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# An importance of diazotrophic cyanobacteria as a primary producer during Cretaceous Oceanic Anoxic Event 2

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## Abstract

In Livello Bonarelli black shale deposited during Cretaceous Oceanic Anoxic Event 2 (OAE-2, ca. 94 Ma), nitrogen isotopic compositions of bulk sediments are in a narrow range from  $-2.7$  to  $-0.7\text{‰}$ . We also determined molecular distribution and nitrogen isotopic compositions of geoporphyrins extracted from the black shale. The nitrogen isotopic compositions of  $C_{32}$  Ni deoxyphylloerythroetioporphyrin (DPEP) and total Ni porphyrins are  $-3.5$  and  $-3.3\text{‰}$ , respectively, leading us to the estimation that the mean nitrogen isotopic composition of photoautotrophic cell was around  $+1\text{‰}$  during the formation of Bonarelli black shale. This value is suggestive of  $N_2$ -fixation a dominant process for these photoautotrophs when assimilating nitrogen. Furthermore, Ni-chelated  $C_{32}$  DPEP, derived mainly from chlorophyll *a* was the highest concentration. Based on these evidence, we conclude that diazotrophic cyanobacteria were major primary producers during that time. The cyanobacteria may be key photoautotrophs during the formation of black shale type sediments intermittently observed throughout the later half of the Earth's history, and hence may have played a crucial role in the evolution of geochemical cycles.

## 1 Introduction

The Oceanic Anoxic Event (OAE; Schlanger and Jenkyns, 1976; Arthur et al., 1985) can be defined as “the time envelope during which the global ocean conditions were propitious for the deposition of organic carbon rich sediments (but not implying global total anoxia of deep-sea masses)” (Arthur and Sageman, 1994). The organic carbon-rich, dark-colored sediments accumulated during the OAEs are called “black shales”, whose color is believed to be originated from organic matter or iron monosulfides. In Cretaceous, six OAEs have been recognized (Kuroda and Ohkouchi, 2006<sup>1</sup>, and ref-

<sup>1</sup>Kuroda, J. and Ohkouchi, N.: Implications of spatiotemporal distribution of black shales deposited during Cretaceous Oceanic Anoxic Event-2, submitted, 2006.

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erences therein). Many studies have discussed the causal mechanisms for the deposition of such organic-rich sediments. They include the sluggish deepwater circulation caused by stratified ocean (e.g., Degens and Stoffers, 1976; Jenkyns, 1980), high biological productivity (e.g., Pedersen and Calvert, 1990), and changes in surface water ecology (Ohkouchi et al., 1997; Sinninghe Damsté and Köster, 1998; Kuypers et al., 2004; Kuroda et al., 2005).

In this study we determined nitrogen isotopic composition of organic matter to understand nitrogen nutrition of biological communities in the surface ocean during the Cretaceous OAE-2, around 94 Ma. Geoporphyrins, molecular markers for photoautotrophs are investigated together with the isotopic composition of bulk sediments. Geoporphyrins are tetrapyrrole molecules with alkyl chains whose structures are strongly suggestive of derivatives of chlorophylls, heme, vitamin B<sub>12</sub>, cytochrome, and some others (Appendix A; e.g., Treibs, 1934; Baker and Louda, 1986; Callot and Ocampo, 2000). Since production of chloropigments in natural environments were estimated to be overwhelmingly large relative to those of other compounds, the nitrogen isotopic composition of geoporphyrins should mainly reflect those of chloropigments (e.g., Baker and Louda, 1986; Hayes et al., 1987; Callot and Ocampo, 2000). Based on these evidences, here we discuss an importance of diazotrophic cyanobacteria in western Tethys Sea during the Cretaceous OAE-2.

## 2 Sample and methods

### 2.1 Samples

We collected “Livello Bonarelli” black shale and adjacent rocks from an outcrop at Gorgo Cerbara in the northern Apennines, Italy. The Livello Bonarelli is characterized by an alteration of millimeter- to centimeter-scale dark and light layers (e.g., Arthur and Premoli-Silva, 1982; Jenkyns et al., 1994; Kuroda et al., 2005) and has been considered to be one of the major representatives of OAE-2. Detailed description of geology

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of this region and the Bonarelli sediments were provided in Arthur and Premoli Silva (1982) and Kuroda et al. (2005), respectively. At the Gorgo Cerbara outcrop, forty-three blocks were sampled from across the 104-cm interval of Bonarelli. In order to obtain “fresh” rocks, 5–20 cm of the outcrop surface was removed before sampling. These block samples were sliced at 1.5 mm intervals (Kuroda et al., 2006<sup>2</sup>) and some of them were supplied for the analysis of nitrogen isotopic composition of bulk sediments. For geoporphyrin analyses, we chose GCB-17 sample, which was collected at 51 cm above the base of Bonarelli.

## 2.2 Extraction, separation, and analyses of geoporphyryns

Detailed procedures and instrument conditions for chloropigment analyses will be described separately (Kashiyama et al., 2006<sup>3</sup>). The pulverized sediments (58.3 g) were Soxhlet-extracted with MeOH/dichloromethane (3:7, v/v) for three days. The total extract was separated by silica gel column chromatography (Aldrich, 200–400 mesh, 1% deactivated with H<sub>2</sub>O) to 8 sub-fractions. Fractions containing porphyrins were easily identified by color bands. Third fraction (N-2b) was eluted with *n*-hexane/dichloromethane (50:50, v/v) to collect a reddish orange-colored band originated from Ni porphyrins. Fifth fraction (N-2d) was eluted with *n*-hexane/dichloromethane (30:70, v/v) to collect a pink-colored band originated from VO porphyrins. Fractions containing geoporphyryns were injected to reversed-phase high-performance liquid chromatography (HPLC). The HPLC system consisted of a solvent delivery system that comprised a binary pump (Agilent G1312), an on-line degasser (Agilent G1322), an autosampler (Agilent G1313A), an on-line photodi-

<sup>2</sup>Kuroda, J., Ogawa, N. O., Taminizu, M., Coffin, M. F., Tokuyama, H., Kitazato, H., and Ohkouchi, N.: Massive volcanism as a causal mechanism for a Cretaceous oceanic anoxic event, submitted, 2006.

