Chapter

20

Nitrogen and carbon isotopic compositions of copper, nickel, and vanadyl porphyrins in Cretaceous black shales

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Abstract

We determined both the nitrogen and carbon isotopic compositions of 17 nickel, copper, and vanadyl alkylporphyrins isolated from the sediments of Cretaceous Oceanic Anoxic Events (OAEs; the Livello Selli and Livello Bonarelli black shales) to investigate processes involved in the preservation of porphyrins based on their isotopic signatures. We observed 1) the $\delta^{15}N$ and $\delta^{13}C$ values were not identical between copper- and nickel-complexed deoxophylloerythroetioporphyrin or between vanadyl- and nickel-complexed porphyrins with identical chemical structures; and 2) although the $\delta^{15}N$ and $\delta^{13}C$ values of all of the nickel-complexed porphyrins fell within a similar isotopic range in each sample, the relative isotopic differences between different species of nickel-complexed porphyrins rarely exhibited any regular relationships. These results suggest that the decomposition routes of the precursory chloropigments differentiated at an early stage of diagenesis (in the surface sediments or water column), implying that physicochemical conditions during preservation of the chloropigments in the surface sediments, as well as the overlying water column, may have significantly fluctuated under OAE conditions.

Key Words: sedimentary porphyrins, carbon isotope, nitrogen isotope, chlorophyll diagenesis, oceanic anoxic event

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INTRODUCTION

Sedimentary porphyrins are biomarkers that potentially serve as a fertile source of information on the activities of past photosynthetic primary producers and hence hold the key to the biogeochemical dynamics of the paleo-ocean. Porphyrins are commonly observed in organic-rich sediments, oil shales, and petroleum, mainly originating in chloropigments (Callot and Ocampo 2000). A variety of sedimentary porphyrins are known, which presumably reflects the structural diversity existing among the original chloropigments. The precursor − product relationships of the porphyrins have been deduced primarily based on structural evidence (e.g., Treibs 1936; Baker and Louda 1986; Callot and Ocampo 2000) and further elaborated by a series of studies on intermediate species (Keely et al. 1990, 1994, 2006; Eckardt et al. 1991; Naylor and Keely 1998, Ocampo et al. 1999; Callot and Ocampo 2000; Goericke et al. 2000; Mawson and Keely 2008). Nonetheless, there have been only a few studies that have attempted to reconstruct the nature of paleo-productivity. This is largely due to an limited understanding of the defunctionalization/transformation processes of the chloropigments in the aquatic environment and in the sediments, resulting in considerable uncertainties in the precursor − product relationships between chloropigments and sedimentary porphyrins.

Isotopic compositions of sedimentary porphyrins could provide independent evidence for elucidating the processes and fates of chloropigments in the aquatic environment as well as in sediments. However, only a limited number of reports have been published on the stable isotopic compositions of individual chloropigments produced by aquatic phototrophs (Sachs et al. 1999; Ohkouchi et al. 2006, York et al. 2007) and on individual sedimentary porphyrins extracted from sedimentary rocks (Hayes et al. 1987; Boreham et al. 1989, 1990; Ocampo et al. 1989; Popp et al. 1989; Chicarelli et al. 1993; Keely et al. 1994; Ohkouchi et al. 2006; Kashiyama et al. 2008a, 2008b; Higgins et al. 2009). It is particularly important that, unlike other lipid biomarkers, these tetrapyrrole compounds contain nitrogen atoms in addition to carbon, which potentially allows dual isotopic characterization (Chicarelli et al. 1993; Ohkouchi et al. 2006; Kashiyama et al. 2008a, 2008b). Both carbon and nitrogen isotopic compositions of sedimentary porphyrins are essentially inherited from their precursors (Ohkouchi et al. 2006, 2008; see also discussion on preservation of carbon isotopomers of tetrapyrroles in Ohkouchi et al. 2010) and are not prone to fractionation during diagenesis. Therefore, dual nitrogen-carbon isotopic characterization of sedimentary porphyrins may be a valuable tool in identifying and/or distinguishing among their sources (Kashiyama et al. 2008b).

In the present study, we determined the nitrogen and carbon isotopic compositions of various species of sedimentary porphyrins from the black shales of the Oceanic Anoxic Events (OAEs), in addition to those reported in Kashiyama et al. (2008a). OAE black shales preserve sedimentary porphyrins in very high concentrations compared to ordinary marine shales and thus were the most suitable materials for compound-specific isotopic

study. As discussed in Kashiyama et al. (2008a), the isotopic compositions of sedimentary porphyrins, and thus the estimated values for the phototrophs, are somewhat shifted from the typical values observed in modern oceans. This likely reflects unusual oceanic conditions during the OAEs, when the abundance and isotopic compositions of the chemical species used in primary production (e.g., dissolved inorganic carbon and nitrogen) may have been different from those observed in common oceanic settings today. Nonetheless, OAE black shales provide an abundant source of information on the processes of preservation based on relative isotopic variations among different species of sedimentary porphyrins. The high abundance of sedimentary porphyrins in the present samples allowed determination of isotopic compositions of even some minor components that might be important in understanding preservation processes, despite their relatively low abundance.

In this chapter, we first briefly summarize previous studies of the precursor − product relationships of the porphyrins. We then discuss our own dataset to highlight unexplored aspects of preservation processes of porphyrins in terms of the isotopic evidence.

The Origin of Sedimentary Porphyrins

Structural types of sedimentary porphyrins and their presumed sources

The majority of sedimentary porphyrins have been categorized into either deoxophyllo erythroetioporphyrin (DPEP) or etioporphyrin (ETIO) types as defined below. The DPEP types are monocycloalkanoporphyrins with a five-membered ring between the C−13 and C−15 carbons (Fig. 1; hereafter referred to as "ring E"), and are represented by DPEP (1a−c). Significantly, although diverse tetrapyrrole macrocycles are known among biomolecules, ring E structure is known exclusively in the chloropigments of photoautotrophs such as chlorophylls *a*−*d* and bacteriochlorophylls *a*−*g* (Fig. 1; see also the summary of chloropigment diversity as well as Fig. 1 and Table 1 in Ohkouchi et al., this volume). Therefore, it is generally accepted that the DPEP types originate from chloropigments of photoautotrophs of the past ("Treibs scheme," Treibs 1936; Baker and Louda 1986; Callot and Ocampo 2000; Keely et al. 2006).

DPEP, a representative of the DPEP-type porphyrins, is among the most abundant sedimentary porphyrins in geologic samples, and retains the common carbon skeleton of all chloropigments (Callot and Ocampo 2000). Although it can, therefore, be derived from any chloropigment, DPEP is considered to have originated primarily from chlorophyll *a*, which is the sole quantitatively significant chloropigment produced by virtually all oxygenic photoautotrophs living in an aerobic atmosphere (i.e., algae and cyanobacteria). Therefore, DPEP has been considered a universal biomarker of photoautotrophs, and its isotopic compositions are considered to represent the average isotopic values of the entire photoautotrophic community (e.g., Kashiyama et al. 2008b).

