^{14}C/^{12}C$ ratio for surface ocean water, Geophys. Res. Lett., 7, 1020–1022, 1980.

Broecker, W. S., Peng, T.-H., Ostlund, G., and Stuiver, M.: The Distribution of Bomb Radiocarbon in the Ocean, J. Geophys. Res., 90, 6953–6970, 1985.

Butzin, M. and Roether, W.: Tritium budget of the South Atlantic, J. Geophys. Res., 109, C03036, doi:10.1029/2002JC001561, 2004.

Carpenter, S. R., Elser, M. M., and Elser, J. J.: Chlorophyll production, degradation and sedimentation: implications for paleolimnology, Limnol. Oceanogr., 31, 112–124, 1986.

Cauwet, G., Déliat, G., Krastev, A., Shtereva, G., Becquevort, S., Lancelot, C., Momzikoff, A., Saliot, A., Cociasu, A., and Popa, L.: Seasonal DOC accumulation in the Black Sea: a regional explanation for a general mechanism, Mar. Chem., 79, 193–205, 2002.

Chamberlain, P. M., McNamara, N. P., Chaplow, J., Stott, A. W., and Black, H. I. J.: Translocation of surface litter carbon into soil by Collembola, Soil Biol. Biochem., 38, 2655–2664, 2006.

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

Chu, P. C., Ivanov, L. M., and Margolina, T. M.: Seasonal variability of the Black Sea chlorophyll-a concentration, J. Marine Syst., 56, 243–261, 2005.

Cociasu, A., Dorogan, L., Humborg, C., and Popa, L.: Long-term ecological changes in Romanian coastal Waters of the Black Sea, Mar. Pollut. Bull., 32, 32–38, 1996.

Druffel, E. R. M.: Bomb radiocarbon in the Pacific: Annual and seasonal timescale variations, J. Mar. Res., 45, 667–698, 1987.

Enting, I. G., Wigley, T. M. L., and Heimann, M.: Future Emissions and Concentrations of Carbon Dioxide: Key Ocean/Atmosphere/Land Analyses, Technical Paper No 31, CSIRO Division of Atmospheric Research, Melbourne, Australia, 1994.

Falkowski, P. G.: Biogeochemistry of Primary Production in the Sea, in: Treatise on Geochemistry, edited by: Heinrich, D. H. and Karl, K. T., Pergamon, Oxford, 185–213, 2003.

Fontugne, M., Guichard, F., Bentaleb, I., Strechie, C., and Lericolais, G.: Variations in ^{14}C Reservoir Ages of Black Sea Waters and Sedimentary Organic Carbon during Anoxic Periods: Influence of Photosynthetic Versus Chemoautotrophic Production, Radiocarbon, 51, 969–976, 2009.

Fuchsman, C. A., Murray, J. W., and Konovalov, S. K.: Concentration and natural stable isotope profiles of nitrogen species in the Black Sea, Mar. Chem., 111, 90–105, 2008.

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

Godwin, H.: Half-life of Radiocarbon, Nature, 195, 984–984, 1962.

Goericke, R., Strom, S. L., and Bell, M. A.: Distribution and sources of cyclic pheophorbides in the marine environment, Limnol. Oceanogr., 45, 200–211, 2000.

Gossauer, A. and Engel, N.: Chlorophyll catabolism – structures, mechanisms, conversions, J. Photoch. Photobio. B, 32, 141–151, 1996.

Gruber, N., Keeling, C. D., and Bates, N. R.: Interannual Variability in the North Atlantic Ocean Carbon Sink, Science, 298, 2374–2378, 2002.

Gulin, S. B., Polikarpov, G. G., Egorov, V. N., Martin, J. M., Korotkov, A. A., and Stokozov, N. A.: Radioactive Contamination of the North-western Black Sea Sediments, Estuar. Coast. Shelf S., 54, 541-549, 2002.

Hoyt, P. B.: Chlorophyll-type compounds in soil. II. their decomposition, Plant Soil, 25, 313–328, 1966.

Huang, T.-C., Chowa, T.-J., and Hwanga I.-S.: The cyclic synthesis of the nitrogenase of Synechococcus RF-1 and its control at the transcription level, FEMS Microbiol. Lett., 50, 127–130, 1988.

Ikemoto, H. and Mitsui, A.: Diazotrophic synchronous growth of a marine unicellular cyanobacterium, Synechococcus sp. strain Miami BG 043511, under aerobic and microaerobic/anaerobic conditions, Microbiology, 140, 2153–2158, 1994.

