DEVELOPMENT OF A SENSITIVE $\delta^{15}N$ ANALYSIS FOR PHOTOPIGMENTS

Nicholas D. Ballew

A Thesis Submitted to the
University of North Carolina Wilmington in Partial Fulfillment
of the Requirements for the Degree of
Master of Science

Department of Chemistry and Biochemistry

University of North Carolina Wilmington

2007

Approved by

Advisory Committee

_____ _____

_____
Chair

Accepted by

_____
Dean, Graduate School

TABLE OF CONTENTS

ABSTRACT

A method for the oxidation and nitrogen isotopic analysis of photopigments (chlorophyll and phaeophytin) has been developed. Homogenous single source materials (spinach and diatom culture) as well as mixed matrix (estuarine water) natural samples were tested with this approach. This method first uses extraction and purification of photopigments by column chromatography, followed by UV irradiation to convert photopigment N to nitrate and nitrite in the presence of hydrogen peroxide. $\delta^{15}N$ of bulk samples and chlorophyll extracts were determined with an Isotope Ratio Mass Spectrometer (IRMS) with an Elemental Analysis (EA) Interface. Quantification of post-oxidation products (nitrate and nitrite) and determination of oxidation efficiency were performed using ion chromatography. For isotopic analysis, the resulting nitrate/nitrite was converted to N_2O at the USGS Reston Stable Isotope Laboratory (RSIL) using a strain of bacteria *Pseudomonas aureofaciens*, which lacks N_2O-reductase activity. The N_2O is analyzed on an IRMS to provide the $\delta^{15}N$ ratio of the product. Comparisons were made between bulk source material (spinach and diatom), the respective extracted photopigments, and the post-oxidation products. Isotopic offsets of homogenous samples were isotopically depleted by a range of 3 to 6 ‰ from bulk to extract and again from extract to post-oxidiation. This stepwise lightening of the isotopic signature was consistent with isotopic fractionation during each of these steps, although no clear Rayleigh fractionation relationship was discernable. Natural mixed matrix samples exhibited irreproducible isotopic results following both extractions and oxidation. We suggest that these results were due at least, in part, to very high amounts of co-extracted compounds potentially extracting other nitrogen sources and/or severely depressing oxidation efficiencies of photopigment nitrogen. Although

isotopic offsets for each step of the mixed matrix samples varied considerably, they all yielded end products which were substantially isotopically depleted (up to -12‰) relative to bulk isotopic values of the starting material.

LIST OF TABLES

Table Page

LIST OF FIGURES

Figure

Page

INTRODUCTION

Humans currently contribute more reactive nitrogen (N) to the biosphere than all other natural N-fixation processes combined (Galloway et al., 2003; Howarth et al., 1996). As continents trend closer towards N saturation, increasing N loads are exported to the ocean margins. Nitrogen availability typically constrains primary production in coastal marine systems, and globally, near-shore habitats currently receive N inputs at historically unprecedented rates (Howarth et al., 2002; Nixon, 1997). Accelerated primary production represents the first link between increased nitrogen inputs and biological response, and rates of autotrophy in and of themselves define the trophic status of a water mass (Nixon, 1995). Determining N source utilization by primary producers (e.g. phytoplankton and benthic microalgae) is thus central to understanding marine ecosystem response to N inputs.

The multiple sources and rapid cycling of nitrogen in these environments create difficulties in attributing recent algal production to specific N sources, or inferring historical patterns in N loading by examining the sediment record. A common approach is to use nitrogen stable isotope analysis ($\delta^{15}N$) of potential sources and of primary producers at natural abundance or ^{15}N-enriched levels. This approach has provided insights into N source contribution to primary production in some systems (McClelland et al., 1997), yet its general utility depends on clear isotopic separation of N-source end-members, and how well microalgae can be isolated from bulk organic matter for isotopic analysis. Isotopic end-member overlap, can be overcome to some extent by in situ, low-level ^{15}N enrichment of a particular N source. Tobias et al., (2001) and (2003a,b) demonstrated that this approach is feasible for a variety of N sources and applicable on kilometer spatial scales in hundreds of thousands of cubic meters of water, but

conclusions are still subject to the fundamental limitation that arises from difficulty in cleanly separating microalgae (phytoplankton) from suspended or deposited organic matter, and to a greater extent from isolating benthic microalgae from sediments.