<sup>3</sup>Kashiyama, Y., Tada, R., Kitazato, H., and Ohkouchi, N.: An improved method for isolation and purification of sedimentary porphyrins by high-performance liquid chromatography for compound-specific isotopic analysis, submitted, 2006.

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ode array detector (Agilent G1315), and a mass detector (Agilent 1100 series SL). This system was coupled to a personal computer with Agilent Chemstation software. The reversed-phase HPLC analyses were performed using three ZORBAX SB-C18 columns (4.6×250 mm; 5 μm silica particle size) connected in series protected by a ZORBAX SB-C18 column (4.6×12.5 mm, 5 μm silica particle size). The solvent gradient was programmed as acetonitrile/H<sub>2</sub>O/pyridine (89.8:10:0.2, v/v) (solvent A) and acetonitrile/pyridine (99.8:0.2, v/v) (solvent B) gradient ((time min, %B): (0, 70) (90, 70) (150, 100) (200, 100)). Solvent flow rate was 1.0 mL min<sup>-1</sup>. HPLC-grade solvents (Wako Pure Chemicals) were used for HPLC and LC/MS analyses after being ultrasonicated for 30 min.

Temporal identifications of the compounds were achieved based on retention time, UV-Vis spectra, and mass spectra. The mass spectra were collected from *m/z* 400–1200 with atmospheric pressure chemical ionization (APCI) mass spectrometry operated in a positive-ion mode (Nakajima et al., 2003). Conditions of APCI were as follows: drying gas flow: 6.0 L min<sup>-1</sup>, nebulizer pressure: 50 psig, drying gas temperature: 350°C, vaporizer temperature: 500°C, capillary voltage (positive): 4000V, and corona current: 5.0 μA. By using a fraction collector, we isolated compounds with great care to collect entire peak to avoid isotopic fractionation (Ohkouchi et al., 2005; Kashiyama et al., 2006<sup>3</sup>).

### 2.3 Isotope analyses

A purified porphyrin and N-2b fraction were transferred to precleaned tin capsules with dichloromethane. The solvent was dried under a N<sub>2</sub> stream and the capsules were carefully folded with forceps. The isotopic composition of sedimentary bulk nitrogen and geoporphyryns were determined by an on-line system of ThermoFinnigan Delta plus XP isotope-ratio mass spectrometry coupled to a flash EA1112 automatic elemental analyzer through a ConFlo III interface (Ohkouchi et al., 2005). The nitrogen isotopic composition is expressed as conventional δ-notation relative to atmospheric N<sub>2</sub>. Based on the repeated measurements of our laboratory standards (proline and

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tyrocine) and a porphyrin standard (octaethylporphyrin, Aldrich), analytical error ( $1\sigma$ ) was estimated to be within 0.2‰.

### 3 Results and discussion

#### 3.1 Nitrogen isotopic composition of bulk sediments in Cretaceous black shales

5 Figure 2 illustrates nitrogen isotopic composition of bulk sediments ( $\delta_{\text{bulk}}$ ) from Bonarelli black shales and limestone just beneath the shale (Kuroda, 2005). In the black shale, the  $\delta_{\text{bulk}}$  values range from  $-0.7$  to  $-2.7\text{‰}$  (average= $-1.9\text{‰}$ ,  $n=23$ ), whereas those of limestone sequence from  $-1.3$  to  $+2.6\text{‰}$  (average= $+0.9\text{‰}$ ,  $n=22$ ). This result basically supports our previous observation that the  $\delta_{\text{bulk}}$  of Bonarelli black shales is confined to a narrow range from  $-2$  to  $0\text{‰}$  (Ohkouchi et al., 1997). They did not find any correlation between total nitrogen and potassium contents as a relative measure of clay minerals in the black shale, and concluded that the nitrogen in the black shale mostly exist as organic nitrogen rather than ammonium nitrogen which is potentially adsorbed on the surface or interlayer of clay minerals (Müller, 1977). Coupling with abundant geohopanooids, derivatives of components of cellular membrane of prokaryotes, in these samples, they concluded that the diazotrophic cyanobacteria could have been major primary producers during the OAE2.

15 In the biological  $\text{N}_2$ -fixation process, the reactant dinitrogen is catalytically converted to an end product ammonium in the cell. This process includes a cleavage of very stable nitrogen-nitrogen triple bond in dinitrogen which theoretically suggests it to have a large isotopic fractionation. However, isotopic fractionation factors experimentally determined based on many laboratory culture experiments and field observations of diazotrophic organisms exhibited much smaller than theoretically anticipated ( $\alpha=1.000$ – $1.002$ , where  $\alpha=^{14}\text{k}/^{15}\text{k}=1-\delta^{15}\text{N}/1000$ ;  $^{14}\text{k}$  and  $^{15}\text{k}$  are rate constants of  $^{14}\text{N}$  and  $^{15}\text{N}$ , respectively) (Hoering and Ford, 1960; Delwiche and Steyn, 1970; Wada, 1980; Wada and Hattori, 1991). It suggests that another chemical step rather than breaking the

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triple bond requires more energy or lacks backward reaction in certain steps of N<sub>2</sub>-fixation (Wada, 1980). Although we still do not understand the detailed mechanism(s) related to isotopic behavior during the N<sub>2</sub>-fixation, the small isotopic fractionation factor in overall N<sub>2</sub>-fixation process strongly contrasts with relatively large isotopic fractionation factors associated with assimilation processes of nitrate, nitrite, and ammonium (Wada and Hattori, 1991). Dissolved dinitrogen in seawater has quite constant isotopic composition around 0.5‰ in the modern ocean (e.g., Miyake and Wada, 1967), so that the cellular nitrogen assimilated through the N<sub>2</sub>-fixation is confined to a narrow and characteristic isotopic range from 0 to -2‰ (Minagawa and Wada, 1986).