Jaoshvili, S.: The rivers of Black Sea, European Environment Agency, Kopenhagen, Denmark, 58 pp., 2002.

Jones, G. A. and Gagnon, A. R.: Radiocarbon chronology of Black Sea sediments, Deep-Sea Res. Pt. I, 41, 531–557, 1994.

Jugaru Tiron, L., Le Coz, J., Provansal, M., Panin, N., Raccasi, G., Dramais, G., and Dussouillez, P.: Flow and sediment processes in a cutoff meander of the Danube Delta during episodic flooding, Geomorphology, 106, 186–197, 2009.

Kanduć, T., Szramek, K., Ogrinc, N., and Walter, L. M.: Origin and cycling of riverine inorganic carbon in the Sava River watershed (Slovenia) inferred from major solutes and stable carbon isotopes, Biogeochemistry, 86, 137–154, 2007.

Kashiyama, Y., Ogawa, N. O., Kuroda, J., Shiro, M., Nomoto, S., Tada, R., Kitazato, H., and Ohkouchi, N.: Diazotrophic cyanobacteria as the major photoautotrophs during mid-Cretaceous oceanic anoxic events: Nitrogen and carbon isotopic evidence from sedimentary porphyrin, Org. Geochem., 39, 532–549, 2008a.

Kashiyama, Y., Ogawa, N. O., Shiro, M., Tada, R., Kitazato, H., and Ohkouchi, N.: Reconstruction of the biogeochemistry and ecology of photoautotrophs based on the nitrogen and carbon isotopic compositions of vanadyl porphyrins from Miocene siliceous sediments, Biogeosciences, 5, 797–816, doi:10.5194/bg-5-797-2008, 2008b.

Kennicutt II, M. C., Bidigare, R. R., Macko, S. A., and Keeney-Kennicutt, W. L.: The stable isotopic composition of photosynthetic pigments and related biochernicals, Chem. Geol., 101, 235–245, 1992.

King, L. L.: A mass balance of chlorophyll degradation product accumulation in Black Sea sediments, Deep-Sea Res. Pt. I, 42, 919–942, 1995.

Krupatkina, D. K., Finenko, Z. Z., and Shalapyonok, A. A.: Primary production and size-fractionated structure of the Black Sea phytoplankton in the winter-spring period, Mar. Ecol.-Prog. Ser., 73, 25–31, 1991.

Kusch, S., Rethemeyer, J., Schefuß, E., and Mollenhauer, G.: Controls on the age of vascular plant biomarkers in Black Sea sediments, Geochim. Cosmochim. Ac., 74, 7031–7047, 2010.

Kuypers, M. M. M., Sliekers, A. O., Lavik, G., Schmid, M., Jorgensen, B. B., Kuenen, J. G., Sinninghe Damste, J. S., Strous,

M., and Jetten, M. S. M.: Anaerobic ammonium oxidation by anammox bacteria in the Black Sea, Nature, 422, 608–611, 2003.

Levin, I., Hammer, S., Kromer, B., and Meinhardt, F.: Radiocarbon observations in atmospheric CO_2: Determining fossil fuel CO_2 over Europe using Jungfraujoch observations as background, Sci. Total Environ., 391, 211–216, 2008.

Liu, K.-K. and Kaplan, I. R.: The eastern tropical Pacific as a source of ^{15}N-enriched nitrate in seawater off southern California, Limnol. Oceanogr., 34, 820–830, 1989.

Matile, P., Hörtensteiner, S., Thomas, H., and Kräutler, B.: Chlorophyll Breakdown in Senescent Leaves, Plant Physiol., 112, 1402–1409, 1996.

Mayer, L. M.: Relationships between mineral surfaces and organic carbon concentrations in soils and sediments, Chem. Geol., 114, 347–363, 1994.

McCarthy, J. J., Yilmaz, A., Coban-Yildiz, Y., and Nevins, J. L.: Nitrogen cycling in the offshore waters of the Black Sea, Estuar. Coast. Shelf S., 74, 493–514, 2007.

McIlvin, M. R. and Altabet, M. A.: Chemical Conversion of Nitrate and Nitrite to Nitrous Oxide for Nitrogen and Oxygen Isotopic Analysis in Freshwater and Seawater, Anal. Chem., 77, 5589–5595, 2005.

McNichol, A. P., Osborne, E. A., Gagnon, A. R., Fry, B., and Jones, G. A.: TIC, TOC, DIC, DOC, PIC, POC – unique aspects in the preparation of oceanographic samples for 14C-AMS, Nuclear Instruments and Methods in Physics Research Section B: Beam Interactions with Materials and Atoms, 92, 162–165, 1994.

