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Anal. Chem., **2009**, 81 (1), 184-192 • DOI: 10.1021/ac8017185 • Publication Date (Web): 25 November 2008

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A Method for Determining the Nitrogen Isotopic Composition of Porphyrins

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We describe a new method for analysis of the nitrogen isotopic composition of sedimentary porphyrins. This method involves separation and purification of geoporphyrins from sediment samples using liquid chromatography and HPLC, oxidation of the nitrogen within porphyrin-enriched fractions using a two-step process, and isotopic analysis of the resulting nitrate using the denitrifier method. By analysis of these degradation products of chlorophylls, we are able to measure an isotopic signature that reflects the nitrogen utilized by primary producers. The high sensitivity of the denitrifier method allows measurement of small samples that contain low concentrations of porphyrins. Extraction of only 50 nmol of nitrogen (nmol N) allows the following five analyses to be made (each on ~10 nmol N): nitrogen concentration, an assessment of potential contamination by nonporphyrin N, and three replicate isotopic measurements. The measured values of δ^{15}N have an average analytical precision of ±0.5‰ (1σ) and an average contribution from Rayleigh fractionation of 0.7‰ from incomplete oxidation of porphyrin N to nitrate. The overall method will enable high-resolution records of δ^{15}N values to be obtained for geological and ecological applications.

Stable isotope ratios of nitrogen in sedimentary organic matter can be used to study nitrogen cycling in present and past marine systems. The marine nitrogen cycle is largely biologically mediated, and biological processes involve a kinetic isotope effect that discriminates between ^{14}N and ^{15}N.[1−4] The nitrogen isotope ratio, or δ^{15}N value, of biomass in the surface ocean reflects the isotopic composition of the source nitrogen, isotope effects involved in its uptake, and any isotopic fractionation that results from surface recycling.[5] Preservation of organic matter in sediments allows use of sedimentary nitrogen isotopic records to study the cumulative effects of oceanic denitrification,[6−9] nitrogen fixation,[10−13] and nitrate utilization in surface waters.[14,15]

To date, most isotopic studies of sedimentary nitrogen involve measurement of bulk organic matter as preserved in marine sediments. However, as illustrated in earlier studies, as organic matter sinks through the water column and is buried, its bulk isotopic composition may be altered.[16,17] During periods of low flux, or in low productivity regions, a slight decrease in the δ^{15}N values of sinking particles with increasing depth is observed, perhaps as a result of adsorption of nonauthigenic nitrogen.[18,19] The opposite has been observed in the Southern Ocean, where the δ^{15}N of sinking particles increases during wintertime low flux periods.[5] Further isotopic changes also occur postdeposition. In sum, changes in δ^{15}N, toward both higher and lower values, may occur as a result of remineralization, winnowing, input from terrestrial sources, and ammonium adsorption onto clay particles.[15,17,20−24] Isotopic alterations are difficult to constrain, yet they are critical factors affecting the use of bulk

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10.1021/ac8017185 CCC: $40.75
Published on Web 11/25/2008

sedimentary N as a paleoceanographic tracer for past surface water conditions.

Here we present a new method for making compound-specific nitrogen isotope measurements on sedimentary geoporphyrins, the tetrapyrrole skeletons of fossil chlorophyll molecules. Chlorophyll is produced in the surface ocean by photoautotrophs and its $\delta^{15}N$ reflects that of the surface nutrient-N pool. By purifying and measuring intact chlorophyll molecules, we ensure that our measurements select for molecules that were formed in surface waters and have not undergone any subsequent alteration. Porphyrin degradation involves breaking carbon−carbon bonds in the tetrapyrrole structure to produce individual pyrrole rings. Any isotopic alteration resulting from porphyrin breakdown could potentially affect the carbon isotopic composition of the remaining pool of porphyrin but not its value of $\delta^{15}N$.

We have developed a method for extraction and purification of porphyrins from sediment samples for subsequent nitrogen isotopic analysis. Traditional combustion-based isotope ratio mass spectrometer (IRMS) measurements require a sample size of 1−10 μmol of N per sample. The methodology presented here, where extracted porphyrin nitrogen is oxidized to nitrate, leverages the small sample requirements of the denitrifier method for our isotopic analysis.[25] The denitrifier method involves isotopic analysis of N_2O that is produced from NO_3^- by naturally occurring denitrifying bacteria that lack nitrous oxide reductase (the enzyme that reduces N_2O to N_2[26]), and only 10 nmol of N is needed for a single isotopic measurement.

EXPERIMENTAL SECTION

Samples. Vanadyl octaethylporphine (VO-OEP) standards were purchased from Frontier Scientific, urea from Fluka Analytical, and antipyrine from Sigma. Chlorophyll *a* (Chl *a*) was extracted from spinach with acetone and purified by reversed-phase (RP) HPLC (Agilent Technologies 1100 Series) on a ZORBAX Eclipse XDB-C_{18} column (4.6 mm × 250 mm, 5 μm; Agilent) using an isocratic flow of CH_3OH/acetone (80:20 v/v) at 1.0 mL/min. VO-OEP was dissolved in dichloromethane (DCM), and chlorophyll was stored in acetone. The natural sediment samples used were Cretaceous-age marine sediments from Ocean Drilling Program Leg 207 Site 1258A and Devonian-age New Albany Shale from outcrop (provided by S. Petsch). All pigment samples were stored at −20 °C in the dark, and exposure to light was minimized during sample processing. Organic solvents were all Burdick & Jackson GC^2 grade.

Estimation of Standard Nitrogen Concentrations. The concentrations of Chl *a* and VO-OEP standard solutions were estimated spectrophotometrically. Absorption spectra of VO-OEP in DCM and Chl *a* in acetone were measured from 450−700 nm using a Beckman Coulter DU 640 spectrophotometer. Chl *a* concentration (in μg/mL) was calculated according to the equation[27]

$$C_{\text{Chl } a} \approx -1.7858A_{647} + 11.8668A_{664} \quad (1)$$

where A_{647} and A_{664} are absorbances at 647 and 664 nm, respectively. The concentration of the VO-OEP solution was calculated using a modified version[28] of the Beer−Lambert law that takes into account the baseline behavior of metalloporphyrin absorbance:

$$A_T = \varepsilon_T l C_{\text{VO-OEP}} \quad (2)$$

where A_T is true absorbance, ε_T is the true extinction coefficient (M^{-1} cm^{-1}), l is the cuvette length (cm), and $C_{\text{VO-OEP}}$ is concentration. A_T is computed according to the following equation:

$$A_T = A_2 - A_1 + \left[(A_1 - A_3)\frac{(\lambda_2 - \lambda_1)}{(\lambda_3 - \lambda_1)}\right] \quad (3)$$

where each subscript represents a peak or trough wavelength in the absorption curve of a metalloporphyrin. For VO-OEP, this equation is

$$A_T = A_{569} - A_{507} + [0.6526(A_{507} - A_{602})] \quad (4)$$

and ε_T = 27 700 M^{-1} cm^{-1}. With the use of the measured concentrations of Chl *a* and VO-OEP, N concentrations of standard solutions are calculated and standards are aliquoted so that final N concentrations of ∼50 μM N are achieved in 1 mL of oxidizing reagent.