The  $\delta_{\text{bulk}}$  values in the Cretaceous black shales were also reported by Rau et al. (1987). They determined the  $\delta_{\text{bulk}}$  of the black shales (they referred them “laminated black marlstone” and “black clay stone”) from DSDP sites 367, 530, and 603 in Atlantic Ocean and found that the  $\delta_{\text{bulk}}$  values are in a range from -2.7 to 0.6‰, which are substantially lower than those of adjacent organic-poor samples (-0.7 to 5.7‰). Based on the isotopic evidence, they concluded that the diazotrophic cyanobacteria could be major primary producers at those times. Recently, Kuypers et al. (2004) reported the  $\delta_{\text{bulk}}$  values of sediments both from OAE-1a from central Italy and OAE-2 from DSDP site 367 in the North Atlantic. In these black shales, they also found 2-methylhopanoids, derivatives of 2-methylhopanepolyols specifically produced as a component of membrane reinforces by some (not all) cyanobacteria and prochlorophytes (Bisseret et al., 1985; Simonin et al., 1996; Summons et al., 1999), leading them to the conclusion that the diazotrophic cyanobacteria were important primary producers during the Cretaceous Oceanic Anoxic Events.

However, we should note two pitfalls when interpreting the  $\delta_{\text{bulk}}$  records. First, as mentioned above, ammonium and organic compounds adsorbed on or fixed in interlayers of clay minerals could partly contribute to the bulk nitrogen in the sediments (e.g., Müller, 1977). Therefore, it potentially alters the primary isotopic signature significantly, even though it may account for a small fraction. Second and more importantly, the decomposition of organic matter in the water column and sediments is quite complex

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processes mediated by a variety of aerobic and anaerobic microbes involving deamination that potentially alter the nitrogen isotopic signature of original organic matter (Macko and Estep, 1984). In fact, many studies have observed that the degradation of organic matter leads to alteration of original nitrogen isotopic signature in both aerobic and anaerobic environments at least in very early stage of diagenesis (Wada, 1980; Altabet and Francois, 1994; Nakatsuka et al., 1997; Freudenthal et al., 2001; Lehmann et al., 2002). Such changes in isotopic signature during the early diagenesis apparently contrast with the view that the sedimentary nitrogen isotopic signature is more or less faithfully record the isotopic composition of organic matter produced in the surface ocean (e.g., Calvert et al., 1992; Altabet and Francois, 1994; Nakatsuka et al., 1995; Farrell et al., 1995). It is mainly based on the field observations that the  $\delta_{\text{bulk}}$  values from surface sediments are in good agreement with those of particulate organic matter in overlying surface water and sinking particles (Altabet and François, 1994; Farrell et al., 1995). Further studies are definitely required for rigorously evaluating the fidelity of  $\delta_{\text{bulk}}$  values as a recorder of organic matter formed in the euphotic zone.

One of the best ways to circumvent the situation is to determine the nitrogen isotopic compositions of molecular markers derived from marine photoautotrophs. For this purpose, geoporphyrins, derivatives of chlorophylls, green photosynthetic pigments produced by algae, cyanobacteria, and higher plants, are one of the best candidates, because not only they are strongly source-specific but also they contain four nitrogen atoms in a single molecule.

### 3.2 Geoporphyrins in Bonarelli black shale

Figure 2 illustrates a total ion chromatogram of reversed-phase HPLC for the “Ni porphyrin fraction” (fraction N-2b) of an extract from the Bonarelli black shale sample. The Ni-chelated porphyrins are the most abundant porphyrin homologue in this sample, whereas VO porphyrins (fraction N-2d) secondly abundant. In the chromatogram of N-2b fraction, the largest peak (Peak 1 in Fig. 2) was temporally assigned as C<sub>32</sub> Ni deoxophylloerythroetioporphyrin (DPEP, Appendix A; Baker et al., 1968) based on the

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interpretation of mass spectrum and comparison of absorption spectrum with previous results (Baker and Louda, 1986; Figs. 3a and b). The tetrapyrrole structure of C<sub>32</sub> DPEP with an exocyclic ring in the southern part of the molecule is closely related to that of chlorophylls, and it has generally been accepted that the C<sub>32</sub> DPEP must derive primarily from them (Baker and Louda, 1986; Callot and Ocampo, 2000). Since carbon number of the porphyrins in our sample ranged mostly from 29 to 33 (Fig. 2), they would be originated mainly from chlorophylls. There are four major types of chlorophylls, namely chlorophylls *a*, *b*, *c*, and *d* (Appendix A). Theoretically, all these chlorophylls are potential precursors of the sedimentary C<sub>32</sub> Ni DPEP. However, we think that the chlorophyll *a* is a dominant precursor. In natural environment, the chlorophyll *a* is far more abundant and widespread relative to other chlorophylls. In the ocean, the chlorophyll *c* is a secondly important chlorophyll, which is produced by various groups of algae including diatoms and dinoflagellates. It is synthesized as a minor form of chlorophyll with abundant chlorophyll *a*. Distributions of the chlorophylls *b* and *d* are more limited in the oceanic photoautotrophs; the former is produced by green algae (Blankenship, 2004), whereas the latter by red algae and cyanobacteria (Larkum and Kühl, 2005). The consideration that the C<sub>32</sub> DPEP was originated from chlorophyll *a*, is consistent with the fact that it is the most abundant porphyrin species in many geological samples (e.g., Baker and Louda, 1986; Boreham et al., 1990; Keely et al., 1990; Sundararaman and Boreham, 1991; Keely et al., 1994). In our Bonarelli black shales, terrestrial contribution was estimated to be minor based on organic geochemical evidence including relative abundance of *n*-alkanes from higher plant wax (Van Grass et al., 1983; Farrimond et al., 1990; Ohkouchi et al., 1997; Kuroda, 2004). Therefore, we conclude them derived mostly from chlorophyll *a* produced by aquatic photoautotrophs.