Minagawa, M. and Wada, E.: Nitrogen isotope ratios of red tide organisms in the East China Sea: A characterization of biological nitrogen fixation, Mar. Chem., 19, 245–259, 1986.

Mollenhauer, G. and Rethemeyer, J.: Compound-specific radiocarbon analysis – analytical challenges and applications, IOP C. Ser. Earth Env., 5, 012006, doi:10.1088/1755-1307/5/1/012006, 2009.

Montoya, J. P., Carpenter, E. J., and Capone, D. G.: Nitrogen fixation and nitrogen isotope abundances in zooplankton of the oligotrophic North Atlantic, Limnol. Oceanogr., 47, 1617–1628, 2002.

Murray, J. W., Fuchsman, C., Kirkpatrick, J., Paul, B., and Konovalov, S. K.: Species and δ^{15}N signatures of nitrogen transformations in the suboxic zone of the Black Sea, Oceanography, 18, 36–47, 2005.

Ocampo, R., Sachs, J. P., and Repeta, D. J.: Isolation and structure determination of the unstable 13^2,17^3-Cyclopheophorbide a enol from recent sediments, Geochim. Cosmochim. Ac., 63, 3743–3749, 1999.

Ogawa, N. O., Nagata, T., Kitazato, H., and Ohkouchi, N.: Ultrasensitive elemental analyzer/isotope ratio mass spectrometer for stable nitrogen and carbon isotope analyses, in: Earth, Life, and Isotopes, edited by: Ohkouchi, N., Tayasu, I., and Koba, K., Kyoto University Press, Kyoto, 339–353, 2010.

Oguz, T.: Long-Term Impacts of Anthropogenic Forcing on the Black Sea Ecosystem, Oceanography, 18, 112–121, 2005.

Ohkouchi, N., Nakajima, Y., Okada, H., Ogawa, N. O., Suga, H., Oguri, K., and Kitazato, H.: Biogeochemical processes in the saline meromictic Lake Kaiike, Japan: implications from molecular isotopic evidences of photosynthetic pigments, Environ. Microbiol., 7, 1009–1016, 2005.

Ohkouchi, N., Kashiyama, Y., Kuroda, J., Ogawa, N O., and Kitazato, H.: The importance of diazotrophic cyanobacteria as primary producers during Cretaceous Oceanic Anoxic Event 2, Biogeosciences, 3, 467–478, doi:10.5194/bg-3-467-2006, 2006.

Ohkouchi, N., Nakajima, Y., Ogawa, N. O., Chikaraishi, Y., Suga, H., Sakai, S., and Kitazato, H.: Carbon isotopic composition of the tetrapyrrole nucleus in chloropigments from a saline meromictic lake: A mechanistic view for interpreting the isotopic signature of alkyl porphyrins in geological samples, Org. Geochem., 39, 521–531, 2008.

Ostlund, H. G. and Dryssen, D.: Renewal rates of Black Sea deep water, Proceedings of the chemical and physical oceanography of the Black Sea, International meeting, University of Göteborg, Göteborg, Sweden, 1986.

Owens, T. G. and Falkowski, P. G.: Enzymatic degradation of chlorophyll a by marine phytoplankton in vitro, Phytochemistry, 21, 979–984, 1982.

Panin, N. and Jipa, D.: Danube River Sediment Input and its Interaction with the North-western Black Sea, Estuar. Coast. Shelf S., 54, 551–562, 2002.

Pearson, A., McNichol, A. P., Schneider, R. J., Reden, K. F. v., and Zheng, Y.: Microscale AMS 14C measurement at NOSAMS, Radiocarbon, 40, 61–75, 1998.

Pennock, J. R., Velinsky, D. J., Ludlam, J. M., Sharp, J. H., and Fogel, M. L.: Isotopic fractionation of ammonium and nitrate during uptake by *Skeletonema costatum*: Implications for δ^{15}N dynamics under bloom conditions, Limnol. Oceanogr., 41, 451–459, 1996.

Repeta, D. J. and Simpson, D. J.: The distribution and recycling of chlorophyll, bacteriochlorophyll and carotenoids in the Black Sea, Deep-Sea Res., 38, S969–S984, 1991.

Reschke, S., Ittekkot, V., and Panin, N.: The Nature of Organic Matter in the Danube River Particles and North-western Black Sea Sediments, Estuar. Coast. Shelf S., 54, 563–574, 2002.

Rho, J., Bauman, A., Boettger, H., and Yen, T.: A search for porphyrin biomarkers in nonesuch shale and extraterrestrial samples, Origins Life Evol. B., 4, 69–77, 1973.