Sedimentary porphyrins without ring E are also commonly observed. Such porphyrins are typified by etioporphyrin III (19) and hence are termed ETIO types. Treibs (1936)

Table 1 ^{1}H NMR signal assignments for compounds 1a, 2, 3a, 5a, 6, 8, 9, 10, 12, 13, 14, and 16 in $CDCl_3$ (chemical shifts in δ ppm).

	1a	2	3a	5a	6	8*	9*	10	12	13	14	16
CH_3-CH_2-3	1.76 (t)	1.75 (t)	–	1.76 (t)	–	1.74 (t)	–	1.73 (t)	1.79 (t)	1.74 (t)	–	1.76 (t)
CH_3-CH_2-8	1.77 (t)	1.95 (t)	1.80 (t)	1.77 (t)	1.67 (t)	1.75 (t)	1.74 (t)	1.73 (t)	1.78 (t)	1.93 (t)	1.77 (t)	1.96 (t)
CH_3-CH_2-17	1.66 (t)	1.67 (t)	1.70 (t)	–	–	1.69 (t)	1.69 (t)	–	1.80 (t)	1.74 (t)	1.75 (t)	–
CH_3-CH_2-3	3.92 (q)	3.92 (q)	–	3.92 (q)	–	3.85 (q)	–	3.86 (q)	3.94 (q)	3.90 (q)	–	3.92 (q)
CH_3-CH_2-8	3.93 (q)	4.12 (q)	3.95 (q)	3.93 (q)	3.93 (q)	3.87 (q)	3.86 (q)	3.86 (q)	3.96 (q)	4.11 (q)	3.93 (q)	4.11 (q)
CH_3-CH_2-17	3.90 (q)	3.90 (q)	3.96 (q)	–	–	3.93 (q)	3.93 (q)	–	3.92 (q)	3.90 (q)	3.91 (q)	–
CH_3-15^2	–	–	–	–	–	–	–	–	–	–	–	1.95 (d)
CH_3-2	3.48 (s)	3.50 (s)	3.63 (s)	3.49 (s)	3.46 (s)	3.41 (s)	3.39 (s)	3.42 (s)	3.49 (s)	3.47 (s)	3.60 (s)	3.48 (s)
CH_3-3	–	–	–	–	3.45 (s)	–	3.39 (s)	–	–	–	–	–
CH_3-7	3.49 (s)	–	3.49 (s)	3.49 (s)	3.51 (s)	3.42 (s)	3.41 (s)	3.42 (s)	3.49 (s)	–	3.46 (s)	–
CH_3-12	3.46 (s)	3.49 (s)	3.51 (s)	3.47 (s)	3.48 (s)	3.53 (s)	3.53 (s)	3.54 (s)	3.63 (s)	3.60 (s)	3.60 (s)	3.46 (s)
CH_3-18	3.46 (s)	3.48 (s)	3.53 (s)	3.61 (s)	3.61 (s)	3.41 (s)	3.41 (s)	3.54 (s)	3.51 (s)	3.47 (s)	3.49 (s)	3.46 (s)
CH_3-15	–	–	–	–	–	4.18 (s)	4.19 (s)	4.23 (s)	–	–	–	–
– CH_2-13^1	4.00 (m)	4.04 (m)	4.06 (m)	4.03 (m)	4.03 (m)	–	–	–	–	–	–	–
– CH_2-13^2	5.22 (m)	5.26 (m)	5.29 (m)	5.13 (m)	5.27 (m)	–	–	–	–	–	–	–
– CH_2-15^1	–	–	–	–	–	–	–	–	–	–	–	4.60, 5.35 (d, d)
– CH_2-15^2	–	–	–	–	–	–	–	–	–	–	–	4.60 (m)
H-3	–	–	8.94 (s)	–	–	–	– 8.94 (s)	–	–	–	–	
H-7	–	8.97 (s)	–	–	–	– 8.98 (s)	–	– 8.95 (s)	–	–		
H-13	–	–	–	–	–	9.16 (s)	9.17 (s)	9.13 (s)	8.96 (s)	8.94 (s)	8.96 (s)	8.95 (s)
H-17	–	–	–	8.96 (s)	8.95 (s)	–	–	9.13 (s)	–	–	–	–
H-5	9.80 (s)	9.76 (s)	9.90 (s)	9.78 (s)	9.83 (s)	9.49 (s)	9.49 (s)	+	9.80 (s)	+	9.76 (s)	9.76 (s)
H-10	9.78 (s)	9.88 (s)	9.83 (s)	9.79 (s)	9.72 (s)	9.54 (s)	9.54 (s)	+ 9.60, 9.64, or 9.65 (s)	9.84 (s)	+ 9.74, 9.77, 9.80, or – 9.92 (s)	9.85 (s)	9.92 (s)
H-15	–	–	–	–		–	–		9.74 (s)	+	9.76 (s)	
H-20	9.77 (s)	9.83 (s)	9.76 (s)	9.85 (s)	9.76 (s)	9.51 (s)	10.50 (s)	+	9.79 (s)	+	9.84 (s)	9.79 (s)

* Analyzed using 400-MHz NMR at Bruker Biospin K. K. All other data obtained using 600-MHz NMR at Tsukuba University.

concluded that etioporphyrin III in coals originates from heme (21), which was supported by the co-occurrence of a presumed intermediate mesoporphyrin IX (20) (Treibs 1934). Decades later, Corwin (1960) suggested thermal cracking of ring E of the DPEP types as the origin of the ETIO types. More recently, biotic and abiotic degradation of chloropigments resulting in cleavage of ring E have been recognized (Watanabe 1993; Naylor and Keely 1998). Two compound-specific carbon isotopic studies of sedimentary porphyrins suggested that 1) only etioporphyrin III exhibited distinct isotopic values from those of the DPEP types and is thus likely to have originated in a unique, non-chlorophyll source; and (2) the other ETIO types had similar isotopic values to the DPEP types, suggesting a chlorophyll origin (Boreham et al. 1989; Ocampo et al. 1989).

Chlorophyll transformation

The Treibs scheme leading from chlorophyll *a* (25) to DPEP has now been elaborated on by identification of various intermediates in recent sediments (Keely et al. 1990, 2006; Eckardt et al. 1991). The earliest stage of chlorophyll transformation occurs in the water column and perhaps in young sediments. The reductive transformation of chlorophyll *a* is largely related to processes (presumably enzymatic) within photoautotrophs (senescence) and/or heterotrophs (herbivory) rather than abiotic reactions (e.g., Harradine et al. 1996; Goericke et al. 2000; Bustillos-Guzmán et al. 2002; Keely 2006; Chikaraishi et al. 2007). Through reductive transformation, chloropigments are converted to a variety of pheopigments (Fig. 2). Similar transformations are observed/expected for other chlorophyll/bacteriochlorophyll species (Keely 2006).