Although bacteriochlorophyll *a* produced mainly by purple sulfur bacteria is also a potential precursor of C<sub>32</sub> Ni DPEP, we think them unlikely to be important contributors. Since they require H<sub>2</sub>S as an electron donor (Imhoff, 1995), the purple sulfur bacteria have generally been observed around O<sub>2</sub>/H<sub>2</sub>S interface occurred relatively shallow (~10 m) portion of the water column (e.g., Overmann et al., 1991; Van Gerner-

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den, 1995; Nakajima et al., 2003). They adapt to grow under strong light environment, whereas the growth and reproduction are strongly limited under low irradiance (Van Gernerden, 1995). As suggested below, during the Bonarelli formation, the O<sub>2</sub>/H<sub>2</sub>S interface should have existed in the lower part of photic zone, where the growth of purple sulfur bacteria must strongly be limited due to the low irradiance.

A small peak 2 in Fig. 2 has a relatively strong absorption maximum at 398 nm with a slight maximum at 500–570 nm (Fig. 3c), suggesting it to be a mixture of porphyrins. The peak has a mass spectrum characterized by several ions including a minor ion at  $m/z$  561 as well as  $m/z$  524, 538, and 540 which may be assigned to molecular/isotope ions of Cu-chelated porphyrins (Fig. 3d). The molecular ion  $m/z$  561 possibly corresponds to  $[M+H]^+$  of C<sub>34</sub> Ni porphyrin. It is a very weak ion accounting for about 20% of the total ion current, but the retention time (ca. 30 min after the C<sub>32</sub> Ni DPEP) of the peak in the HPLC chromatogram supports this idea (Fig. 2). Theoretically, the C<sub>34</sub> porphyrin should be derived from bacteriochlorophyll *d* or *e* homologues (Callot et al., 1990; Gibbison et al., 1995), with the former having long alkyl chains at C-8 and C-12, while the latter having a long alkyl chain at C-8, an ethyl chain at C-12, and an extra methyl chain at C-20 (Appendix A). Our observation is consistent with Pancost et al. (2004) which reported methyl isobutyl maleimide, a degradation product of bacteriochlorophylls *d* and *e*, in OAE-2 sediments from central Italy and Tunisia. Since both bacteriochlorophylls *d* and *e* are distributed only in strictly anaerobic green sulfur bacteria (e.g., Otte et al., 1993; Glaeser et al., 2002; Nakajima et al., 2003), the presence of derivatives of these bacteriochlorophylls indicates that at least the base of photic zone was anaerobic at these sites during the OAE-2. This consideration supports previous observation of Sinninghe Damsté and Köster (1998) which reported derivatives of isorenieratene, a carotenoid pigment of green sulfur bacteria in the Bonarelli black shale. Since the estimated amounts of both C<sub>34</sub> porphyrin and isorenieratene derivatives in Bonarelli are small (2 ng gC<sub>org</sub> and 11 μg gC<sub>org</sub>, respectively), the contribution of green sulfur bacteria to the primary production was substantially small and the O<sub>2</sub>/H<sub>2</sub>S interface would have existed deeper part of the photic zone.

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### 3.3 Nitrogen isotopic composition of geoporphyrins in the Bonarelli black shales

Since a chromatographic peak of C<sub>32</sub> Ni DPEP is clearly separated from other peaks with baseline resolution (Fig. 2), we succeeded baseline-to-baseline isolation of C<sub>32</sub> Ni DPEP by a preparative technique which minimized the isotopic fractionation associated with the isolation step (Bidigare et al., 1990). The nitrogen isotopic composition of C<sub>32</sub> Ni DPEP is -3.5‰. We also determined the nitrogen isotopic composition of total Ni porphyrins (total fraction of N-2b), giving data (-3.3‰) close to that of C<sub>32</sub> Ni DPEP. In this sample, non-geoporphyrin compounds could “contaminate”, which potentially alter the isotopic signature. Nevertheless, we think that the nitrogen contained in this fraction would be derived mainly from geoporphyrins. In the silica gel column chromatography, we collected only a narrow band colored by Ni porphyrins. Furthermore, the nitrogen-containing, solvent-extractable compounds are functionalized mainly to amide, amine, and cyano groups. Generally, the polarities of these molecules are substantially larger than those of Ni porphyrins and eluted in more polar sub-fractions during the silica gel column chromatography.

To properly interpret the nitrogen isotopic record of sedimentary porphyrins, we have to understand the factors controlling that of chlorophyll during the biosynthesis and potential alteration of isotopic signature during the diagenetic transformation from chlorophyll to geoporphyrin(s). In Fig. 4, we illustrated a synthetic pathway of chlorophyll *a* in the cell, which is basically the same as that of other chloropigments including bacteriochlorophylls (Senge and Smith, 1995). A unique precursor for all the biologically synthesized tetrapyrroles including chlorophylls is 5-aminolevulinic acid (ALA, 3). Therefore, the nitrogen atoms in chloropigments are originated exclusively from those in ALA. In case of chlorophylls, the ALA is synthesized from L-glutamate (1) through C5 pathway (e.g., Beale, 1993) (Fig. 4). In the C5 pathway, the glutamate is transformed to glutamate-1-semialdehyde (GSA, 2) through activated form of glutamate (Glutamyl-tRNA). Then, the amino group at position C-2 of GSA is replaced by one at C-1, yielding ALA (Beale, 1993; Kannangara et al., 1994). It has been proved that the

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ALA was formed from GSA by *intermolecular* nitrogen transfer (Mau and Wang, 1988; Mayer et al., 1993) catalyzed by GSA aminotransferase. Therefore, the nitrogen isotopic composition of ALA is theoretically derived from ammonium which is aminated to GSA. Subsequently, 8 molecules of ALA condensate to form uroporphyrinogen III (5), which is a precursor of tetrapyrrole structure of chlorophylls. Although four ammonium were removed in this step, the process should not produce significant isotope fractionation because bonds to pyrrolic nitrogen are neither formed nor broken (Sachs et al., 1999). Once the uroporphyrinogen III is formed, an only step related to the nitrogen atoms in the tetrapyrrole structure is a branch between Mg-chelation (to form chlorophylls) and Fe-chelation (to form heme) which occurs in the late stage of chlorophyll synthesis (Walker and Willows, 1997). Although it was not strictly verified, this metalation catalyzed by Mg-chelatase may not have isotopic fractionation, since Mg branch receives overwhelmingly large quantity of precursors (protoporphyrin) (Beale, 1993). Furthermore, recent experiments inserting metal (chemically but not enzymatically) in the tetrapyrrole structure in our laboratory exhibited little or no isotopic fractionation (Kashiyama et al., 2006<sup>3</sup>). Overall, the nitrogen isotopic composition of chlorophylls should theoretically be equivalent to or quite similar with that of ALA, and hence that of ammonium aminated to GSA.