Porphyrin Extraction and Concentration. Total lipid extracts (TLE) were collected from natural samples using accelerated solvent extraction (ASE 200; Dionex Corporation) in CH_2Cl_2/CH_3OH (90:10, v/v). TLEs were then separated into two bulk fractions (flash fractions F1 and F2; Figure 1) based on polarity using a Biotage FLASH+ 12i system with a SiO_2 column (KP-SIL 12 mm × 250 mm, 40−63 μm). F1, which is concentrated in Ni-chelated porphyrins, was eluted using 45 mL of hexane, applied as 30 mL followed by 15 mL. F2, which is concentrated in VO-chelated porphyrins, was eluted with 60 mL of DCM/hexane (50:50, v/v) in two aliquots of 30 mL. These conditions were optimized to provide the highest elution efficiency of porphyrin at the minimum solvent polarity.

Preparative High-Performance Liquid Chromatography. Flash fractions were further purified using normal-phase (NP) HPLC (Agilent 1100 series) on a ZORBAX SIL column (4.6 mm × 250 mm, 5 μm) with hexane and ethyl acetate as running solvents to generate porphyrin-concentrated fractions (PCFs). F1 was eluted in a gradient from 100% hexane to 50% hexane/50% ethyl acetate in 30 min and F2 in a gradient from 100% hexane to 100% ethyl acetate in 30 min. Flow rates were 1.0 mL/min. Porphyrin peaks were detected by absorbance at 393 and 405 nm for Ni- (F1) and VO- (F2) porphyrins, respectively. The entire

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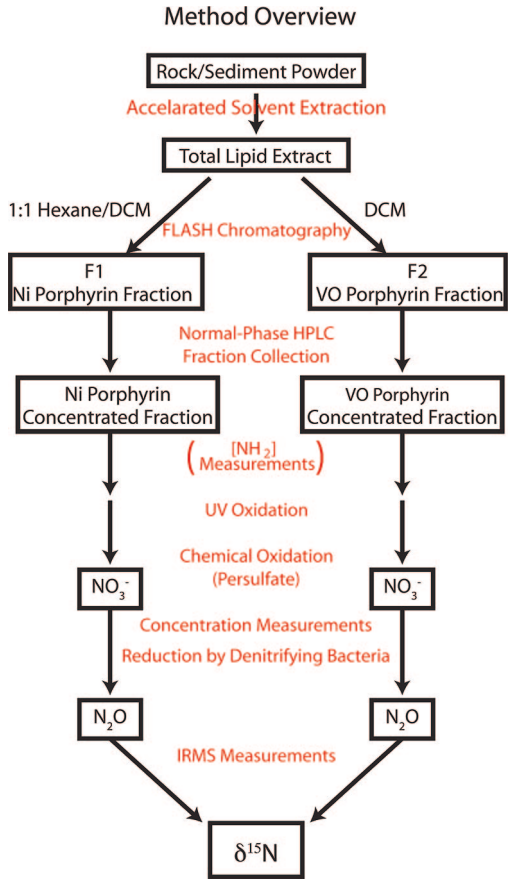

Figure 1. Procedure for nitrogen isotopic measurement of sedimentary porphyrins. Specifics of each step are described in the text.

porphyrin elution window was collected using a fraction collector (Agilent), with time-based collection. The collection window for F1 was from 8.5 to 12.5 min (4 min long). The collection window for F2 was either from 11 to 14 min (3 min long), from 11 to 18 min (7 min long), or from 11−22.5 min (11.5 min long), depending on which porphyrins were present in the sample. Most samples analyzed required only the 3 min (F2) or 4 min (F1) window. Porphyrin concentrations were estimated from peak areas at 393 and 405 nm (for F1 and F2, respectively) multiplied by a conversion factor determined as follows: for a series of test environmental samples, chromatograms of absorbance at 393 and 405 nm were manually integrated, generating area units of milliabsorbance units min (mAU min). These samples were then oxidized, and NO_3^- concentrations were measured as nmol N/sample. The conversion factor is the average ratio of the integrated absorbance to nmol N ($\sim 4 \times 10^{-4}$ nmol N mAU^{-1} min^{-1} for our test samples). This conversion factor was used to estimate [N] of PCFs to within a factor of two for preparation of 50 nmol samples.

Analytical High-Performance Liquid Chromatography. Natural samples were first analyzed by analytical RP-HPLC-MS (Agilent 1100 series LC/MSD) with an atmospheric pressure chemical ionization (APCI) source to ensure that the absorbance peaks at 393 and 405 nm observed in preparative HPLC correspond to the elution of identifiable porphyrins. Aliquots were separated using a ZORBAX XDB C_{18} column (4.6 mm × 250 mm, 5 μm) with a solvent program consisting of acetonitrile (ACN), H_2O, ethyl acetate, tetrahydrofuran (THF), and acetic acid (Table S-1 in the Supporting Information; modified after Kashiyama et al.[29]). The flow rate was 1.0 mL/min. Absorbance was measured at 393 and 405 nm. Total ion chromatograms were analyzed for ion masses corresponding to common Ni- and VO-chelated etioporphyrins and deoxophylloerythroetioporphyrins (DPEP) with carbon numbers ranging from 25−32 (Table S-2 in the Supporting Information).

Amine Content Analysis. To estimate the quantity of non-porphyrin nitrogen present in various fractions, we used a fluorescence assay to measure the concentrations of primary amines.[30,31] Samples dissolved in isopropyl alcohol (IPA) were reacted with a solution containing the chromophore naphthalene-2,3-dicarboxyaldehyde (NDA; Sigma), which in the presence of CN^- reacts with primary amines to form a 1-cyano-2-substituted-benz[*f*]isoindole derivative that is more stable than traditional *o*-phthalaldehyde (OPA) derivatives. This compound is highly fluorescent when excited at 410−450 nm. Reactions were prepared according to Siri et al.[31] Reagents included 43 mM KCN in nanopure H_2O, 5 mM NDA in CH_3OH, and 125 mM borate buffer at pH 8.7. All reagents were stored at 4 °C in the dark and prepared several hours before the assay reaction.