Further transformations converting the fairly reactive pheopigments to stable, fully defunctionalized porphyrins (Fig. 2) are likely abiotic and occurring over geologic time scales (Keely et al. 1990; Keely 2006). However, direct evidence of presumed intermediates at this stage is particularly limited (Keely et al. 1990, 1994), which has prevented complete understanding of reaction mechanisms, causes, and timing. Uncertainties involve keto-reduction, full aromatization of the porphyrin structure, complex formation with various metals, and, more importantly in our context, loss or transformation of peripheral groups at specific positions of the tetrapyrrole structure, presumably reflecting structural variety among the original chloropigments. This last reaction is the key to relating the structural variation observed among sedimentary porphyrins to specific chloropigments (and hence to specific photoautotrophs), but remains somewhat hypothetical. We describe examples below and in Fig. 3 and also summarize previous studies providing isotopic evidence.

Unique functional groups such as the C−7 formyl group of chlorophyll *b* (27) or the C −17 carboxyvinyl group of chlorophylls *c* (30 − 32) can be selectively lost during early diagenesis and possibly yield 7-nor-DPEP (2) or 17-nor-DPEP (5a, b), respectively (Fig. 3b, d; Chicarelli and Maxwell 1984; Verne-Mismer et al. 1988). Hence, these DPEP compounds can be regarded as biomarkers specific to chlorophyll *b*- and chlorophyll *c*-producing algae, respectively. The absence of the C−3 methyl group in compounds such as 3-nor-DPEP (3a, b) may have resulted from the loss of the C−3 formyl group of chlorophyll *d*

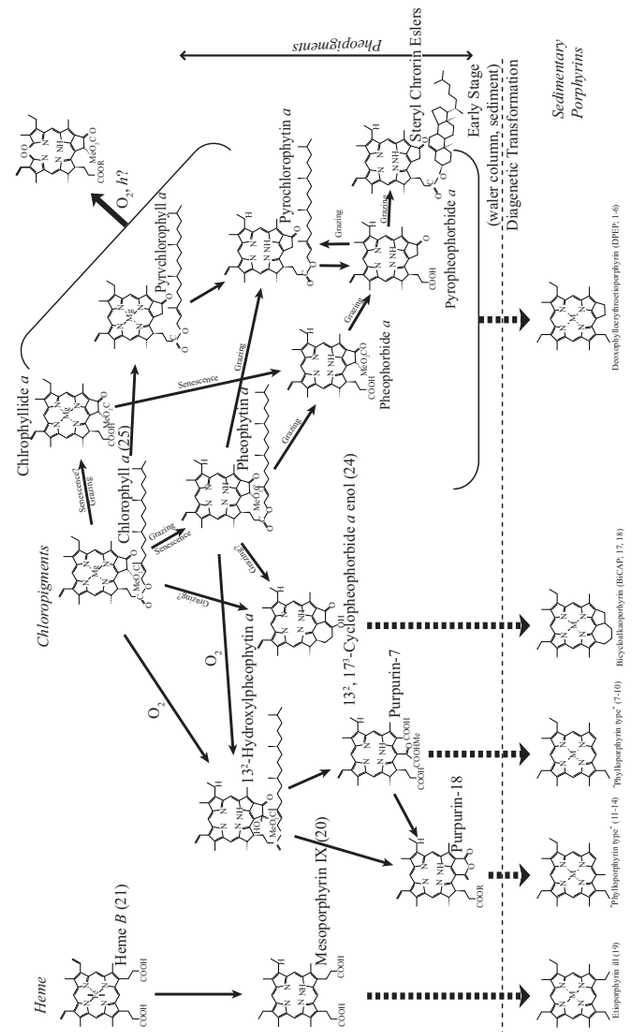
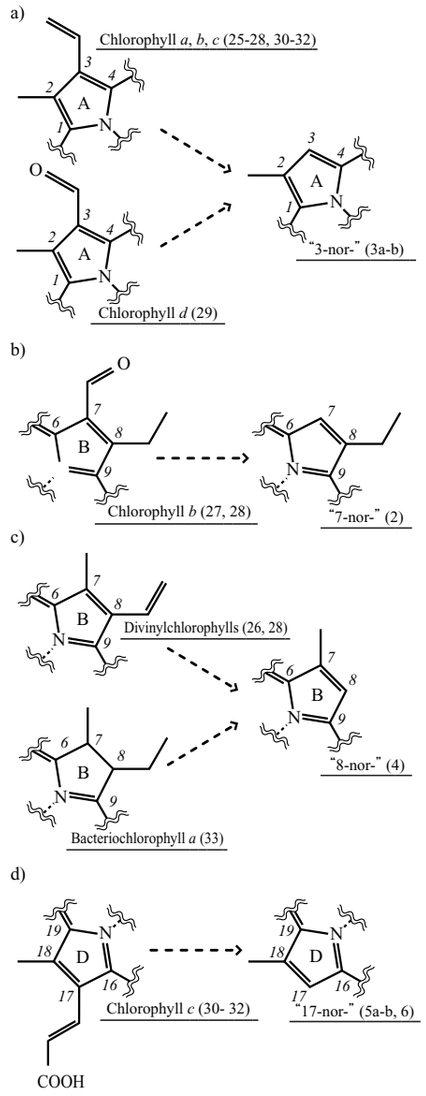


Fig. 2. Various transformation reactions of chloropigments and hemes represented by those of chlorophyll *a* and heme *B*. The early stage of transformation occurring in the water column and surface sediments is relatively well studied, whereas the later-stage reactions are poorly understood. Chloropigments are reductively defunctionalized into a variety of magnesium-free compounds (pheopigments) through primarily enzymatic processes retaining ring E, are oxidatively altered into pheopigments without ring E, or are photo-oxidatively decomposed after cleaving of the tetrapyrrole macrocycle (Mühlecker et al. 1993). The early-stage processes that are responsible for generation of various types of sedimentary porphyrins have been deduced based on structural similarity with known intermediate pheopigments.