It has been known that the nitrogen isotopic composition of chlorophylls is somewhat depleted in <sup>15</sup>N relative to the whole cell (Chikaraishi et al., 2005). Figure 5 compiles the nitrogen isotopic relationship between chlorophylls (chlorophylls *a* and *b*) and cell of aquatic photoautotrophs (Sachs et al., 1999; Goericke and Montoya, unpub. results). It shows that the nitrogen isotopic composition of chlorophylls is depleted in <sup>15</sup>N by 4.8±1.4‰ (1σ, n=20) relative to the cell. This <sup>15</sup>N depletion suggests that nitrogen in other compounds in the cell like proteins and DNA are substantially enriched in <sup>15</sup>N relative to chlorophylls. However, at this moment, we still do not have little information on the nitrogen isotopic distribution in the cell to verify it. Further investigations are required for the refinement of the isotopic relationship between chlorophylls and cell to more critically discuss the nitrogen isotopic record in the chlorophylls.

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Moreover, the diagenetic transformation from chlorophylls to porphyrins is potentially a process alter the original isotopic record. The transformation pathways from chlorophylls to porphyrins in the water column and sediments have long been investigated and several schemes have been proposed (e.g., Baker and Louda, 1986; Eckardt et al., 1991; Callot and Ocampo, 2000; Ohkouchi et al., 2005a). They are a series of complex chemical and microbial processes including defunctionalization, condensation, and metallation. However, all these processes except for demetallation (Mg loss) and metal insertion (Ni chelation) are not related to the nitrogen atoms in tetrapyrrole structure. During the Mg-loss process, we observed no isotopic discrimination in a modern meromictic lake (Ohkouchi et al., 2005b). In our laboratory experiments, we have recently observed little isotopic fractionation associated with Ni chelation in octaethylporphyrin (Kashiyama et al., 2006<sup>3</sup>). Therefore, we think that the nitrogen isotopic signature of geoporphyrins is directly inherited from chlorophylls.

Applying above discussion, we reconstructed the nitrogen isotopic composition of photoautotrophic cell of  $\sim +1\%$ . This cell is significantly depleted in  $^{15}\text{N}$  relative to those generally observed in that assimilated nitrate, but close to the range of diazotrophs as described above. Together with the consideration that the chlorophyll *a* could be a major precursor for these geoporphyrins, we conclude that the diazotrophic cyanobacteria were major photoautotrophs during the formation of Bonarelli black shale.

To our knowledge, two reports have determined the nitrogen isotopic compositions of porphyrins/chlorins in geological samples. In Triassic Serpiano oil shale, Chicarelli et al. (1993) determined the nitrogen isotopic compositions of 7 porphyrin species and found the range of  $\delta^{15}\text{N}$  values between  $-3.4$  and  $-3.0\%$ . They concluded that “cyanobacterial fixation of  $\text{N}_2$  may have been the main cause of  $^{15}\text{N}$  depletion”. If applying that the nitrogen isotopic difference between chlorophyllide portion of chlorophylls and whole cell is about 5 (Fig. 5), the reconstructed isotopic composition of primary producer should have been 0 to 2%. In Mediterranean sapropels, Sachs and Repeta (1999) determined the nitrogen isotopic compositions of pyropheophorbide *a* isolated from sapropel layers (S2, S3, and S4) from eastern Mediterranean Sea deposited dur-

ing the late Quaternary and found them around  $-5\%$ . They concluded that the “nitrogen fixation supply a substantial fraction of new nitrogen” at those periods. The conclusions lead by these two independent studies coincide with what we concluded above.

### 3.4 Cyanobacteria hypothesis

5 The cyanobacteria are a diverse group of gram-negative prokaryotes which are unicellular organisms lacking cellular differentiation. All cyanobacteria contain chlorophyll *a* and undergo photosynthesis, generating oxygen. Many, though not all, cyanobacteria have an ability to fix dinitrogen using nitrogenase, an enzyme that catalyzes the reduction of dinitrogen to ammonium (e.g., Bergman et al., 1997). In the modern ocean,  
10 the genus *Trichodesmium* (mainly *Trichodesmium thiebautii* and *Trichodesmium erythraeum*) primarily inhabits surface waters of oligotrophic tropical and subtropical regions where the seawater temperature of surface mixed layer is higher than  $25^{\circ}\text{C}$  (Carpenter, 1983; Capone et al., 1997). This genus often forms extensive surface blooms in western boundary currents like Kuroshio or Gulf Stream where the water  
15 column is characterized by low nutrient and vertically very stable with the mixed layer of around 100 m (e.g., Marumo and Asaoka, 1974; Carpenter and McCarthy, 1975; Carpenter and Romans, 1991; Capone et al., 1997; Dupouy et al., 2000). Furthermore, some other unicellular cyanobacteria, especially for two genera, *Synechococcus* and *Prochlorococcus* have also been found at  $10^3$  and  $10^5$  cells  $\text{mL}^{-1}$ , respectively, in  
20 oligotrophic oceans and accounts for significant fraction of primary production in some regions of tropical-subtropical Atlantic and Pacific (e.g., Campbell et al., 1997; Ting et al., 2002). Recent studies have reported that the unicellular diazotrophic cyanobacteria played a significant role in nitrogen cycle in oligotrophic ocean (Zehr et al., 2001; Montoya et al., 2004). Ecology of these planktonic cyanobacteria remains largely unknown  
25 and factors controlling cyanobacterial blooming is still a matter of debate (e.g., Pearl, 1996; Bianchi et al., 2000). However, nutrient other than nitrogen, either phosphorus or iron is potentially a limiting factor for the formation of cyanobacterial bloom (Gruber and Sarmiento, 1997; Fuhrman and Capone, 2001; Dyhrman et al., 2006). It has been

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known that the growth and reproduction of diazotrophic cyanobacterium require large amounts of iron and molybdenum, because the nitrogenase contains 28 to 36 iron and 2 molybdenum atoms in a single enzyme to facilitate electron transfer reactions (Hardy and Havelka, 1975; Raven, 1988). Therefore, high input of iron could be one of the major factors to control the blooming of N<sub>2</sub>-fixing diazotrophic cyanobacterium in the ocean (Falkowski, 1997).