Evaporated porphyrin samples were dissolved in 360 μL of IPA in amber vials, sonicated, and then mixed with 360 μL of water. Aliquots of 120 μL of KCN, 120 μL of borate buffer, and 40 μL of NDA in that order were added to the samples to achieve a final reaction volume of 1 mL. Valine and glycine standards (Sigma) in concentrations ranging from 100 nM to 2 mM N were prepared to create a standard curve. Valine and glycine were chosen because their fluorescence levels are on the low and high end, respectively, when reacted with NDA.[31] After addition of reagents, all samples were vortexed and kept at room temperature for 30 min. Fluorescence was measured in 96-well plates using a Spectramax Gemini XS spectrofluorometer with an excitation wavelength of 442 nm and emission detection at 480 nm.

Oxidation of Porphyrins to Nitrate. HPLC-collected PCFs were oxidized to nitrate in a two-step process (Figure 1). Samples containing ~50 nmol porphyrin-N were aliquoted into 9 mm quartz tubes, and ~3 mL of DCM were added. The tubes were exposed to UV light for 6 h in a Baker SterileGuard II biological cabinet. Samples were then evaporated and transferred to 1.5 mL vials with Teflon-lined septa screw caps, and the solvent was evaporated fully under N_2 gas. Fresh persulfate oxidizing reagent (POR) was prepared during this interval. $K_2S_2O_8$ that had been recrystallized three times was added to a 0.15 M NaOH solution to make a final $K_2S_2O_8$ concentration of 0.05 M.[32] In all cases, Barnstead Nanopure H_2O was used. A volume of 1 mL of POR was added to each sample vial, and the vials were capped. The samples were then autoclaved for 30 min at 121 °C.

Nitrate Concentrations. Nitrate concentrations were measured by reduction to NO followed by chemiluminescent detection of NO using an Antek Nitric Oxide Detector model 7020 or a

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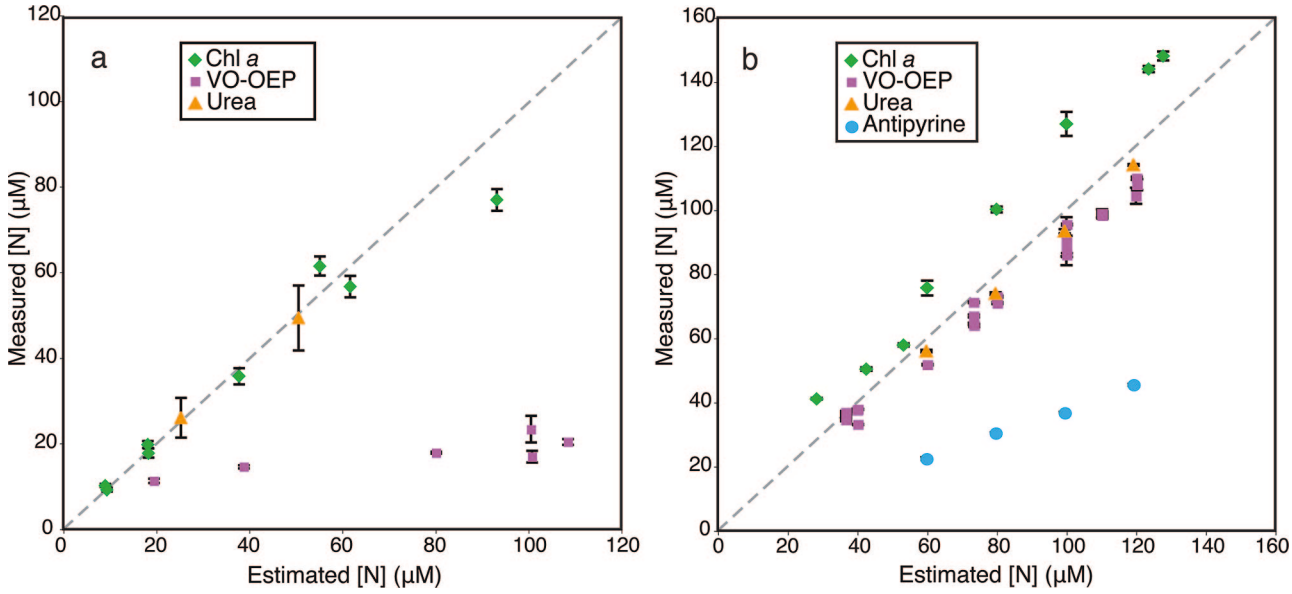

Figure 2. Oxidation yields for Chl *a*, VO-OEP, urea, and antipyrine that were oxidized using POR (a) without the initial exposure to UV light and (b) with exposure to UV light. Dashed lines represent 100% oxidation yield.

Monitor Labs Inc. N Oxides Analyzer model 884.[33] $NaNO_3$ and KNO_3 solutions in concentrations ranging from 5 to 100 μM NO_3^- were used to make standard curves for yield measurements. Samples and standards were injected in either 100 or 200 μL aliquots.

Isotopic Analysis. The appropriate volume of sample calculated to contain 10 nmol N was used for each $\delta^{15}N$ measurement by the denitrifier method.[25] *Pseudomonas chlororaphis* (ATCC no. 13985, formerly *P. aureofaciens*) or *Pseudomonas chlororaphis* (ATCC no. 43928), denitrifying bacteria that lack the enzyme nitrous oxide reductase, were used to reduce nitrate quantitatively to nitrous oxide. N_2O mass ratio analyses were performed on a Finnigan Delta PLUS XP (WHOI) or a Thermo Scientific Delta V (URI) isotope ratio mass spectrometer using gas purification and inlet systems similar to Casciotti et al.[34] Masses 44, 45, and 46 were used to compute the $^{15}N/^{14}N$ ratio after correcting m/z 45 for the contribution from $^{14}N^{14}N^{17}O$.[25] Isotopic measurements were standardized to the N_2 reference scale by parallel analyses of USGS32 and USGS34[35] (Delta PLUS XP) and IAEA N3[36] (Delta V), internationally recognized KNO_3^- reference materials.

Isotopic values for bulk samples of VO-OEP were measured at the UC Davis Stable Isotope Facility. VO-OEP samples containing 50−90 μg of N were evaporated under N_2 in tin capsules. Samples were analyzed using a PDZ Europa ANCA-GSL elemental analyzer interfaced to a PDZ Europa 20-20 IRMS (Sercon Ltd.) as described at http://stableisotopefacility.ucdavis.edu/CNMethod1.html. Samples were calibrated to in-house standards that are calibrated against NIST standard reference materials IAEA-N1, IAEA-N2, and IAEA-N3.