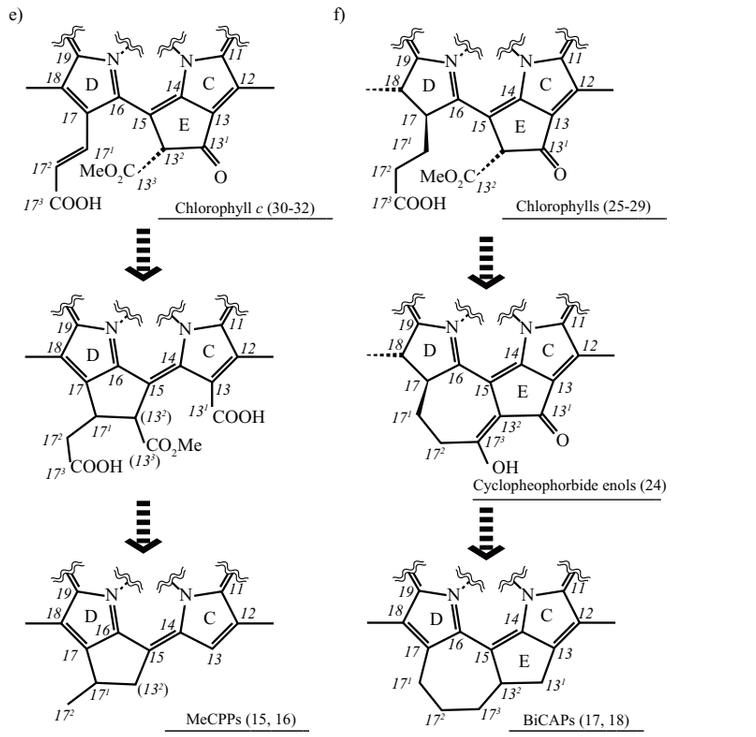


Fig. 3. Hypothetical transformation paths of peripheral functional groups of chloropigments into known sedimentary porphyrins lacking peripheral alkyl groups at specific positions **(a–d)** or having unusual ring structures **(e, f)**. See text for explanation of each path. The validity of these relationships remains to be tested.

(Fig. 3a; Keely et al. 2006; Kashiyama et al. 2008b), although it can also be explained by defunctionalization of the C−3 vinyl group of other chlorophylls (Fig. 3a; Fookes 1983). Certain sedimentary porphyrins have a methylcyclopentane ring between the C−15 and C −17 positions that was presumably formed by rearrangement of the peripheral substituents involving ring E and the C−17 carboxyvinyl group of chlorophylls *c* (30 − 32) (methylc yclopentanoporphyrin, MeCPP, 15; Fig. 3e; Dougherty et al. 1970). Thus, MeCPPs are regarded as biomarkers specific to chlorophyll *c*-producing algae. Other major porphyrins include bicycloalkanoporphyrins (BiCAPs; 17), which are formed after intermediates such as cyclopheophorbide *a* enols (24), chlorophyllones (22), and mesochlorophyllones (23) (Fig. 3f; Mawson and Keely 2008). These presumed intermediates are probably formed from any chlorophyll with herbivorous grazing (Harris et al. 1995; Goericke et al. 2000), although there may be abiotic processes that produce them in sediments (Keely 2006).

Kashiyama et al. (2008b) discussed the origins of 3-nor-, 8-nor-, and 17-nor-DPEPs (3b, 4, 5b) as well as BiCAP (18) based on comparisons of their compound-specific nitrogen and carbon isotopic compositions to those of DPEP (1c). Assuming that DPEP was derived from common chlorophylls, hence reflecting the isotopic signatures of the entire phototrophic community, distinctive isotopic values exhibited by 8-nor- and 17-nor-DPEP relative to DPEP suggested their rather unique origins. In particular, 8-nor- and 17-nor-DPEP likely originated from divinylchlorophylls (26, 28) (or bacteriochlorophyll *a*, 33; Kashiyama et al. 2007b) and chlorophylls *c* (30−32), respectively. On the other hand, the isotopic values of 3-nor-DPEP were not distinguishable from those of DPEP, suggesting that it may have as non-specific an origin as does DPEP. Isotopic compositions of BiCAP were apparently distinguishable from those of DPEP, suggesting that they represent distinct assemblies of phototrophs. Thus, BiCAPs as well as the above precursory bicycloalkano-pigments likely represent specific populations within the photoautotrophic community. However, structure-based analysis concluded that both DPEP and BiCAP are derived from common chlorophylls such as chlorophyll *a*. This further implies that isotopic variability among sedimentary porphyrins is not only attributable to differences in their precursory pigments (i.e., taxonomic differences) but also to differences in representativeness of the phototrophic community (i.e., spatial, ecospatial, or temporal variations).

Materials and Methods

Geological background and samples

The Livello Selli and Livello Bonarelli black shales are organic-rich pelagic shales occurring within a thick mid-Cretaceous chalk bed. They were formed during the OAEs (Schlanger and Jenkyns 1976), the periods when production by N_2-fixing cyanobacteria predominated over the stratified, dominantly anaerobic water column (Ohkouchi et al. 1997, 2006; Kuypers et al. 2004; Kashiyama et al. 2008a). The Livello Selli and Livello Bonarelli black shales represent the two most intensive formations of globally distributed unusually organic-rich sediments, namely, OAE−1a (early Aptian) and OAE−2 (late Cenomanian), respectively. Further detailed geological backgrounds of the studied sections are described in Kuroda et al. (2005) and Kashiyama et al. (2008a). The samples analyzed in this study were obtained from the upper part of the Livello Selli black shale (Fig. 4a) and six levels within the Livello Bonarelli black shale (Fig. 4b).

Isolation and purification of porphyrins

Isolation and purification of sedimentary porphyrins were conducted according to the methods described in Kashiyama et al. (2007a, 2008a, b). Briefly, we Soxhlet-extracted 0.3−1 kg of pulverized sediments with chloroform: methanol (70: 30, v/v) for ~72 hr. Two subfractions with an intense reddish color were isolated from the total lipid extracts using silica gel column chromatography. The fractions contained copper-nickel alkylporphyrins

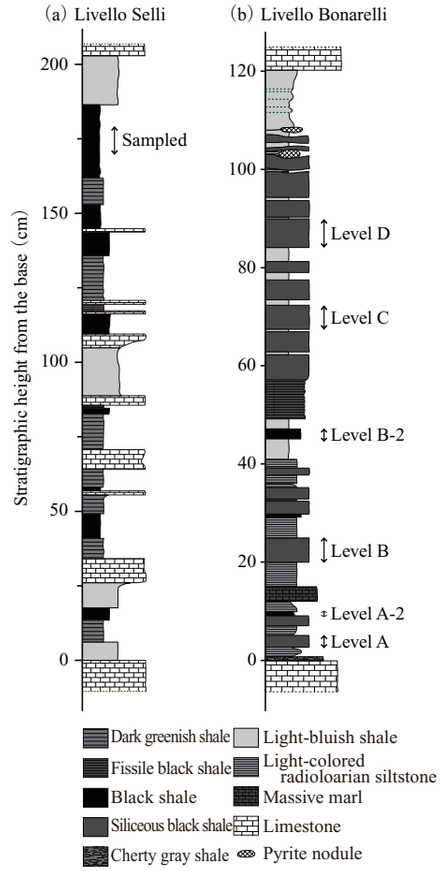


Fig. 4. Stratigraphic columns for **(a)** the Livello Selli black shale and **(b)** the Livello Bonarelli black shale at the Gorgo Cerbara section, central Italy. Sedimentary porphyrins within the black shale were analyzed 172−178 cm from the base of the Livello Selli and from four discrete stratigraphic levels within the Livello Bonarelli: Level A: 2−5 cm, Level A−2: 9−10 cm, Level B: 20−25 cm, Level B−2: 44−46 cm, Level C: 67−72 cm, and Level D: 84−89 cm, as measured from the bottom of the shale. Figure modified after Kashiyama et al. (2008a).

and vanadyl alkylporphyrins, respectively, eluted in that order by solvents with increasing polarity. Porphyrins in these fractions were further purified using reverse-phase open column chromatography prior to HPLC analysis and isolation.