Microalgal sample contamination with bulk organic matter of a different $\delta^{15}N$ is typically one of the largest sources of error in establishing isotopic fidelity between nitrogen sources and sinks (i.e. algae). This error is introduced both into the application of $\delta^{15}N$ techniques used to study 'modern' autotrophic processes and into investigation of historical shifts in N source support of primary production. In the study of modern systems, microalgal separation is particularly problematic in coastal habitats dominated by sediment autotrophy through benthic microalgae. Because the organic fractions of microalgal cells rapidly break down after mortality during cell sinking and / or soon after deposition, paleo-reconstruction of past autotrophic N-utilization cannot be accomplished through physical separation of intact cells. Instead, much of this inference has been derived from interpretation of ^{15}N analysis of bulk sediment organic matter (Altabet et al., 1999; Pride et al., 1999; Higginson et al., 2003). While this approach can yield a reasonable proxy for phytoplankton N contributions in the open ocean, the bulk sediment ^{15}N is still subject to alteration from various isotope fractionating processes during diagenesis (Lehman et al., 2002; Montoya, 1994; Sachs et al., 1999), and contributions from non photosynthetic organic N inputs along continental margins (Eadie et al., 1994). Reconstructions of past N source contributions to paleo-production based on $\delta^{15}N$ in sediments are subject to substantial re-interpretation when $\delta^{15}N$ bulk sediment profiles are reanalyzed with more diatom specific biomarkers (Pantoja et al., 2002; Robinson et al., 2004; Sigman et al., 1999). A truly algal-specific and diagenesis recalcitrant measurement of $\delta^{15}N$ in the sediment record would

remove some of the ambiguity associated with interpretation of past patterns of nitrogen utilization based on $\delta^{15}N$ preserved in bulk sediments.

The goal of this project is to develop an improved technique for measuring the $\delta^{15}N$ of primary producer-derived N in modern and buried sediments by using a photopigment biomarker proxy. The proposed approach builds upon existing pigment isolation approaches by integrating a new ^{15}N analytical technique that uses $1/100^{th}$ the amount of N mass previously required.

The advantage of using photopigment biomarkers (e.g. $\delta^{15}N$ of chlorophyll and phaeophytin) is twofold: 1) The approach permits clear separation of photosynthetically-derived N from a large matrix of sediment and water column organic matter in modern systems; 2) The N-containing tetrapyrrole ring is preserved in sediments long after cell mortality and offers a more precise (autotroph-specific) indicator of paleo-N source than bulk sediments.

Existing methodologies for isolating microalgae for $\delta^{15}N$ analysis rely on density or size differences between algal cells and non-photosynthetic organic matter. In turbid, highly net heterotrophic estuaries, upwards of 90% of suspended organic nitrogen may be non-algal (Tobias et al., 2003a) thus limiting the use of density-fractionated seston as a robust substitute for microalgal $\delta^{15}N$. Phytodetrital or benthic microalgal separation from sediments poses a similarly difficult problem (Currin et al., 1995). Even after multiple density separations, benthic microalgal recovery from sediment may remain poor. Techniques that rely on cell motility (Tobias et al., 2003a) account for only specific species (motile species) which may reside in a geographically limited distribution. Insufficient separation of benthic microalgae is particularly problematic in ^{15}N tracer studies where sample contamination may yield substantial underestimates of N turnover rates (Tobias et al., 2003a).

Combining stable isotope analysis with carbon-containing biomarkers (e.g. lipid biomarkers) has been used extensively to trace algal carbon sources in sediments on recent to extended timescales (Boschker et al., 1998; Canuel 2001). However, integrating $\delta^{15}N$ with N-containing biomarkers remains rare. Few examples exist for N biomarkers in general and two that have been integrated with isotope analysis and used in ecosystem scale inquiry (D-alanine, and diaminopimelic acid) are bacterial biomarkers (Tobias et al., 2003b; McClelland and Montoya, 2002). They cannot specifically address microalgal N utilization or contribution to the sediment record. Photosynthetic pigments are perhaps the only biomarkers available for sourcing nitrogen from microalgae or tracking microalgal derived N through early diagenesis. A few attempts to use $\delta^{15}N$ of pigments as algal biomarkers have previously occurred (Sachs et al., 1999; Pantoja et al., 2002). In each of these instances it was demonstrated that the pigments could be extracted and analyzed with good isotopic accuracy and with a reproducible isotopic offset between photopigment and the whole microalgal cell. However, in each of these cases the amount of sample (i.e. N mass) necessary for isotopic analysis was so large that it has precluded widespread use of the approach. The technique outlined in this project employs recent advances in isotopic NO_3-N analysis that can quantify $\delta^{15}N$ on approximately $1/100^{th}$ the nitrogen mass previously required. This method can be broken down into 5 steps: 1) pigment extraction and purification; 2) oxidation of pigment N; 3) $\delta^{15}N$ isotopic analysis of bulk extracts, 4) $\delta^{15}N$ analysis of oxidized pigments 5) preliminary application to terrestrial and marine derived samples.