Around the Cenomanian-Turonian boundary, the sea surface temperature was estimated to be substantially higher than that of the present (e.g., Wilson and Norris, 2001). Even in the high latitudes of both Northern and Southern Hemispheres, it has been estimated to be as high as 20°C or more (Huber, 1998; Jenkyns et al., 2004). The strong stratification in the water column as suggested by the existence of derivatives of bacteriochlorophyll *e* and isorenieratene (Sinninghe Damsté and Köster, 1999) would have strongly prevented the supply of nutrient from the deeper water to surface water. Such warm and oligotrophic surface water in the Cretaceous Oceanic Event resembles to the modern oligotrophic surface water in the tropical and subtropical ocean. It could be, therefore, suitable for the inhabitation of cyanobacteria in the surface oxic layer of the water column at that time. Recently, Dumitrescu and Brassell (2005) observed abundant 2β-methylhopanes and 2β-methylhopanones in OAE-1a sediments from Pacific Ocean (Shatsky Rise, ODP Leg 198) and concluded that the cyanobacteria could have largely contributed to them.

#### 4 Conclusions and implications

Geoporphyrin distribution and nitrogen isotopic compositions of geoporphyrins from the Bonarelli black shales deposited at Cenomanian-Turonian boundary, Cretaceous suggested that the diazotrophic cyanobacterium was a dominant source of its organic matter. This cyanobacteria hypothesis appears to explain several features of the Bonarelli black shales including high C/N ratio and sacked-shape organic matter (Ohkouchi et al., 2003). Nevertheless, our argument in this study strongly relies on a limited number

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of data and it should be verified with more data in future.

The black shales and “black shale like sediments” including sapropels have been observed not only in Cretaceous, but also in Quaternary (e.g., Arthur and Sageman, 1994; Mediterranean, Cita et al., 1977; Black Sea, Ross et al., 1970; Japan Sea, Oba et al., 1991; Tada and Irino, 1999), Jurassic (e.g., Stein et al., 1986; Jenkyns, 1988; Harries and Little, 2000), and Paleozoic (e.g., Cluff, 1980). Furthermore, in the early Proterozoic, up to 30% (generally 10%) of total shale is organic-rich “black shales” (Condie et al., 2000). It appears that the organic-rich, black-colored sediments are intermittently observed at least later half of Earth’s history. Although at this moment we do not know that the causal mechanisms for these black shales are identical between them, some of these events have been estimated to be cyanobacterial origin (Sachs and Repeta, 1999; Dumitrescu and Brassell, 2005). If the cyanobacteria hypothesis can be extended to all other black shale type sediments, they have played a crucial role in the evolution of biogeochemical cycles even in the later half of the Earth’s history.

Microscopic observations of Archean sedimentary rock (~3.5 billion years) have indicated that the cyanobacteria were the first photosynthesizing organism in Earth’s history (e.g., Schopf, 1994). They are believed to be a major primary producer around 1 billion years after their appearance (Rothschild and Mancinelli, 1990). The oceanic environments during the Cretaceous OAEs may have been similar with those of Archean when atmospheric O<sub>2</sub> level was substantially lower than today. Oxygen deficiency in oceanic environment occurred intermittently may have given rise to reincarnation of surface water ecology.

Based on the compilation of Bois et al. (1982), the Cretaceous strata would have been source rocks for ca. 30% of the world oil reservoirs. It has been proposed that much of the Cretaceous petroleum had its origin in organic matter deposited during OAEs (Arthur and Schlanger, 1979). It leads us to a consideration that the major source organisms for oil could have been cyanobacteria. This consideration is consistent with what was found by Orrison et al. (1984) that hopanoids, membrane components of prokaryotes, are ubiquitous and abundant in crude oil. Although hopanoids are widely

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produced by eubacteria and Ourrison et al. (1984) concluded that the eubacteria rather than cyanobacteria could be a major origin of petroleum based on the evidence, it does not necessarily rule out the possibility of cyanobacterial origin since they also produce hopanoids as membrane reinforces (Rohmer et al., 1984; Bisseret et al., 1985; Summons et al., 1999).

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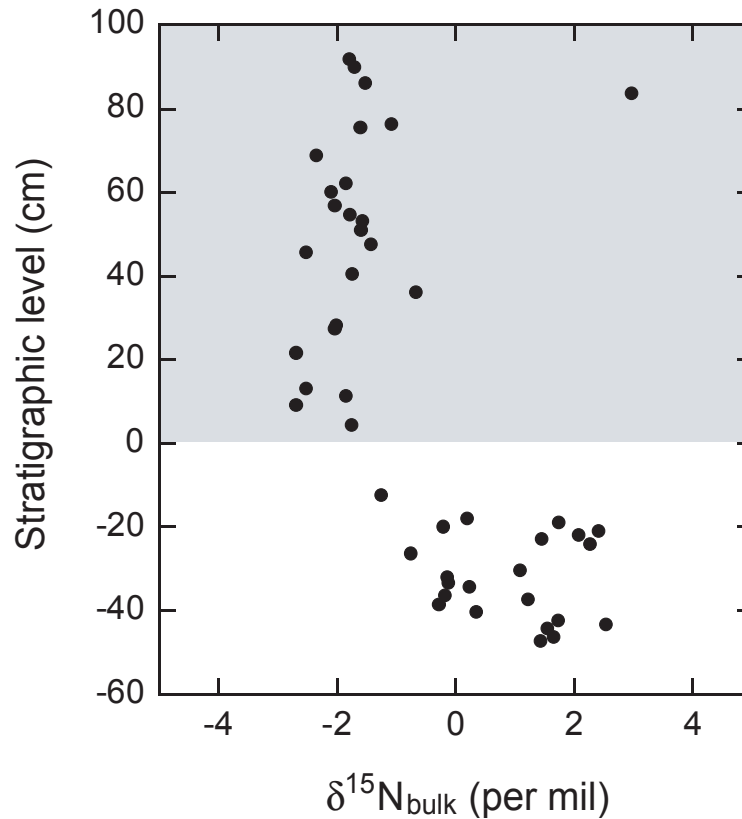
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**Fig. 1.** Stratigraphic variation of nitrogen isotopic composition of bulk sediments ( $\delta_{\text{bulk}}$ ). Shaded area indicates Livello Bonarelli black shale horizon.