Individual porphyrins were separated and isolated following multi-step HPLC methods for copper-nickel porphyrins described by Kashiyama et al. (2007a, 2008a) and for vanadyl

porphyrins described by Kashiyama et al. (2007a, 2008b). These multi-step methods using distinctive HPLC conditions (i.e., reversed- and normal-phase HPLC) allowed complete isolation of each porphyrin species from the others with baseline resolution, by which we avoided chromatographic isotopic fractionation (Filer 1999). The HPLC system (Agilent 1100 series) comprised a binary pump, an on-line degasser, an autosampler, a total temperature controller for HPLC columns (POLARATHERM™ Series 9000; Selerity Technologies Inc., Salt Lake City, UT), and an on-line photodiode array detector (DAD), as well as being optionally equipped with a fraction collector and a mass selective detector (MSD) connected via an atmospheric pressure chemical ionization (APCI) interface. The system was coupled to a personal computer on which Agilent Chemstation software was installed. The HPLC conditions can be found in Kashiyama et al. (2008a, b).

The purities of the isolated, isotopically analyzed porphyrins were estimated to be >95% based on the areas of the absorption chromatograms (at 392 nm for nickel porphyrins, 398 nm for copper porphyrins, and 408 nm for vanadyl porphyrins). The ^{1}H NMR spectra of the isolated porphyrins indicated little non-porphyrin impurity.

Structural assignment of porphyrins

The structural identification of nickel-complexed DPEP has been reported previously (Kashiyama et al. 2008a). The structures of the isotopically analyzed nickel porphyrins other than DPEP were determined based on the MSD mass spectra and the ^{1}H NMR spectra (Table 1) with reference to previously reported spectra of known structures (Fookes 1983, 1985; Serebrennikova et al. 1987; Verne-Mismer et al. 1990; Ocampo et al. 1992). The vanadyl porphyrins were identified through chromatographic analysis with reference to our laboratory standards (isolated from the middle Miocene Onnagawa Formation and structurally determined in Kashiyama et al. 2008b). The isotopically analyzed copper porphyrin (Cu DPEP) was identified through chromatographic analysis with reference to our Cu DPEP standard prepared from its vanadyl complex, originally obtained from the Onnagawa Formation (Kashiyama et al. 2008b) after transmetallation.

Nitrogen and carbon isotopic analyses

The nitrogen and carbon isotopic compositions of individual porphyrins were determined by a ThermoFinnigan Delta plus XP isotope-ratio mass spectrometer coupled to a Flash EA1112 automatic elemental analyzer via a Conflo III interface (EA/IRMS; Ogawa et al., this volume). Nitrogen and carbon isotopic compositions are expressed in conventional δ-notation vs. atmospheric N_2 (AIR) and Pee Dee belemnite (PDB) standards, respectively. In practice, the nitrogen and carbon isotopic compositions were calibrated against an isotopically defined laboratory standard (nickel octaethylporphyrin; $\delta^{15}N = +0.86‰$ and $\delta^{13}C = -34.17‰$; Aldrich Chemical Co., Milwaukee, WI). The detailed procedure is described in Kashiyama et al. (2008a) and Ogawa et al. (2010).

Analytical uncertainties due to instrumental conditions were estimated to be 0.12−0.17‰ for $\delta^{15}N$ and 0.08−0.22‰ for $\delta^{13}C$ (1σ; ranges reflect the uncertainty determined on different days). Analytical uncertainties caused by the separation and purification processes were estimated as 0.41‰ for $\delta^{15}N$ and 0.10‰ for $\delta^{13}C$ (1σ; Kashiyama et al. 2008a). We thus concluded that the overall analytical uncertainties for the data reported here were 0.4‰ for $\delta^{15}N$ and 0.2‰ for $\delta^{13}C$ (1σ).

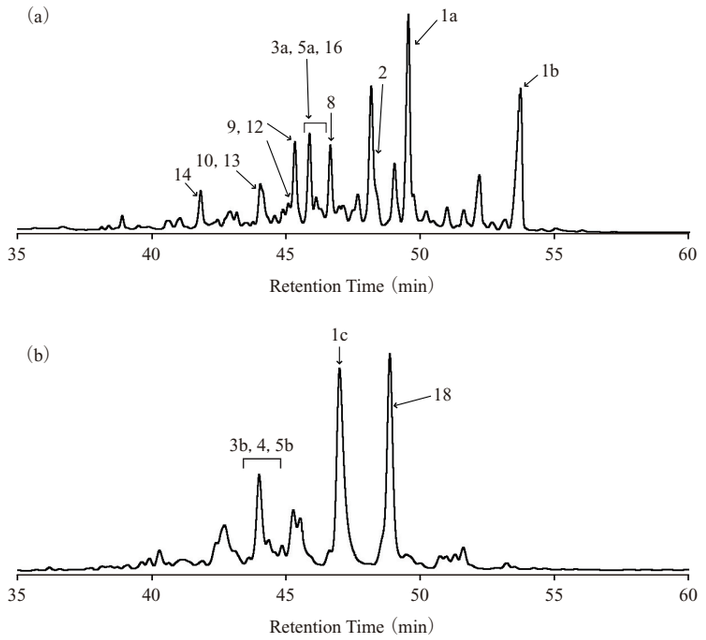


Fig. 5. Representative reverse-phase HPLC/DAD chromatograms of **(a)** the Ni-, Cu-alkylporphyrin fraction (sample from Level B of the Livello Bonarelli black shale; absorption at 392 nm) and **(b)** the VO-alkylporphyrin fraction (sample from Level D of the Livello Bonarelli black shale; absorption at 408 nm). Numbers refer to the chemical structures illustrated in Fig. 1.

Results

All of the samples from the Bonarelli black shale contained substantial amounts of Cu- and Ni-complexed alkylporphyrins with a relatively minor amount of vanadyl-complexed alkylporphyrins (hereafter VO-porphyrins). The samples from the Selli black shale contained Ni-porphyrins as the primary component with relatively minor amounts of Cu- and VO-porphyrins.