METHODS

The method development undertaken in this project involves 5 key steps. These concepts are: (1) pigment extraction and purification; (2) pigment oxidation and post-oxidation analysis; (3) isotopic analysis of bulk extracts, (4) isotopic analysis of post-oxidized N; (5) application of method to terrestrial and marine samples

General Procedures

Polyethylene disposable gloves (VWR International, #32915-188) were worn when handling all reagents, buffers, and samples. All digital pipet tips, plastic and glass scintvials and quartz test tubes were placed in a 10% hydrochloric acid bath and soaked overnight. After soaking, all containers and tips were rinsed thoroughly with Milli-Q water and dried in a designated clean space. All volumetric glassware and caps were rinsed several times with Milli-Q water prior to making solutions. All quartz screw cap tubes were heated in a Fisher Scientific IsoTemp Muffle Furnace for 6 hours at 445˚C prior to use.

Reagents and Standards

Sodium nitrate and sodium nitrite were obtained from Sigma (St. Louis, MO). Water was purified using a Millipore Q-water system (Millipore Corp., Bedford, MA) and used to prepare all solutions. Reagent grade acetone and hexane from Sigma (St. Louis, MO) were used to extract photopigment from spinach, diatoms, and sediment samples. Concentrated hydrochloric acid (Reagent Grade) was used to prepare the 10% acid bath. Unless otherwise noted, all chemicals used in this project were reagent grade from VWR International, West Chester, PA.

Photopigment Extraction/Purification

Photopigments from a terrestrial source (spinach) and from marine sources (cultured diatoms and creek water suspended particulate organic matter (SPOM)) were used in the study.

Spinach Extraction

Organically grown spinach (450 g) was blended in a Waring Commercial Blender HGB-SS (Model N# 36BL23) using 250 mL of a 1:1 mixture of methanol:dicholoromethane for 3 minutes. The supernatant was filtered through cheesecloth and transferred to a 500 mL separatory funnel. The filtered spinach was washed with 50 mL of acetone, followed by subsequent 10 mL aliquots of acetone, hexane, and ether. This supernatant was filtered through cheesecloth and transferred to the same separatory funnel.

After adding 25 mL of saturated aqueous NaCl to the separatory funnel, two layers formed. The aqueous layer was drained from the separatory funnel and the organic layer was transferred into a 250 mL round bottom flask. The aqueous layer was returned to the separatory funnel and extracted with 50 mL of hexane to ensure complete extraction of photopigments. The aqueous layer was removed and the organic layer was combined with the initial extract in a round bottom flask. The extract was concentrated to dryness under reduced pressure with the Buchi Rotavapor R-3000 and a Cole-Parmer Aspirator Pump (Model # 7049-00).

Diatom Extraction

Artificial sea water (ASW) was prepared using a standard recipe (Appendix A). Approximately 8 L of ASW were added to a 3.5 gallon Pyrex glass carboy (VWR) and autoclaved for 30 minutes on the liquid cycle of an autoclave to sterilize the media. After the ASW cooled overnight to room temperature, 10 mL of a *Thalassiosira Weisfloggi* (*T.*

Weisfloggi) diatom culture was added to the stock solution. The carboy was placed in a Percival Incubator at 16˚C with constant fluorescent lighting.

To measure diatom growth, 5 mL aliquots were obtained daily, transferred to a Pyrex cuvette and measured on the AK – 10 fluorometer to measure the growth rate of the diatom culture. Once the culture growth rate leveled off, 5 additional mL of the nitrogen stock and silicate stock solutions were added to the culture. This was added to increase growth since silicate and nitrogen are the limiting elements in diatom growth. Once diatom growth ceased, diatoms were filtered onto a muffle furnaced GF/D glass fiber filter using a GeoPump.

The filter was placed in an amber glass jar with 200 mL of 90% acetone saturated with $MgCO_3$ overnight to extract the photopigments from the diatoms. The purification procedure used in the spinach extraction was applied to the diatom extraction with proportionately less solvent.