RESULTS AND DISCUSSION

Oxidation Yields for Nitrogen Standards. Chemical oxidation alone is sufficient for conversion of certain nitrogen-containing compounds to nitrate,[10] including Chl *a* and urea (Figure 2a). However, yields for VO-OEP were <60% with only POR oxidation. This incomplete oxidation is not due to saturation of the oxidizing reagent but to kinetic inhibition of organic matter oxidation, as demonstrated by increased oxidation yields under longer incubations.[32] Nydahl[32] calculated the conversion ratios for complete oxidation of various compounds and found that organic mixtures containing C and N are quantitatively oxidized with molar ratios of $K_2S_2O_8$ to organic matter (C + N) of 10:1. In our application, a sample containing, for example, 12.5 nmol of C-32 DPEP (50 nmol N) that is 50% pure (containing 50% nonporphyrin organic compounds by mass) would have a molar ratio of $K_2S_2O_8$ to C + N of >50:1, well within the range of quantitative oxidation.

To improve conversion yields of VO-OEP and other porphyrins, we employed a two-step process that includes UV and chemical oxidations (for method overview see Figure 1). Porphyrins are less polar than chlorophyll, and their incomplete oxidation by POR results from their insolubility in the aqueous reagent. When oxidized without initial UV exposure, porphyrin samples are visibly immiscible with the aqueous oxidizing reagent. UV exposure appears to increase the aqueous solubility of porphyrins, through partial oxidation and/or fragmentation, and after UV treatment they appear completely soluble in $K_2S_2O_8$ solution.

This two-step oxidation resulted in yields consistently greater than 82%, with an average of 91% for VO-OEP (Figure 2b). Yet, while the VO-OEP and urea yields range from 82−96%, Chl *a* oxidation yields appear to be >100%. These high yields for Chl *a* are probably due to a combination of two factors. First, pigment oxidation yield calculations depend strongly on correct estimation of initial pigment concentrations. In this study, we use the modified Beer−Lambert equations specific to each compound to achieve the greatest accuracy possible. However, chlorophylls are also especially sensitive to light and pH, and once collected, degradation of the Chl *a* standard would decrease the spectro-

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photometrically measured concentrations. This is a likely cause of the apparent underestimation of the true values for these standards (Figure 2b, *x*-axis), especially considering that these samples were reanalyzed several months after they were originally handled for Figure 2a. Second, Chl *a* was extracted from spinach using acetone, which contains high levels of background N (see next section). This also may have contributed to the apparent yields greater than 100%. In contrast, the urea standard was prepared gravimetrically (as opposed to spectrophotometrically); the VO-OEP standards appeared less time-sensitive than Chl *a*, were handled in smaller aliquots than Chl *a*, and never were exposed to acetone. Yields for urea and VO-OEP are therefore considered more reliable.

Antipyrine was measured for comparison with the results of Knapp et al.[10] who measured yields of ~40% for antipyrine when oxidized using POR without UV. However, we found essentially identical yields following our procedure (Table S-4 in the Supporting Information), indicating that antipyrine is not easily oxidized by either treatment.

Background N Contributions. To quantify the procedural blanks, we analyzed various reagents used throughout the sample prepration and processing steps (Figure S-1 in the Supporting Information). The N blank of POR, is minimized by recrystallization of $K_2S_2O_8$, which absorbs NH_4^+ into its crystal structure.[32] With triple recrystallization before preparation of POR, this blank has been measured as 0.86 μM on average (range from 0.40 to 1.38 μM) or 0.86 nmol N in the 1 mL of POR solution used to oxidize ~50 nmol of porphyrin N.

In a sample for isotopic measurement, the total background nitrogen potentially consists of accumulated solvent blanks from extraction, flash chromatography, and HPLC (n_{sol}), plus the above contribution from POR (n_{por}). However, the HPLC step may reset the solvent contribution by removing some of the accumulated extraction and flash chromatography blanks, meaning that the only blanks would be associated with the HPLC solvent volume (n_{hplc}) and n_{por}. Regardless, acetone has the highest N blank of all solvents tested (~2 nmol N/mL), presumably because its properties as a powerful solvent for organic polymers allow it to leach trace nitrogen from substances it contacts before or during sample processing. Other polar solvents, including those used for RP-HPLC, also contained significant nitrogen blanks (CH_3COOH, H_2O, and ACN all >0.3 nmol N/mL). In contrast, the solvents used both in NP-HPLC and for sediment extractions by ASE (hexane, DCM, and CH_3OH) have smaller blanks (≤0.2 nmol N/mL). We thus chose to perform all separations in normal phase mode.

When the total potential accumulated blank is calculated based on the solvent volumes used in extraction, flash chromatography, and HPLC, the value of n_{sol} is approximately 1.0 nmol N (F1) or 0.8 nmol N (F2) for a typical HPLC-collected sample containing 50 nmol N. If the blanks accumulate, adding n_{por} results in a total expected blank of 1.8−2.0 nmol N. However, direct measurement of fraction-collected HPLC solvent after blank (30 μL of DCM) injections suggests that the HPLC solvent blank for F1 and F2 is ~0.14 nmol N/mL, which would make the total blank (n_{hplc} plus n_{por}) equal to ~1.3−1.4 nmol N. To correct for this blank, n_{por} is measured for each set of oxidations, and n_{hplc} is assumed to be 0.14 nmol N/mL, scaled for collection window length.

Isotope mass balance must be used to correct measured values of $\delta^{15}N_{NO_3}$ for these contributions. However, all measured blank concentrations (<2 nmol N in a 1 mL sample) were too small to measure the $\delta^{15}N$ value of the POR blank directly. Its $\delta^{15}N$ value also cannot be measured by dilution series of porphyrin standards, because the potential imprecision in quantification of the porphyrin standards results in an uncertain slope and intercept. Instead we assumed the blank has a $\delta^{15}N$ value near atmospheric N_2 (0‰) and assigned a large range of uncertainty to this choice (±10‰, 1σ). Using these numbers, we subtracted the blank contribution from all data and report uncertainties using full propagation of error (Table S-3 and Supporting Information text). Since VO-OEP standards have a $\delta^{15}N$ value of −12.44 ± 0.24‰ (as measured by EA-IRMS), correction with a 0‰ blank results in estimated $\delta^{15}N$ values of porphyrin-derived NO_3^- that are ~0.2‰ lower than raw $\delta^{15}N$ measurements. For a sample that is more isotopically similar to an atmospheric N_2 blank, we expect blank-corrected $\delta^{15}N$ values to resemble measured $\delta^{15}N$ values more closely.