Fig. 5a shows a typical chromatogram of the Ni-Cu porphyrin fraction from the Bonarelli

Table 2 Nitrogen and carbon isotopic compositions of individual Ni- and Cu-porphyrins isolated from Cretaceous black shales.

	Livello Bonarelli												Livello Selli	
	Level A		Level A-2		Level B		Level B-2		Level C		Level D			
	$\delta^{15}N$	$\delta^{13}C$	$\delta^{15}N$	$\delta^{13}C$	$\delta^{15}N$	$\delta^{13}C$	$\delta^{15}N$	$\delta^{13}C$	$\delta^{15}N$	$\delta^{13}C$	$\delta^{15}N$	$\delta^{13}C$	$\delta^{15}N$	$\delta^{13}C$
Ni 3-nor-PYRR (14)	−4.0	−21.4	−5.7	−19.0	−5.1	−19.1	−4.6	−16.4	n.d.	n.d.	−5.2	−18.3	n.d.	n.d.
Ni 7-nor-PYRR (13)	−5.0	−20.7	−4.7	−18.8	n.d.	n.d.	−5.1	−16.6	n.d.	n.d.	−5.2	−21.2	−4.4	−18.6
Ni 17-nor-PHYL (10)	n.d.	n.d.	−6.7	−17.2	n.d.	n.d.	−4.9	−18.4	n.d.	n.d.	−9.1	−17.6	−5.3	−18.1
Ni 3-Me-PHYL (9)	−6.5	−22.0	−4.1	−17.8	n.d.	n.d.	−4.4	−16.4	n.d.	n.d.	−4.6	−17.1	−3.3	−19.4
Ni 3-Me-17-nor-DPEP (6)	−7.8	−27.2	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	−6.0	−18.5	−1.3	−20.0
Ni 3-nor-DPEP (3a)	n.d.	n.d.	−4.2	−19.3	−5.0	−19.5	−6.0	−16.9	−4.9	−17.5	−5.7	−17.7	−4.6	−18.0
Ni 7-nor-MeCPP (16)	−5.0	−21.4	−4.1	−17.6	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	−5.3	−20.1	−5.5	−23.3
Ni PHYL (8)	−5.5	−20.3	−2.2	−21.7	−7.1	−19.2	n.d.	n.d.	−5.8	−17.9	−6.6	−17.9	−3.1	−17.7
Ni 17-nor-DPEP (5a)	n.d.	n.d.	−4.1	−20.2	n.d.	n.d.	−6.6	−16.8	n.d.	n.d.	−6.5	−17.5	−2.3	−18.1
Ni 7-nor-DPEP (2)	−2.9	−21.2	−2.3	−19.5	−4.5	−20.2	−3.2	−17.2	−5.6	−17.7	−3.2	−18.2	n.d.	n.d.
Ni DPEP (1a)	−4.9	−20.5	−5.0	−19.6	−5.6	−19.6	−4.4	−16.9	−6.6	−17.9	−5.5	−18.0	−3.9	−18.9
Cu DPEP (1b)	−5.1	−20.1	−4.6	−18.4	−5.2	−18.5	−4.9	−17.9	−5.7	−16.3	−5.4	−16.9	n.d.	n.d.

PYRR: porphyrins structurally related to pyrroporphyrin XV (11) (absence of an alkyl group at the C−13 and C-15 positions).
PHYL: porphyrins structurally related to phylloporphyrin XV 7 (absence of an alkyl group at the C−13 position and presence of a methyl group at the C−15 position).

Table 3 Nitrogen and carbon isotopic compositions of individual VO-porphyrins isolated from Cretaceous black shales.

	Level D	
	$\delta^{15}N$	$\delta^{13}C$
VO DPEP (1c)	−5.2	−18.8
VO 3-nor-DPEP (3b)	−4.5	−18.1
VO 8-nor-DPEP (4)	−5.7	−18.3
VO 17-nor-DPEP (5b)	−8.6	−17.4
VO 13^2,17-cycloheptanoDPEP (18)	−3.5	−17.6

black shale. The relative abundances of the major nickel porphyrins did not significantly vary among samples, but the ratios of Ni- and Cu-porphyrins varied among samples. We thus isolated 4−11 species of Ni-porphyrins for isotopic analysis from each sample (Table 2), including those already described in the previous report (Ni-DPEP; Kashiyama et al. 2008a). We also isolated Cu-DPEP from all samples of the Bonarelli black shale, which is usually the most abundant alkylporphyrin in these samples. Fig. 5b shows the chromatogram of the VO-porphyrin fraction from Level D of the Bonarelli black shale. Five varieties of VO-porphyrins were isolated for isotopic analysis from this sample only (Table 3).

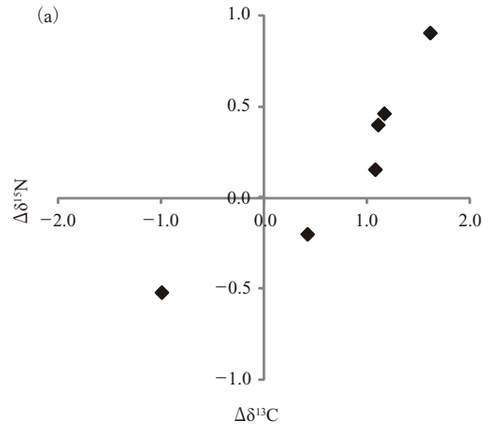
Discussion

Comparison of isotopic compositions among nickel, vanadyl, and copper porphyrins

Our results suggest that nitrogen and carbon isotopic compositions are not identical between porphyrins with an identical carbon skeleton but complexed with different metals. Fig. 6a shows that Ni-DPEP (1a) and Cu-DPEP (1b) are isotopically differentiated by as much as 0.9‰ for $\delta^{15}N$ and 1.6‰ for $\delta^{13}C$ between pairs from the same samples. These values are larger than the estimated analytical errors and are thus considered significant primary signals. Furthermore, Cu-DPEP, VO-DPEP (1c), VO−3-nor-DPEP (3b), and VO−17-nor-DPEP (5b) were also isotopically distinct from the Ni-analogues of these species (Fig. 6b).

It has generally been thought that metallation of sedimentary porphyrins occurs at the latest stage of diagenetic modification (e.g., Keely et al. 1990). If this was the case, the isotopic compositions of porphyrins with different metals but with the same structure would be identical. Little isotopic fractionation during metallation reactions has been observed in laboratory experiments (Kashiyama 2006), although this does not fully exclude the possibility of isotopic fractionation during metallation under natural conditions over a geological timescale. However, the observed differentiation of carbon isotopic composition is not easily explained by such fractionation effects during formation of different metal complexes from identical sources, because the carbon atoms of these alkylporphyrins are neither exchangeable nor directly involved in metal-complex formation. Instead, our results imply that the Cu-, Ni-, and VO-porphyrins in these samples were derived from different sources.