Photopigment Separation

Post-extraction, TLC analysis (Merck) was performed on each extract to determine the presence of phaeophytin and chlorophyll. TLC plates were analyzed under long range UV light and stained with 10% H_2SO_4/ethanol to determine the presence of lipids. Column chromatography using SiO_2 (250-400 mesh, 2 x 15 cm column, Selecto Scientific) and a 2:1 acetone:hexane mixture was performed to purify photopigments. After column chromatography, column fractions containing pure photopigments were combined and concentrated under reduced pressure for HPLC analysis.

HPLC Analysis

Chromatographic analyses were performed to assess photopigment purity from the column chromatography extracts using a HP (Agilent) HPLC Model 1100 series, equipped with a G1311A Quartenary Pump, a Rheodyne 7725i autoinjector, and a G1315A Photo Diode Array (PDA). The chromatographic system was controlled by a computer using the ChemStation Revision A.10.02 software. Chromatography was carried out on a reversed phase Luna 100 mm x 4.60 mm 3 μ C18(2) Phenomenex column with a pore size of 100 Angstroms protected by a guard column.

The mobile phase consisted of 2 components: solvent A, methanol:acetone (7:3); and solvent B, Milli-Q water. The pigments were eluted beginning with a 70% isocractic elution of A for 1 minute, followed by a linear gradient increase to 95%A from 1-5 minutes. This was followed by a linear gradient increase to 100% A over 5-20 minutes, isocratic elution with 100% A for 5 minutes, a linear gradient decrease to 70%A from 25-29 minutes and finally a 1 min isocratic elution with 70% A prior to the next injection. The solvent flow rate was 1.0 mL/min. The injection loop volume was 200 uL. The eluate was scanned with the PDA at selected wavelengths of 432, 450, 470, 652 and 656 nm to determine the presence of photopigments.

Spectroscopic Calculations

Once the sample was found to be pure via HPLC, 25 uL of a photopigment standard was dissolved in 1.25 mL of ether or acetone and quantitatively transferred to a quartz 1.0 cm cuvette (Fisher Scientific 14-385-926A) for spectrophotometric analysis on a Cary 1E UV/Vis Spectrophotometer in single beam mode (Varian, Inc.) with WinUV Version 2.00(25) software. The spectrum of the sample was obtained from 350 to 750 nm. Using Beer's Law, molarity of

the photopigment extract was obtained using the molar absorptivity of photopigment a at 663 nm. Using the molecular weight of photopigment, the concentration of the extract, in grams/L, allows for determination of the isotopic ratio of the extract using IRMS-EA. However, it was found that this approach overestimated the amount of photopigment nitrogen). N content determined with the IRMS-EA was used subsequently for calculation of N concentration of the purified extract.

Pigment Oxidation

Two methods, persulfate oxidation and UV oxidation with H_2O_2, were used to determine which method would be better to oxidize photopigments from spinach extracts. Because UV/H_2O_2 oxidation was more successful than persulfate oxidation, so UV/H_2O_2 oxidation was used on the diatom matrices.

Conversion of photopigments by UV oxidation was performed in a stainless steel UV oxidation chamber that was fabricated locally. The chamber was equipped with a 1200 W Mercury Arc lamp (Ace Glassware 7825-40). Samples were oxidized in GE 214 Semiconductor grade fused quartz screw thread test tubes (12 mL) manufactured by Technical Glass Products, Inc (Model Number SC18). Caps were Teflon lined. Aluminum foil was used on both the inside and the outside of the caps to prevent sample contamination.

Photopigment samples, containing 1.2 x 10^{-7} moles of nitrogen dissolved in 1:2 hexane:acetone, were pipetted into quartz test tubes that had been heated in a muffle furnace at 445˚C for 6 hours. The solvent was blown to dryness under UHP Argon. Post-evaporation, 5 mL of Milli-Q and 20 uL 30% hydrogen peroxide (H_2O_2, Fisher#H323-500) were added to each container. Tops of test tubes were then covered with muffle-furnace combusted aluminum foil to

prevent contamination from materials inside of the cap. Then, the tubes were screwed tight with Teflon lined standard caps. The caps were covered with aluminum foil to prevent UV degradation of the cap. Samples were oxidized in the UV chamber for 4 hours and kept sealed for 12 hours post-UV irradiation to allow maximal conversion from the $NO_{2(g)}$ intermediate to NO_2^- and NO_3^- ions.