Sachs, J. P., Repeta, D. J., and Goericke, R.: Nitrogen and carbon isotopic ratios of chlorophyll from marine phytoplankton, Geochim. Cosmochim. Ac., 63, 1431–1441, 1999.

Sanger, J. E.: Fossil pigments in paleoecology and paleolimnology, Palaeogeogr. Palaeocl., 62, 343–359, 1988.

Siani, G., Paterne, M., Arnold, M., Bard, E., Mativier, B., Tisnerat, N., and Bassinot, F.: Radiocarbon Reservoir Ages In The Mediterranean Sea And Black Sea, Radiocarbon, 42, 271–280, 2000.

Sigman, D. M., Altabet, M. A., McCorkle, D. C., Francois, R., and Fischer, G.: The δ15N of nitrate in the Southern Ocean: Nitrogen cycling and circulation in the ocean interior, J. Geophys. Res., 105, 19599–19614, 2000.

Sigman, D. M., Granger, J., DiFiore, P. J., Lehmann, M. M., Ho, R., Cane, G., and van Geen, A.: Coupled nitrogen and oxygen isotope measurements of nitrate along the eastern North Pacific margin, Global Biogeochem. Cy., 19, GB4022, doi:10.1029/2005GB002458, 2005.

SooHoo, J. B. and Kiefer, D. A.: Vertical distribution of phaeopigments-II. Rates of production and kinetics of photooxidation, Deep-Sea Res., 29, 1553–1563, 1982.

Spooner, N., Getliff, J. M., Teece, M. A., Parkes, R. J., Leftley, J. W., Harris, P. G., and Maxwell, J. R.: Formation of

mesopyrophaeophorbide a during anaerobic bacterial degradation of the marine prymnesiophyte Emiliania huxleyi, Org. Geochem., 22, 225–229, 1995.

Stuiver, M. and Polach, H. A.: Reporting of ^{14}C Data, Radiocarbon, 19, 355–363, 1977.

Stuiver, M., Reimer, P. J., and Reimer, R.: CALIB – radiocarbon calibration, version 6.0 (http://intcal.qub.ac.uk/calib/), 2010.

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

Sun, M., Lee, C., and Aller, R. C.: Anoxic and oxic degradation of 14C-labeled chloropigments and a 14C-labeled diatom in Long Island Sound sediments, Limnol. Oceanogr., 38, 1438–1451, 1993b.

Taguchi, S., Laws, E. A., and Bidigare, R. R.: Temporal variability in chlorophyll a and phaeopigment concentrations during incubations in the absence of grazers, Mar. Ecol.-Prog. Ser., 101, 45–53, 1993.

Toggweiler, J., Dixon, K., and Bryan, K.: Simulations of Radiocarbon in a Coarse-Resolution World Ocean Model 1. Steady State Prebomb Distributions, J. Geophys. Res., 94, 8217–8242, 1989.

Tolmazin, D.: Changing Coastal oceanography of the Black Sea. I: Northwestern Shelf, Prog. Oceanogr., 15, 217–276, 1985.

Tyler, J., Kashiyama, Y., Ohkouchi, N., Ogawa, N. O., Yokoyama, Y., Chikaraishi, Y., Staff, R. A., Ikehara, M., Bronk Ramsey, C., Bryant, C., Brock, F., Gotanda, K., Haraguchi, T., Yonenobu, H., and Nakagawa, T.: Tracking aquatic change using chlorin-specific carbon and nitrogen isotopes: The last glacial-interglacial transition at Lake Suigetsu, Japan, Geochem. Geophy. Geosy., 11, Q09010, doi:10.1029/2010GC003186, 2010.

Uysal, Z.: Pigments, size and distribution of Synechococcus spp. in the Black Sea, J. Marine Syst., 24, 313–326, 2000.

Uysal, Z.: Vertical distribution of marine cyanobacteria Synechococcus spp. in the Black, Marmara, Aegean, and eastern Mediterranean seas, Deep-Sea Res. Pt. II, 53, 1976–1987, 2006.

Velinsky, D. J., Fogel, M. L., Todd, J. F., and Tebo, B. M.: Isotopic fractionation of dissolved ammonium at the oxygen-hydrogen sulfide interface in anoxic waters, Geophys. Res. Lett., 18, 649–652, doi:10.1029/91GL00344, 1991.

Zeebe, R. E. and Wolf-Gladrow, D.: CO_2 in seawater: Equilibrium, kinetics, isotopes, Elsev. Oceanogr. Serie., 65, 1–346, 2001.