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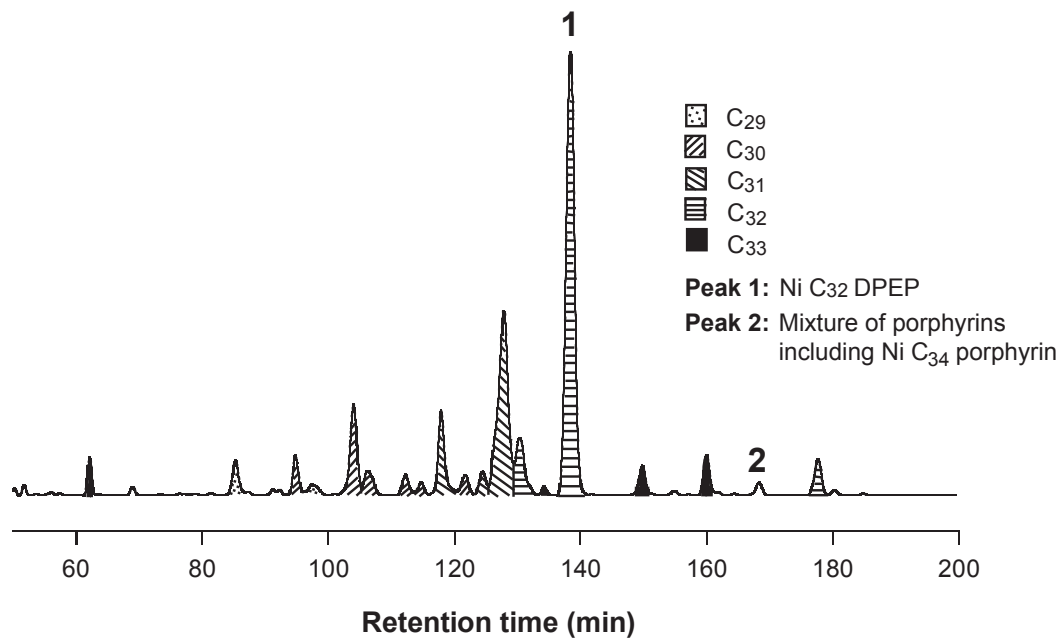
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**Fig. 2.** Total ion chromatogram for Ni porphyrin fraction (fraction N-2b) in sample GCB-17.

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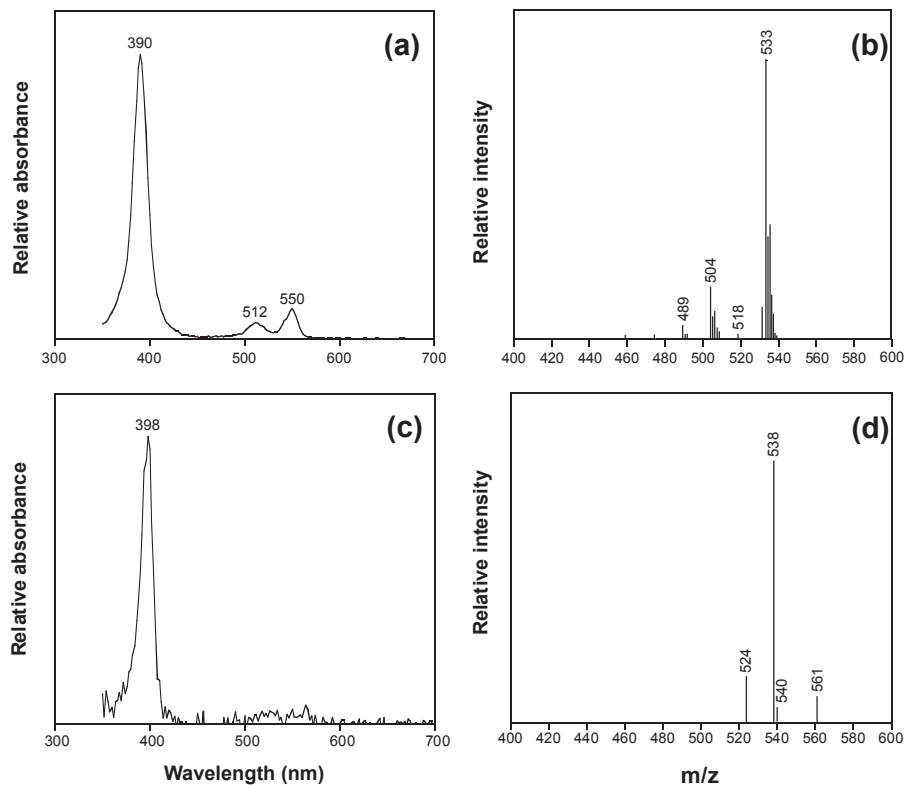
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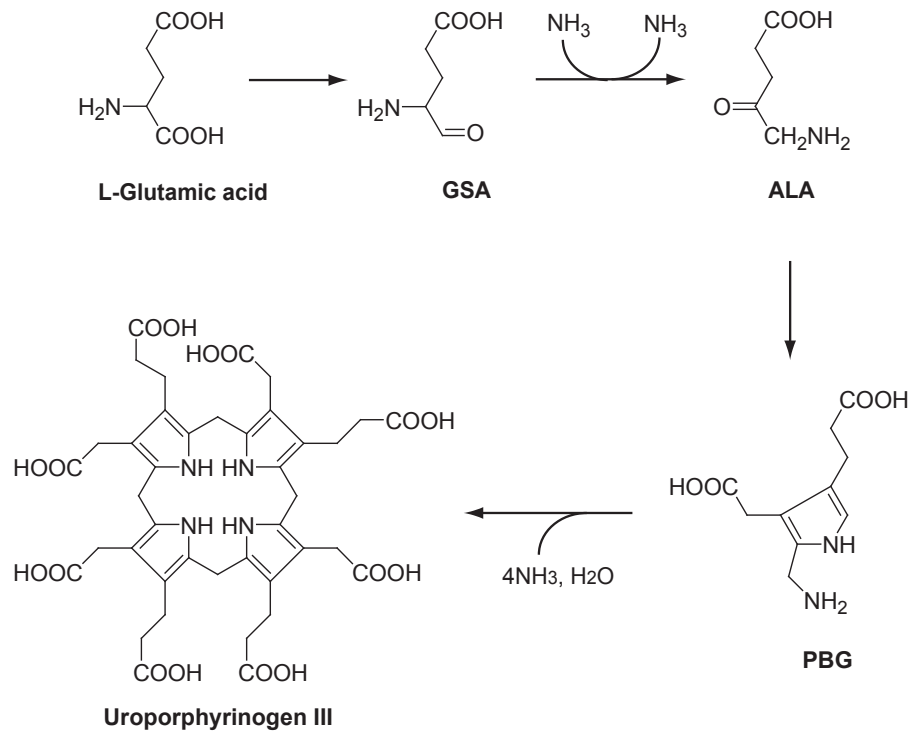
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**Fig. 3.** Absorption spectra and APCI mass spectra for peak 1 (a and b) and 2 (c and d) of chromatogram shown in Fig. 2.