Post-Oxidation NO_x measurement and Determination of Oxidation Efficiency

All analyses of the oxidized products were performed on a Dionex DX-600 Gradient Ion Chromatography system, equipped with an autoinjector, a thermostatted column compartment and a GP50 Gradient pump with a vacuum degasser. Peaknet 6 software for the Dionex IC system was used for all chromatographic analyses. Instrumental parameters were as follows: Eluent = 8.0:1.0 Carbonate:Bicarbonate, Data Collection Rate = 2.0, Temperature Compensation = 1.7, DS3 Temperature = 35ºC, Suppressor Type = ASRS 4mm, Suppressor Current = 43 μamp, Flow Rate = 1.0 mL/min, Run Time = 10.00 min.

An IonPac AS14A-7um analytical column (4 x 250 mm) with an IonPac AG14A-7um Guard Column (4 x 50 mm) was used for all samples. Samples (200 μL) were manually injected onto the column. Nitrate and nitrite standards (1 μM, 5 μM, 10 μM, 20 μM, and 50 μM) were prepared daily from more concentrated standards to determine calibration curves.

Oxidation efficiency was determined by combining the concentration of nitrate and nitrite in each sample to calculate total moles of oxidized organic N. This value was divided by initial moles of organic N provided by EA analysis of purified extract.

IRMS-EA Analysis

Total sample nitrogen mass, % nitrogen, $\delta^{15}N$, and the carbon:nitrogen mass ratio of extracted/purified photopigments from terrestrial and marine sources were measured using continuous flow EA-Isotope Ratio Mass Spectrometry. This system consisted of a Thermo Delta V Plus Isotope Ratio Mass Spectrometer with a Costech ECS 4010 Elemental Combustion System Elemental Analysis Interface.

To perform the analysis, an aliquot of the photopigment standard that would provide approximately 2 μmoles of nitrogen was carefully blotted onto muffle-furnaced GF/D grade glass fiber filters. Once the blotting was complete, the filter was transferred to an acid-washed scintvial and placed inside of a vacuum oven to evaporate the solvent from the filter. After the filter was completely dry, it was carefully packaged in a 5 x 9 mm pressed tin capsule (Costech).

During each run, glutamic acid standards were prepared from two isotopically labeled reference materials (USGS-40 and USGS-41). Standards were weighed on a MX5 (Mettler Toledo) microbalance and quantitatively transferred into a tin capsule for elemental analysis. The mass of each standard bracketed the N content of the photopigment samples.

At the end of each sample run, a mass chromatogram is produced which provides the $^{15}N/^{14}N$ and the $^{13}C/^{12}C$ isotopic ratios and the elemental composition of each sample. With this data, a calibration curve is made from which the nitrogen and the carbon content of each photopigment sample can be extrapolated.

Isotopic Analysis of Post Oxidized N

After IC analysis, the samples were quantitatively transferred to 25 mL acid-washed scintvials. All scintvials were tightly sealed and shipped to the USGS Reston Stable Isotope

Laboratory (RSIL) in Reston, Virginia. At the RSIL, $\delta^{15}N$ of the post oxidized N ($NO_2^- + NO_3^-$) was measured using the 'denitrifier method,' which converts nitrate and nitrite to N_2O. This is accomplished by using a strain of denitrifying bacteria, *Pseudomonas aureofaciens*, which lacks N_2O-reductase activity. The bacteria quantitatively convert nitrate and nitrite to N_2O. The N_2O is cryofused in dry ice and isopropanol trap and released as a pulse to the Delta XP IRMS thru a modified Gas Bench Interface. The $\delta^{15}N$ is calculated from the distribution of mass/charge ratios (m/z) of 44, 45, and 46 after accounting for the $^{18}O/^{16}O$ composition in the N_2O.

The experimental protocol and system design is detailed in Sigman, D.M. et al., 2001.

Method Application to Mixed Media Natural Samples

To test the applicability of the method on natural samples, the method was applied to a sample containing two types of marine samples; *T. Weisfloggi* diatoms and natural creek water samples from Hewletts Creek, NC. Mixtures of natural (Particulate Organic Matter-laden) water and cultured diatoms of known isotopic composition were tested.

First, creek water (25L) was collected from Hewletts Creek at location 34˚11' N, 77˚51' W on May 21st , 2007. The creek water was stored in 15 liter Nalgene carboys for 1 week in a dark room. This storage procedure terminated all actively growing phytoplankton. Second, artificial sea water (8L) was prepared, using the protocol listed in Appendix A, and 1 liter of *T. Weisfloggi* diatoms was added to grow a diatom solution. The concentration of the diatom culture was determined to be 21.4 ug chl a/liter. Once the diatoms were grown for a week, the solutions used in Table 1 were prepared.