Induced Isotopic Fractionation. Potential fractionation factors for the oxidation reaction were calculated assuming a Rayleigh distillation model. Figure 3a shows a color contour of expressed fractionation ($\delta_{VO\text{-}OEP} - \delta_{NO_3}$) versus oxidation yield (f) for a variety of potential fractionation factors (α) according to the Rayleigh equation:

$$(1-f)^{\alpha} = 1 - \frac{f(\delta_p + 1000)}{\delta_0 + 1000} \qquad (5)$$

where f corresponds to the oxidation yield (fraction of VO-OEP oxidized to NO_3^-, from 0 to 1), α is the fractionation factor ($\alpha_{NO_3^-/porphyrin} = (^{15}N/^{14}N)_{NO_3^-} / (^{15}N/^{14}N)_{porphyrin}$), δ_p is the $\delta^{15}N$ value of oxidized VO-OEP (measured as NO_3^-), and δ_0 is the actual value of $\delta^{15}N$ of the VO-OEP standard (as measured by EA-IRMS). The expressed fractionation equals $\delta_0 - \delta_p$. Superimposed on this contour plot are the blank-corrected values of f and $\delta_0 - \delta_p$ from a dilution series of VO-OEP oxidations. The black data points represent VO-OEP oxidized using the above method. Samples in gray were an early batch prepared at nonoptimized conditions including half-concentration POR, shelf storage of >3 months prior to measurement, addition to denitrifying bacteria at too high a volume, and possible dilution to the wrong concentration. The imprecision in these results accordingly reflects the need for careful control of all of the above conditions. Error bars reflect 1σ standard deviations of replicate [NO_3^-] and $\delta^{15}N$ measurements; the latter are corrected for exogenous N by isotopic mass balance, as discussed above, with full propagation of error.

All samples processed with the more concentrated POR fall within fractionation factor (α) values between 0.9955 and 1.0000, which corresponds to a maximum fractionation of ≤4.5‰ between substrate and product in the oxidation reaction. More specifically, with the use of the measured value of $\delta_0 = -12.44$‰ for VO-OEP, most data overlap $\alpha = 0.997$ (Figure 3b). For VO-OEP oxidations using this method, all oxidation yields were >80%, which corresponds to expressed fractionations of <1.2‰, assuming the Rayleigh model with $\alpha = 0.997$. The average oxidation

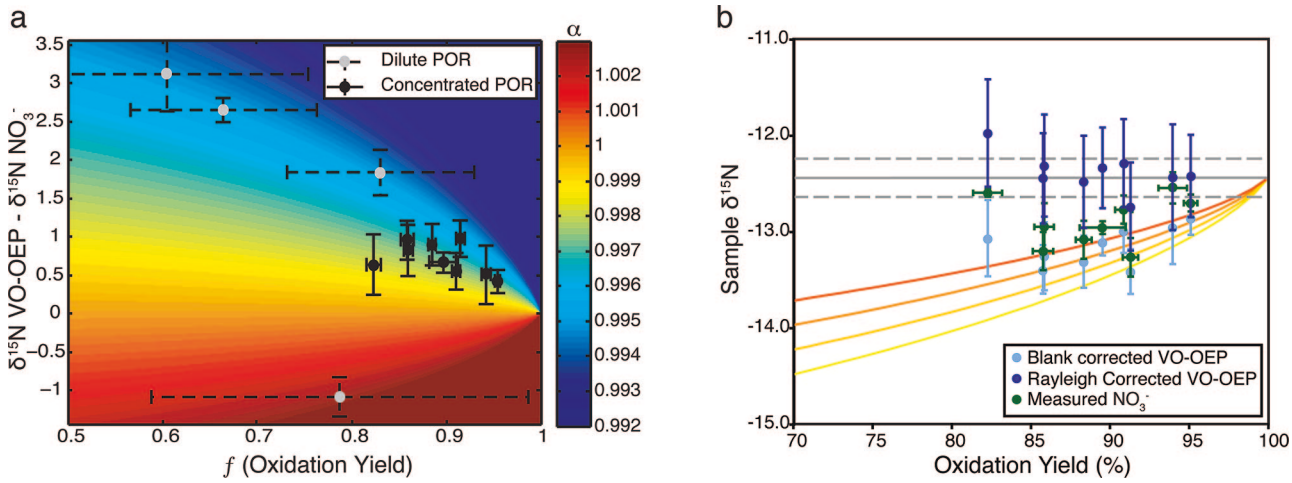

Figure 3. Calculation of fractionation factor (α) involved in oxidation of VO-OEP standard to NO_3^-. (a) Expressed fractionation, calculated as the difference between EA-IRMS-determined $\delta^{15}N$ value of VO-OEP and the $\delta^{15}N$ value of NO_3^- measured using the denitrifier method, as a function of oxidation yield (*f*). Data are superimposed over a plot of α calculated for $\delta_{VO\text{-}OEP} - \delta_{NO_3}$ and *f* according to the Rayleigh fractionation model. Black data points correspond to samples oxidized using the method presented, and gray data points represent samples prepared under nonoptimized conditions (see text). Error bars correspond to blank-corrected isotopic uncertainty and standard deviation of concentration measurements (both 1σ). (b) The same data (aqua) superimposed on Rayleigh fractionation curves corresponding to α values determined in part a but also showing additionally the raw data before correction for background N (green) and after correction for both background N and Rayleigh fractionation (dark blue).

yield is 91.0%, resulting in oxidized porphyrin samples that are on average 0.71‰ more negative than the true $\delta^{15}N$ of the sample (at $\alpha = 0.997$). This is calculated assuming that the blank correction already applied to the samples using isotopic mass balance is correct. If the fractionation factor is calculated for the data before subtraction of a contribution from exogenous N, the average observed fractionation is 0.48‰ (Figure 3b), effectively the same as the instrumental precision (±0.5‰). This would correspond to a negligible value of α of only 0.998; however, we believe this would underestimate the true fractionation associated with the oxidation process.

When the individual standards are corrected for Rayleigh fractionation, according to their individual oxidation yields and a uniform α of 0.997, their corrected $\delta^{15}N$ values average −12.38 ± 0.53‰ (Figure 3b). In most cases for environmental samples, the true porphyrin N concentration cannot be established, however, precluding exact calculation of yield. In that case, we recommend that a correction factor of +0.7 ± 0.4‰ be applied, corresponding to Rayleigh fractionation with $f = 0.91$ and $\alpha = 0.997$, and that this correction factor be propagated through with the instrumental error and blank error to yield an aggregate uncertainty. An uncertainty of 0.4‰ corresponds to the range of expressed fractionations for $\alpha = 0.997 \pm 0.001$ and $f = 0.8$. Though the average oxidation yield of VO-OEP is 91%, a lower value of *f* was chosen to allow for a potentially wider range of oxidation yields for environmental samples.