These results further suggest that metallation events occur at an early stage of chlorophyll-porphyrin diagenesis (cf. Junium et al. 2008). Ohkouchi et al. (2005) reported Cu-chelated bacteriochlorophylls *e* from relatively young sediments (~5 ka) of an anoxic lake. They inferred that elevated concentrations of Cu^{2+} in the anaerobic bottom/pore water had led to incorporation of Cu into the bacteriochlorophylls in the earliest stage of diagenesis. The abundant occurrence of Cu porphyrins in the Bonarelli black shale may thus be the consequence of strongly reduced bottom water conditions in the Livello Bonarelli basin



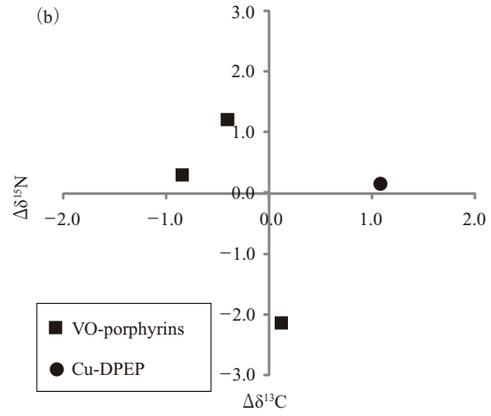


Fig. 6. Relative isotopic variations obtained for various sedimentary porphyrins. **(a)** $\Delta\delta^{15}N$ and $\Delta\delta^{13}C$ isotopic differences for Cu-DPEP porphyrin relative to Ni-DPEP for each sample. **(b)** $\Delta\delta^{15}N$ and $\Delta\delta^{13}C$ isotopic differences for various VO-porphyrins and Cu-DPEP porphyrin relative to Ni-DPEP for the sample from Level D of the Livello Bonarelli black shale.

during OAE−2 (e.g., Kuroda et al. 2005).

Isotopic variability among various porphyrin species and their sources

Both nitrogen and carbon isotopic compositions of Ni-porphyrins exhibited stratigraphically similar trends in a broad sense (Fig. 7). The trends generally followed those of Ni-DPEP (1a) reported previously (Kashiyama et al. 2008a). The significance of these trends has been already discussed there, and is summarized below.

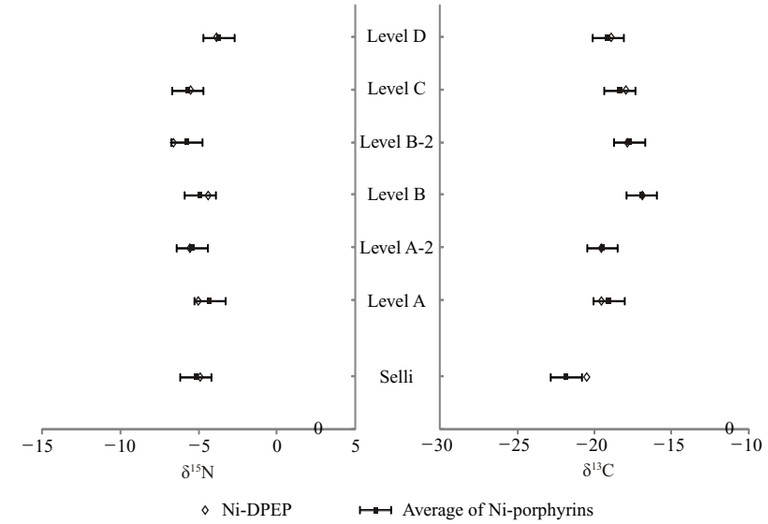


Fig. 7. Nitrogen and carbon isotopic compositions of Ni-DPEP and average values for all Ni-porphyrins. Error bars indicate ranges for various Ni-porphyrins (1σ).

Because DPEP is mainly derived from chlorophyll *a* produced by the majority of aerobic phototrophs, the isotopic compositions of DPEP presumably reflect mean values for the entire photoautotrophic community in the paleo-ocean (Ohkouchi et al. 2006). The tetrapyrrole nucleus of chlorophyll, which corresponds to DPEP, is depleted in ^{15}N by 4.8 ± 1.4‰ (1σ, n = 20) relative to the cell (Ohkouchi et al. 2006), whereas it is enriched in ^{13}C by 1.8 ± 0.8‰ (1σ, n = 18) relative to the cell (Ohkouchi et al. 2008). Therefore, the isotopic compositions of the original photoautotrophic biomass to be reconstructed are approximately 4.8‰ higher in $\delta^{15}N$ and approximately 1.8‰ lower in $\delta^{13}C$ than those determined for Ni-DPEP.

Accordingly, the mean $\delta^{15}N$ value of the entire photoautotrophic community throughout the relevant time period was estimated to be characteristically low (−2 to +1‰). This is a typical range for the $\delta^{15}N$ values of N-fixing photoautotrophs (Wada and Hattori 1976), chiefly cyanobacteria in oxygenated surface waters, in contrast to the nitrate-assimilating photoautotrophs, which should strongly reflect the relatively elevated $\delta^{15}N$ values of nitrate in the ocean (~ + 6‰; e.g., Miyake and Wada 1967). On the other hand, the $\delta^{13}C$ value of the photoautotrophic community was generally elevated, most likely reflecting either β-carboxylation and/or active transport of bicarbonate into the cell, which are typical metabolic activities for cyanobacteria (Ogawa and Kaplan 2003; Kashiyama et al. 2008a, 2008b). In addition, the apparent trend of $\delta^{13}C$ increasing upward stratigraphically within the Bonarelli black shale should partly reflect the positive shift in the $\delta^{13}C$ values of dissolved inorganic carbon, due to preferential sequestration of ^{12}C during global deposition

Table 4 Average nitrogen and carbon isotopic compositions of PHYL/PYRR types and DPEP types from each sample.

		PHYL/PYRR type			DPEP type		
		Average	1σ	*n*	Average	1σ	*n*
Livello Bonarelli							
Level A	$\delta^{15}N$	−5.3	1.0	4	−5.2	2.4	3
	$\delta^{13}C$	−21.1	0.7		−23.0	3.7	
Level A-2	$\delta^{15}N$	−4.7	1.7	5	−3.9	1.1	4
	$\delta^{13}C$	−18.9	1.7		−19.7	0.4	
Level B	$\delta^{15}N$	−6.1		2	−5.0	0.6	3
	$\delta^{13}C$	−19.2			−19.7	0.4	
Level B-2	$\delta^{15}N$	−4.8	0.3	4	−5.1	1.6	4
	$\delta^{13}C$	−17.0	0.9		−17.0	0.2	
Level C	$\delta^{15}N$	−5.8		1	−5.7	0.9	3
	$\delta^{13}C$	−17.9			−17.7	0.2	
Level D	$\delta^{15}N$	−6.1	1.8	5	−5.4	1.3	5
	$\delta^{13}C$	−18.4	1.6		−18.0	0.4	
Livello Selli	$\delta^{15}N$	−4.0	1.0	4	−3.0	1.5	4
	$\delta^{13}C$	−18.5	0.7		−18.7	0.9	

of the organic-rich black shale (Kuroda et al. 2007; Kashiyama et al. 2008a). Kashiyama et al. (2008a) thus concluded that N_2-fixing cyanobacteria significantly contributed to primary production during OAEs in the western Tethys Sea.