Carboy	Liters of Diatom Solution	Liters of Creek Water
A	0	8
B	1	7
C	2	6
D	4	4
E	8	0

Table 1: Mixture ratios of Hewletts Creek water and diatom culture used for experiment

After mixing, each carboy was filtered using a peristaltic pump (Geopump) through a GF/D glass fiber filter. Once filtration was complete, each filter was placed into a freezer overnight with 250 mL of acetone to completely remove all photopigments from the GF/D filters. As with the other pigment samples, total N mass, and isotopic value were determined on the bulk extracts from each mixture using IRMS-EA. An aliquot of each mixture was subjected the UV oxidation process (as described above). Percent recovery and isotopic value on the resulting post-oxidation NO_x was measured using the denitrifier method.

RESULTS

To demonstrate the efficacy of this method, the following factors were analyzed: photopigment separation and purification; oxidation optimization and analysis, comparison of $\delta^{15}N$ of bulk photopigment from spinach and diatom cultures; $\delta^{15}N$ of post-oxidized nitrogen, and applications of the method.

Pigment Separation and Purification

TLC analysis of the spinach and the diatom extracts prior to column chromatography showed 2 visibly distinct spots; the first spot was olive green with a retention factor, R_f, value of 0.65 while the second spot was emerald green with an R_f value of 0.38. 10% H_2SO_4/ethanol staining of TLC plates under long range UV light also showed the presence of lipids.

After column chromatography, chlorophyll a and phaeophytin a were combined and samples were analyzed by HPLC to determine extract composition. During HPLC analysis, two peaks were observed with detection at 660 nm (Figure 7). Using the photo diode array detector, it was determined that the first peak, at 19.51 minutes, was chlorophyll a. The second peak, at 25.24 minutes, was determined to be phaeophytin a.

Oxidation

Persulfate vs. UV/H_2O_2 Oxidation

Two oxidation methods, persulfate oxidation and UV/H_2O_2 irradiation, were used to convert the nitrogens in photopigments to NO_x. Persulfate oxidation successfully converted glycine standards to NO_x with high efficiency, but did not consistently convert the nitrogens in

photopigments to NO_x. Poor oxidation efficiencies, <20%, were typically seen due to solubility problems between the photopigment extract and water. Post-oxidation, green globular particulates were seen floating in the solution. Numerous attempts to increase the solubility of the photopigments via increasing the reaction time, increasing the amount of persulfate, and heating the photopigment solution prior to oxidation, proved unsuccessful.

The second oxidation method, UV/H_2O_2, consistently oxidized various nitrogen standards including photopigments, glutamic acid, and phaeophytin with high efficiency. Solubility problems were not encountered with UV/H_2O_2 photolysis. While our measure of efficiency ($[NO_3^-] + [NO_2^-]$) was high with UV/H_2O_2, some samples showed that the final end products consisted of both nitrate and nitrite. The relative proportion was related to the quantity and vendor of H_2O_2 used, irradiation time of the samples and post-oxidation equilibration of the sample.

The amounts of nitrate and nitrate in the product were determined using ion chromatography. Sample calibration curves for the 6 nitrate and nitrite standards (1 to 50 uM) using a 200 μL injection loop are shown in the Appendix B.

Optimizing the UV/H_2O_2 Method

Optimum UV irradiance time was determined by irradiating a solution containing 5 μM photopigments in 200 μL of 30% H_2O_2, and 5 mL of Milli-Q in an oxidation chamber at times varying from 2 to 24 hours. Oxidation efficiency was calculated by adding the concentrations of $[NO_2^-]$ and $[NO_3^-]$ and dividing the value by the total concentration of nitrogen, as determined by EA.