Collection of Porphyrin Fractions from Natural Samples. Previous studies have shown that there is little difference between $\delta^{15}N$ values of most individual porphyrin compounds that are purified from a single natural sample.[37] Therefore, we developed a preparative HPLC method for rapid collection of geoporphyrins that allows for cocollection of multiple compounds, rather than collection of individual peaks as resolved by analytical HPLC of PCFs (Figure 4a,c). PCF samples F1 and F2, containing predominantly Ni- and VO-porphyrins, respectively, were eluted in aggregate using slightly different normal-phase gradients (Figure 4b,d). Rather than optimizing the chromatography to achieve baseline resolution, the programs deliberately were compressed to minimize the time window (and therefore solvent volume) over which each fraction was collected. This strategy is consistent with the dual goals of maximizing throughput while minimizing exogenous nitrogen contamination. Each injection contains 15−40 nmol of porphyrin N, and each run takes 30 min, plus 20 min for column re-equilibration. Conservatively, sufficient material can be collected in three injections each for F1 or F2 to permit isotopic analysis in triplicate (30 nmol N), confirm minimal amine contamination (10 nmol N; see below), and quantify NO_3^- concentration yields (~10−15 nmol N). This corresponds to 3 h of HPLC running time per fraction, allowing for the collection of up to eight different F1 or F2 samples per day under continuous operation.

Amine Content of Lipid Fractions. Amine contents were determined for six different PCFs. These PCFs then were compared to amine concentrations for the corresponding total lipid extracts and pre-HPLC flash fractions. Samples tested included F1 and F2 samples from six different horizons of a Cretaceous ODP core. We assume here that the samples contain only amine and tetrapyrrole (porphyrin) nitrogen and report all samples in common units of percent of total nitrogen as amine (Figure 5). Data are plotted as the NH_2 concentrations calculated using the average of the valine and glycine standard curves.

Our data show a reduction in $[NH_2]$ as samples are purified from TLE to flash fraction to PCF in most samples, with all samples showing significantly lower NH_2 concentrations in PCFs when compared to TLEs. TLEs have between 4.7 ± 1.4 nmol of NH_2 per 100 nmol of porphyrin N. Flash fractions

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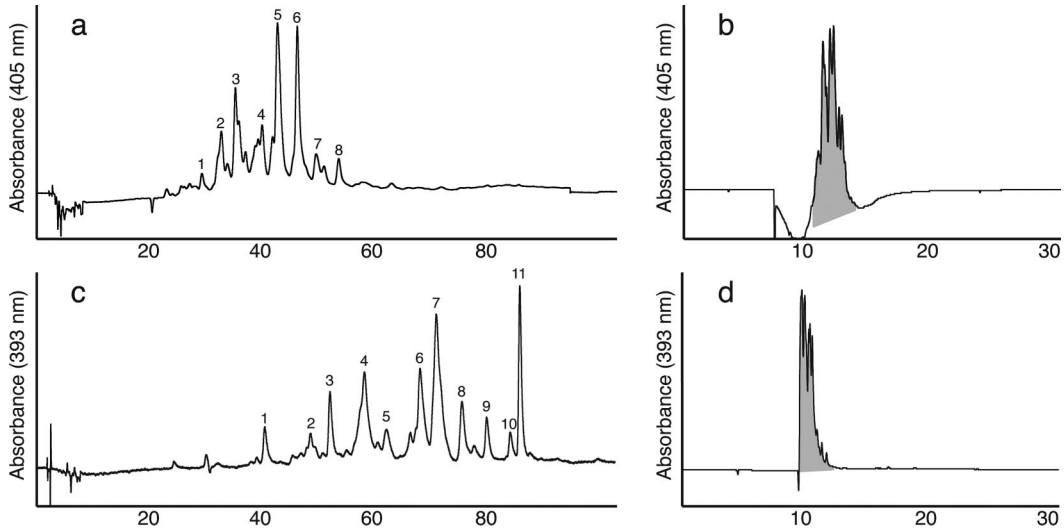

Figure 4. HPLC chromatograms of environmental porphyrin-concentrated fractions (PCFs). F2 samples are a test extract of Devonian-age New Albany Shale (a, b), and F1 samples are a test extract of Cretaceous ODP sediment (c, d). Parts a and c are analytical RP-HPLC chromatograms. See Table S-2 in the Supporting Information for peak identification by HPLC−MS. Parts b and d are preparative NP-HPLC chromatograms, with the fraction collection windows shaded.

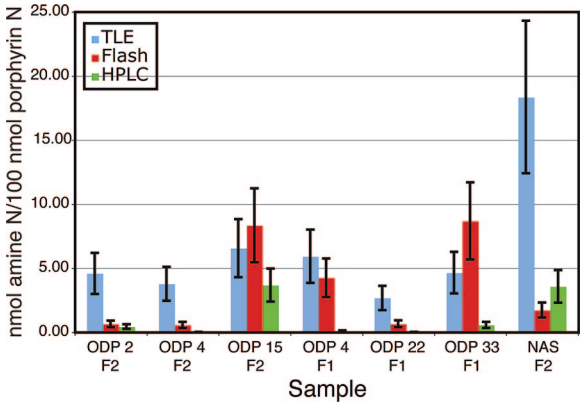

Figure 5. Fluorescent assay measurements of [NH_2] in three ODP F1 samples and three ODP F2 samples. Each sample is measured as TLE, flash fraction, and PCF. Data shown are concentrations as calibrated to the average fluorescence of valine and glycine standards, with error bars representing the valine- and glycine-calculated values for lower and upper limits.

have an average of 3.9 ± 3.9 nmol of NH_2 per 100 nmol of porphyin N, and HPLC purified samples have 0.8 ± 1.4 nmol of NH_2 per 100 nmol of porphyrin N. While this assay is not sensitive to nitrogen bound in other functional groups, much of the nitrogen found in sedimentary organic matter is amine-bound, and this assay allows us to estimate at least part of the nonporphyrin nitrogen content of sedimentary samples. In particular, molecules containing other more polar N functionalities such as amides are likely excluded during the relatively apolar flash chromatography step. Thus amine quantification likely is a good proxy for nonporphyrin N in the purified PCFs. However, since there is no way to effectively assess the direction of isotopic bias introduced by this fraction of nonporphyrin N, it cannot be corrected by isotopic mass balance. It is considered an additional, nonquantifiable uncertainty in results measured from environmental samples. If we assume, however, that 2% of any sample is amine N, even an amine background that differed by 10‰ from porphyrin N would contribute only a 0.2‰ offset to a final reported $\delta^{15}N$ value.