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**Fig. 4.** A partial pathway for chlorophyll synthesis in the cell.

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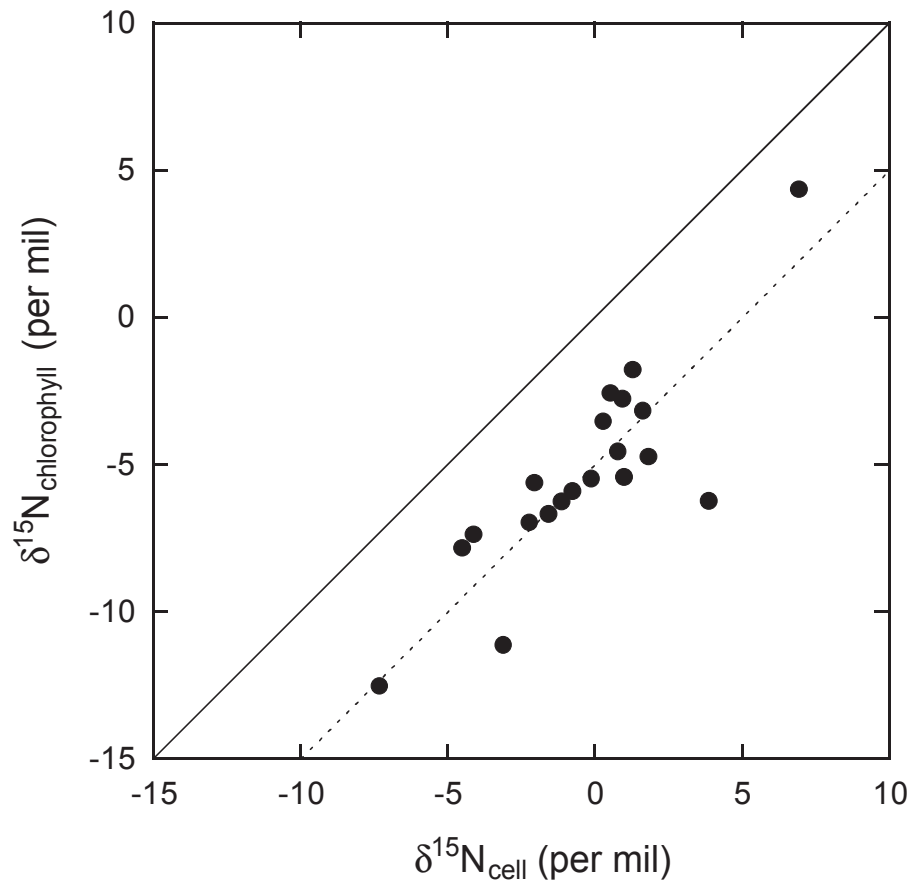
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**Fig. 5.** Isotopic relationship between cellular nitrogen and chlorophyll nitrogen (Sachs et al., 1999; Goericke and Montoya, unpub. results).

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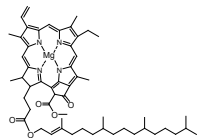
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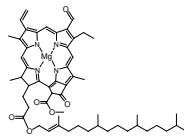
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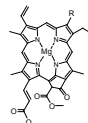
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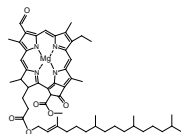
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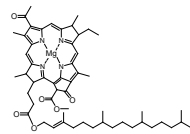
III. Chlorophyll *c*



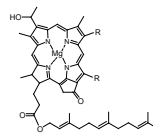
IV. Chlorophyll *d*



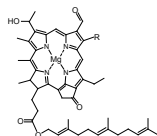
V. Bacteriochlorophyll *a*



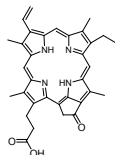
VI. Bacteriochlorophyll *d*



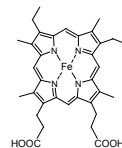
VII. Bacteriochlorophyll *e*



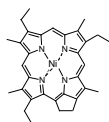
VIII. Pyropheophorbide *a*



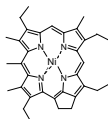
IX. Heme



X. C<sub>32</sub>Ni DPEP



XI. C<sub>34</sub>Ni porphyrin



Note: This structure is one of the candidates for C<sub>34</sub> Ni porphyrin

Fig. A1. Structures of compounds described in the text.

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