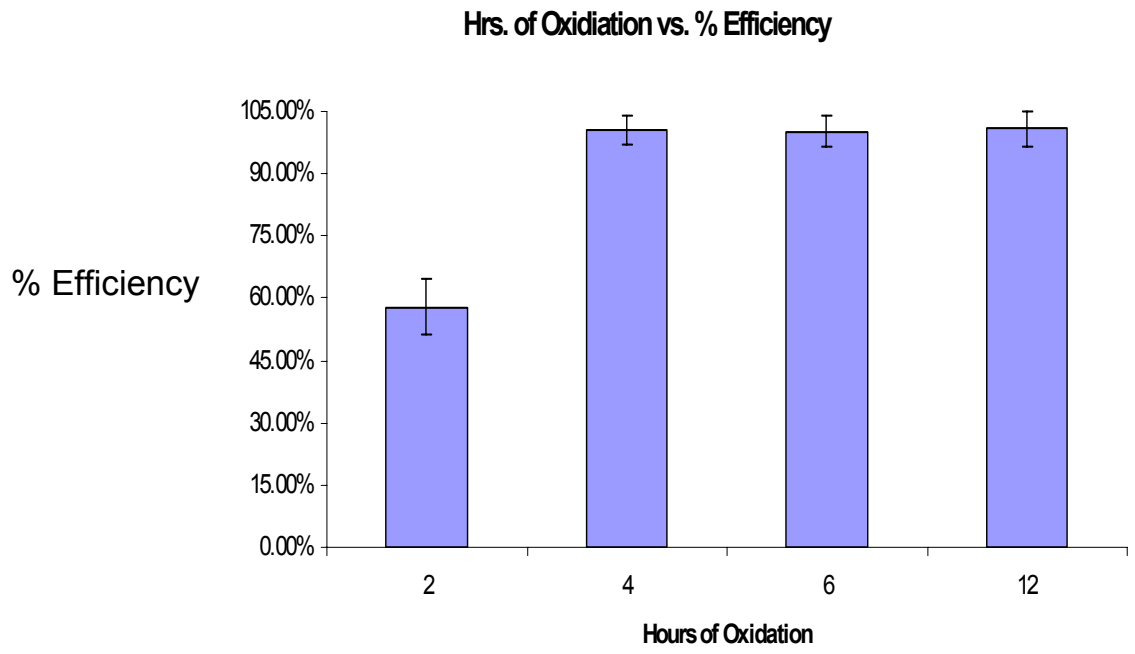

Figure 1: Comparing oxidation efficiency (μmoles of NO_x divided by μmoles of pre-oxidized nitrogen) of photopigment samples (5 uM photopigment, 200 uL 30% H_2O_2) as a function of UV irradiation time.

The optimum irradiance time was 4 hours (Figure 1). This time was deemed optimal because an irradiance time of 2 hours produced a poor percent conversion of photopigment N to nitrate. The % conversion to NO_x was 100% for all times of 4 or more hours.

Two sets of experiments (n = 6) were performed to determine the optimum amount of peroxide for the reaction while minimizing the amount of background. For the first set of experiments, the reaction matrix was a predetermined amount of peroxide, 5 mL of Milli-Q, and a reaction time of 4 hours. The sample matrix for the second set experiments was a pre determined amount of peroxide, 5 μM photopigment, 5 mL of Milli-Q, and a reaction time of 4 hours. Various

amounts of 30% H_2O_2 (0 to 200 µL) were used in the reaction. All samples were analyzed via ion chromatography and the results are shown in Figure 2.