Table 1. $\delta^{15}N$ Data from Natural Samples

sample[a]	$\delta^{15}N \pm 1\sigma^b$ (‰)	$\delta^{15}N \pm 1\sigma^c$ (‰)
ODP3-F1-Flash 1×	−2.71 ± 0.50	−2.64 ± 0.50
ODP3-F1-Flash 2×	−4.20 ± 0.55	−3.30 ± 0.55
ODP3-F1-Flash 4×	−5.20 ± 0.42	−4.08 ± 0.42
ODP3-F1-HPLC 1×	−3.50 ± 0.60	−2.77 ± 0.60
ODP3-F1-HPLC 4×	−4.12 ± 0.48	−3.12 ± 0.48
ODP1-F2-Flash	−6.23 ± 0.41	−6.17 ± 0.41
ODP1-F2-HPLC 2×	−5.84 ± 0.52	−5.77 ± 0.52
ODP1-F2-HPLC 4×	−6.47 ± 0.62	−6.38 ± 0.62
ODP2-F2-HPLC 1×	−5.41 ± 0.43	−5.34 ± 0.43
ODP2-F2-HPLC 2×	−5.51 ± 0.54	−5.40 ± 0.54
ODP2-F2-HPLC 4×	−5.12 ± 0.93	−5.05 ± 0.93
NAS-F2-Flash 1×	−2.36 ± 0.41	−2.29 ± 0.41
NAS-F2-Flash 2×	−2.23 ± 0.50	−0.94 ± 0.50
NAS-F2-Flash 4×	−3.32 ± 0.52	−1.56 ± 0.52
NAS-F2-HPLC 1×	−2.29 ± 0.50	−2.23 ± 0.50
NAS-F2-HPLC 2×	−2.40 ± 0.73	−1.63 ± 0.73
NAS-F2-HPLC 4×	−2.33 ± 0.62	−1.36 ± 0.62

[a] Sample and concentration factor (1×, 2×, 4×). [b] Blank-corrected $\delta^{15}N$ values, adjusted for Rayleigh fractionation of constant 0.7‰, based on an assumed $f = 0.9$ for all samples and $\alpha = 0.997$. Errors include blank-propagated measurement uncertainty and a Rayleigh correction uncertainty of 0.4‰. [c] Blank-corrected $\delta^{15}N$ values, adjusted for Rayleigh fractionation based on individually calculated f, assuming the highest yield for a dilution series is $f = 0.9$ and $\alpha = 0.997$. Errors include blank-propagated measurement uncertainty and a Rayleigh correction uncertainty of 0.4‰.

Results for Natural Samples. We used our method to measure porphyrin $\delta^{15}N$ values for three test samples of organic-rich Cretaceous ODP sediment and one sample of Devonian organic-rich shale from outcrop (NAS). For NAS and two of the ODP sediment samples, the data were compared to $\delta^{15}N$ values measured for flash fractions (also measured using the denitrifier method; Table 1). Both the flash and HPLC fractions are highly concentrated in porphyrin nitrogen, so the $\delta^{15}N$ of these samples are expected to reflect primarily the ^{15}N content of porphyrins.

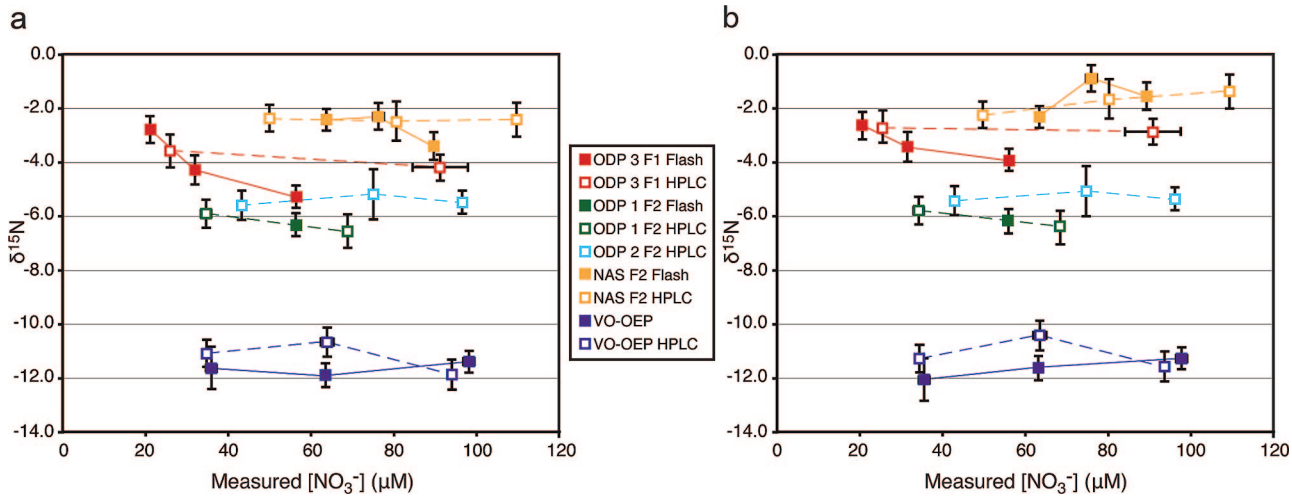

Figure 6. δ^{15}N measurements of PCFs and flash fractions from four environmental samples, nonpurified VO-OEP standard, and VO-OEP standard collected by HPLC. Each set of samples was prepared at three different N concentrations in the oxidizing reagent (1×, 2×, 4×). Data points for each dilution series are connected by lines. (a) Data are plotted with a +0.7‰ correction to account for Rayleigh fractionation assuming an oxidation yield of 90% and $\alpha = 0.997$ for all samples. (b) Data are plotted with Rayleigh fractionation corrections using oxidation yields that are based on estimated concentrations. For environmental samples where the expected [N] is not known, the highest oxidation yield in each dilution series is assumed to be 90%, and the oxidation yields of other samples in the dilution series are calculated by standardizing to this value.

In order to test the precision of our method, natural samples were analyzed in triplicate at three different concentrations. Preparing samples as a dilution series can demonstrate the presence of a blank, as well as any isotopic fractionation from incomplete oxidation that occurs with increased sample concentration. Some dilution series (ODP3 F1, ODP1 F2) show a decrease in the measured δ^{15}N values at higher sample concentrations, which is more significant for flash fractions than for PCFs (Figure 6a). Sample concentration here refers to the total amount of porphyrin-N oxidized with 1 mL of POR, thus the difference in these samples only is the ratio of sample/POR. The decrease in δ^{15}N for flash fractions probably reflects the greater fractionation that is expressed during incomplete oxidation (smaller value of *f*; Figure 3), suggesting that the oxidation may be kinetically inhibited in the presence of high amounts of C. In contrast, the δ^{15}N values of PCFs are approximately equivalent at different concentrations. This argues for oxidation of samples at the minimum total concentration necessary for isotopic analysis in triplicate, plus supplemental measurements.