The results of the present work suggest that all of these Ni-porphyrins were derived from similar biological sources to DPEP (1a), thus similarly representing the average isotopic values of the entire marine photoautotrophic community. It is important to note that the porphyrins preserved in marine sediments should primarily be derived from chloropigments of aquatic photoautotrophs, because any chlorophyll produced by terrestrial plants is decomposed both metabolically and abiotically before reaching the aquatic sedimentary basin (Baker and Louda 1986; Sanger 1988; Keely et al. 1990; Eckardt et al. 1991; Chikaraishi and Naraoka 2005; Kashiyama et al. 2008b).

The ranges of isotopic compositions for sedimentary porphyrins with phylloporphyrin XV (7)-type structures (8−10; hereafter PHYL types) and those with pyrroporphyrin (11)-type structures (12−14; hereafter PYRR types) were indistinguishable from those of the DPEP types (Table 4). PHYL and PYRR types are specific varieties of ETIO types and thus do not have ring E, unlike the DPEP types. However, the PHYL and PYRR types do not have an alkyl group at the C−13 position, whereas etioporphyrin III is characterized by the presence of a C−13 ethyl group that is presumably inherited from the precursor heme structure (20). In particular, PHYL types are characterized by the presence of a C−15 methyl group, which is apparently unrelated to the structures of etioporphyrin III or heme. Callot and Ocampo (2000) proposed a scheme for the opening of ring E of chlorophylls

assuming an intermediate possessing a fragile functional group (34; such as purpurin−7 in Fig. 2; Airs et al. 2000). This scheme could reasonably produce PHYL and PYRR types after defunctionalization of the carboxylic groups and/or the ketoester (Fig. 2). Our results on nitrogen and carbon isotopic compositions are consistent with their scheme as well as carbon isotopic data reported by Boreham et al. (1989) and strongly suggest that the PHYL and PYRR types are also derived from chloropigments after the opening of ring E in an early stage of degradation.

7-nor-DPEP (2) was constantly enriched in ^{15}N relative to DPEP (1a) by 1.7 ± 0.7‰ (1 σ, n = 6) in each sample. Because most varieties of chloropigments including chlorophyll a (25), the most common pigments in all oxygenic phototrophs, are potential sources for DPEP, the isotopic signature of DPEP should represent the entire phototrophic biomass. Therefore, the regular isotopic difference observed for 7-nor-DPEP relative to DPEP can be regarded as evidence that 7-nor-DPEP is derived from a specific taxonomic group of the phototrophs (Kashiyama et al. 2008b). Chicarelli and Maxwell (1984) first reported 7-nor-DPEP and suggested that the absence of the methyl group at the C−7 position was attributable to defunctionalization of the C−7 formyl group present in chlorophyll b. Verne-Mismer et al. (1990) suggested that defunctionalization of the C−7 methoxycarbonyl group of chlorophyll c_3 (32) could also contribute to the production of 7-nor-porphyrins. Our isotopic evidence supports the above arguments, namely, that 7-nor-DPEP was derived from chlorophyll b (27) of green algae or chlorophyll c_3 of certain varieties of algae such as haptophytes, dinoflagellates, or diatoms (Zapata et al. 2006). It further suggests that, although Kashiyama et al. (2008a) demonstrated that diazotrophic cyanobacteria played a significant role in primary production during the OAEs, at least some part of the production was also contributed by algae. The 1.7‰ enrichment in the ^{15}N of 7-nor-DPEP may reflect the utilization of a relatively ^{15}N-enriched inorganic nitrogen source, such as nitrate from deep water, in addition to the nitrogen supplied through N_2-fixation by co-existing cyanobacteria.

No other regular isotopic differences such as that observed between 7-nor-DPEP (2) and DPEP (1a) were identified for any combination of Ni-porphyrins in the present dataset. For example, among the seven samples, 7-nor-PYRR (13) and 7-nor-MeCPP (16) exhibited no regular isotopic relationships to either to DPEP or 7-nor-DPEP, or to each other. In the conventional structure-based hypothesis, however, all of these Ni 7-nor-porphyrins would be considered to have originated from porphyrins with a fragile functional group, such as chlorophyll b (27) with the C−7 formyl group. In contrast, our results suggest that each Ni 7-nor-porphyrin represents a different spectrum of the photosynthetic biomass. Similarly, the three Ni 17-nor-porphyrins (5a, 6, 10) and the two Ni 3-nor-porphyrins (3a, 14) exhibited no systematic relationships, also suggesting different representation of the source biomass for each compound.

We tentatively consider that such inconsistencies between structural and isotopic observations are partly a reflection of stratigraphic variability as well as compositional heterogeneities in the sediments. Apparently, the fate of the precursor pigments, such as

preservation or loss of ring E, differed depending on factors such as the water column chemistry (e.g., Eh/pH conditions) during deposition as well as the organic or inorganic matrix in which the pigments were contained, which may have differentiated the chemical (micro-) environment to which the pigments were exposed during early diagenesis. For the Bonarelli black shale, the thickness of each sample comprising a single lithofacies ranged from 1−6 cm, which represents ~3−46 kyr assuming a sedimentation rate of 1.3−3.1 m myr^{-1} (Kuroda et al. 2007). Thus, the ocean during the OAEs may have experienced kiloyear- to sub-kiloyear-scale fluctuations in the physicochemical conditions (e.g., Eh/pH) of the basin bottom as well as the profile of the overlying water column (cf. Junium et al. 2008).

Conclusions

We determined nitrogen and carbon isotopic compositions of 17 Ni-, Cu-, and VO-porphyrins from two Cretaceous OAE black shales from central Italy. At first glance, all of the isotopic values appear to fall within the spectrum of chlorophyll-derived sedimentary porphyrins represented by DPEP (1a−c). However, a detailed evaluation reveals significant isotopic variations between different species of porphyrins. Significant differences were observed even between porphyrins with identical structures but different metals, suggesting that each variety of metalloporphyrin originated partly or entirely from different sources and hence carries different paleoenvironmental information. Most of the isotopic differences were irregularly variable from one sample to another among the various Ni-porphyrins. Such variability may reflect high instability in paleoenvironmental conditions; the resolution of isotopic signals as well as the structural signatures of sedimentary porphyrins may have been obscured by the limits imposed by the sample resolution.

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