Similarly, these data also show that purification of PCFs by HPLC is essential. While both flash fractions and PCFs contain minimal nonporphyrin N, flash fractions show a greater variation between replicate oxidized samples in measured values of δ^{15}N. Figure 6a shows all environmental sample data plotted with a 0.7‰ Rayleigh correction, whereas in Figure 6b the correction factors are calculated individually for each sample, assuming a maximum oxidation yield of 90% for each dilution series, with other *f* values standardized according to their estimated yields. For both methods of Rayleigh fractionation correction, we assume an uncertainty in the correction factor of 0.4‰, to account for a range of potential oxidation efficiencies. Correcting the data in this way removes some of the isotopic depletion for higher concentration flash samples.

The variability in the data suggests that flash fractions, like large samples, experience problems with oxidation efficiency. The higher concentration of other, non-nitrogen containing molecules in flash fractions may be saturating the oxidizing reagent or inducing some form of kinetic inhibition during oxidation. Without a Rayleigh-fractionation correction, the δ^{15}N values measured for dilution series of flash samples have a 2.5‰ range, whereas the range is much smaller within the dilution series of HPLC-purified PCFs (0.6‰). Obtaining the maximum relative concentration of porphyrin within PCFs and minimizing the total material oxidized appears necessary for preventing fractionation associated with the measurement. Additionally, consistency of replicate isotopic measurements taken from a single sample decreases as the time between oxidation and isotopic analysis increases. We have found that samples measured within a month of oxidation have less variability in δ^{15}N among replicates. We suggest that oxidized porphyrin samples be isotopically analyzed within 4 (ideally <2) weeks of preparation to prevent isotopic alteration.

Application. On the basis of the nitrogen yields of PCFs obtained from our samples, we are able to estimate the porphyrin concentrations of natural sedimentary materials. The organic rich (~10% total organic carbon (TOC)) shale samples used for this study contain ~20 nmol of porphyrin N per gram of original material. To prepare oxidation reactions that contain ~50 nmol of total porphyrin N, ~2.5 g of these samples is required, thus this method allows for the analysis of small sediment samples if they are organic-rich. Assuming that porphyrin concentrations scale with TOC content, our data suggest that for a typical sediment containing 2.5% TOC, a sample size of 10 g would be required for δ^{15}N analysis, and samples containing <1% TOC also might be feasible to analyze if 50 g of material were available.

Another advantage of this method is the ability to rapidly generate large data sets. The first porphyrin δ^{15}N measurements were made using column chromatographic purification of geoporphyrins followed by combustion-based isotopic analy-

sis.[17,38,39] This EA-IRMS approach typically requires large samples of nitrogen (≥1 μmol of N). In combination, these techniques involve increased sample preparation time and require the processing of up to 2 orders of magnitude more material than our method. Other approaches to $\delta^{15}N$ analysis of porphyrins involve purification by HPLC, followed by degradation of porphyrins to maleimides and analysis by gas chromatography−isotope ratio mass spectrometry.[40] While the maleimide approach for porphyrin $\delta^{15}N$ analysis requires ∼5× smaller samples (9.6 nmol N for triplicate analysis), our measurements are performed on a porphyrin mixture rather than on individually purified porphyrins, minimizing the amount of sediment needed as well as the preparation time. We estimate that to measure 20 samples in triplicate should take approximately 3 weeks using our method, with the bulk of the preparation time equally distributed between HPLC purification and flash separation. Additionally, we are able to routinely screen our samples for nonporphyrin nitrogen contamination using the amine fluorescence analysis, which consumes about 10% of each sample.

The method presented here should have numerous applications in paleoceanography. Changes in the availability of fixed nitrogen in the oceans may affect the strength of the biological pump. The biological pump exports carbon from surface waters, and reconstructions of marine nitrogen biogeochemistry in the past may improve our understanding of the global carbon cycle. Our method can be used to measure nitrogen isotopes of porphyrins from marine sediments and sedimentary rocks of various ages. Since PCFs are derived from a mixture of porphyrins, measured isotope values represent an integrated surface-ocean signal. Nonchlorophyll compounds such as hemes may contribute to sedimentary porphyrins, but the ratio of chlorophyll to heme in biological samples argues against a significant isotopic contribution from heme sources.[41] Analysis of the carbon isotopic composition of different etioporphyrin compounds in two oil shales demonstrated a 4‰ depletion of ^{13}C in C_{32} etioporphyrin, indicating a different, potential heme source of this compound.[42] However, in these oil shales, C_{32} etioporphyrin contributes <2% and, in some cases, <0.01% of porphyrin contents. RP-HPLC-MS analysis of our test samples showed an absence of C_{32} etioporphyrin (Table S-2 in the Supporting Information).

Although the method has been developed for the analysis of geoporphyrins, a slight modification of the chromatographic steps would allow for the analysis of nondegraded chloropigments of recent age, from both sediments and the water column. The small-sample capability also will have applications to samples that are organic carbon-poor, to samples whose bulk nitrogen isotopic composition may have been compromised by diagenetic processes, or to samples in which diatoms are absent, which would prohibit the analysis of matrix-protected N found within diatom microfossils.[16,15,43]

CONCLUSIONS

We have developed a method that allows for rapid analysis of $\delta^{15}N$ values of sedimentary porphyrins. Porphyrins are obtained from lipid extracts of small quantities of sediment (approximately 2−50 g), and isotopic data is obtained in triplicate isotopic analyses, using 10 nmol N per measurement. Each sample in total requires ∼50 nmol N for concentration measurements, triplicate isotope measurements, and assessment of purity. Using porphyrin standards, we have determined that porphyrin oxidation is consistent with Rayleigh fractionation with $\alpha \sim 0.997$. For environmental samples where oxidation yields are unknown, we suggest that a standard correction of 0.7‰ be applied, corresponding to an average oxidation yield of 90%. This method is useful for historical reconstruction of the marine nitrogen cycle in sedimentary samples that may have undergone isotopic alteration during diagenesis.

ACKNOWLEDGMENT

This work was supported by funding from the David and Lucille Packard Foundation as well as NSF Grants EAR-0311937 and OCE-0825269 to A.P. We would like to thank S. Carter, J. Husson, and K. McElhoney for laboratory assistance and members of the Summons laboratory, especially A. Kelly, for assistance with ASE. We thank two anonymous reviewers for their comments.

SUPPORTING INFORMATION AVAILABLE

Additional information as noted in text. This material is available free of charge via the Internet at http://pubs.acs.org.

Received for review August 15, 2008. Accepted October 31, 2008.

AC8017185

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