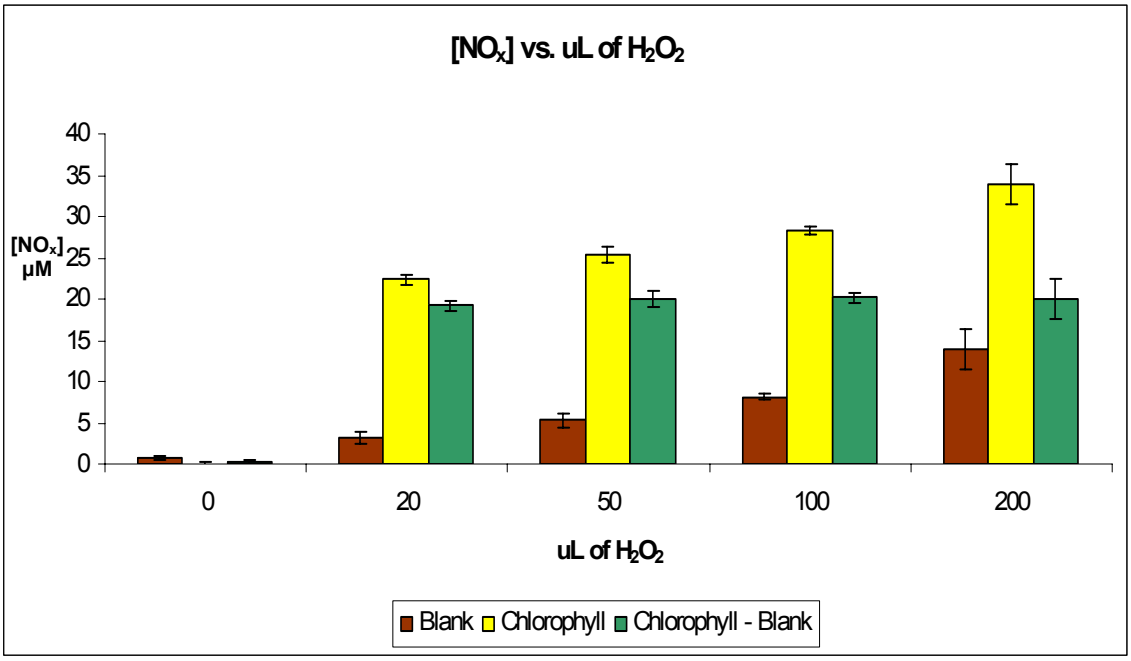

Figure 2: Comparing [NO_x] of oxidized samples (4 hrs. UV Irradiation with Milli-Q (Blank) or 5 µM photopigment and Milli-Q) at different volumes of 30% H_2O_2

The reaction was unsuccessful without peroxide, but once any H_2O_2 was added to the matrix, the oxidation efficiency increased. Furthermore, above 20 µL of H_2O_2, the addition of more H_2O_2 simply translated to a higher blank, without increased oxidation efficiency. It was seen that for each µL of H_2O_2 added to the matrix, the background increased by 0.063 µM NO_x. To minimize blanks without impacting oxidation efficiency, 20 µL was chosen as the optimum amount of peroxide.

Experiments (n=4) were performed to determine whether different sources of peroxide would produce different blanks. Two bottles of 30% H_2O_2 were purchased from VWR International and Fisher Scientific. 5 mL of Milli-Q and predetermined amounts of 30% H_2O_2 peroxide were

added to quartz test tubes. The samples were irradiated for 4 hours in the UV digestion chamber and then analyzed by ion chromatography.

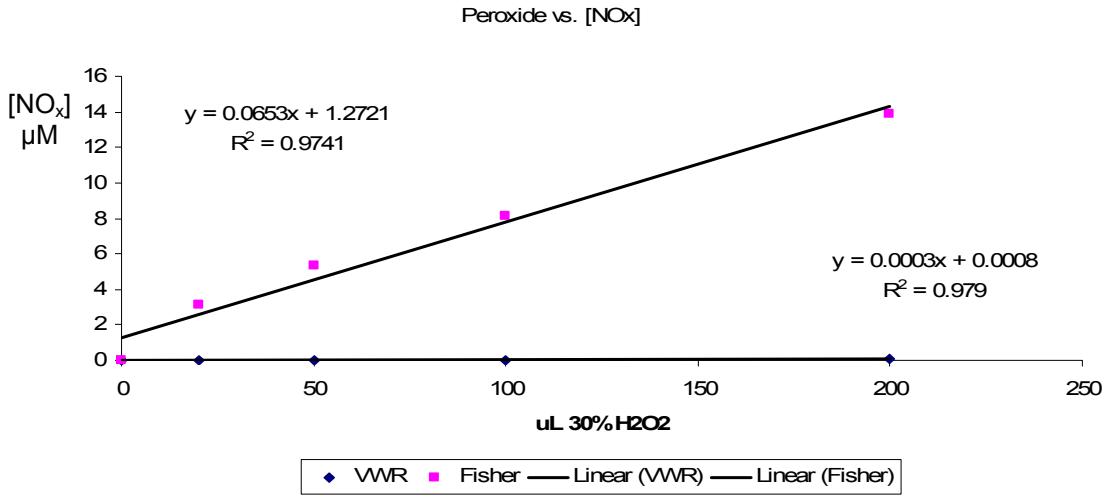

Figure 3: Comparing concentration of NO_x in oxidized samples (Milli-Q and 30% H_2O_2 with using 2 different manufacturers of peroxide over a range of 0 to 200 uL (n=3)

This experiment clearly demonstrates that H_2O_2 from different manufactures provided drastically different blanks. The H_2O_2 from Fisher produced a blank of 65 nM NO_x per uL while the peroxide from VWR provided a blank of 0.3 nM NO_x per uL of peroxide. Therefore, peroxide from Fisher produced a blank that was 210 times higher than the blank from the VWR reagent. It was not known if the reagent age, stabilizer or another quality of the peroxide was responsible for the difference. Since both types of peroxide provided the same % oxidation efficiency, peroxide from VWR was the preferred peroxide for this reaction and subsequently used for all oxidations.

Post-Oxidiation Equilibrium

Experiments were performed to determine the amount of time necessary to allow for maximum conversion of initial oxidiation products, which contains the NO radical, to form NO_3^- and NO_2^-. Samples, post-oxidation, were kept in the UV digestion chamber for pre-determined amounts of time and removed for analysis via ion chromatography. The results are listed in Figure 4.

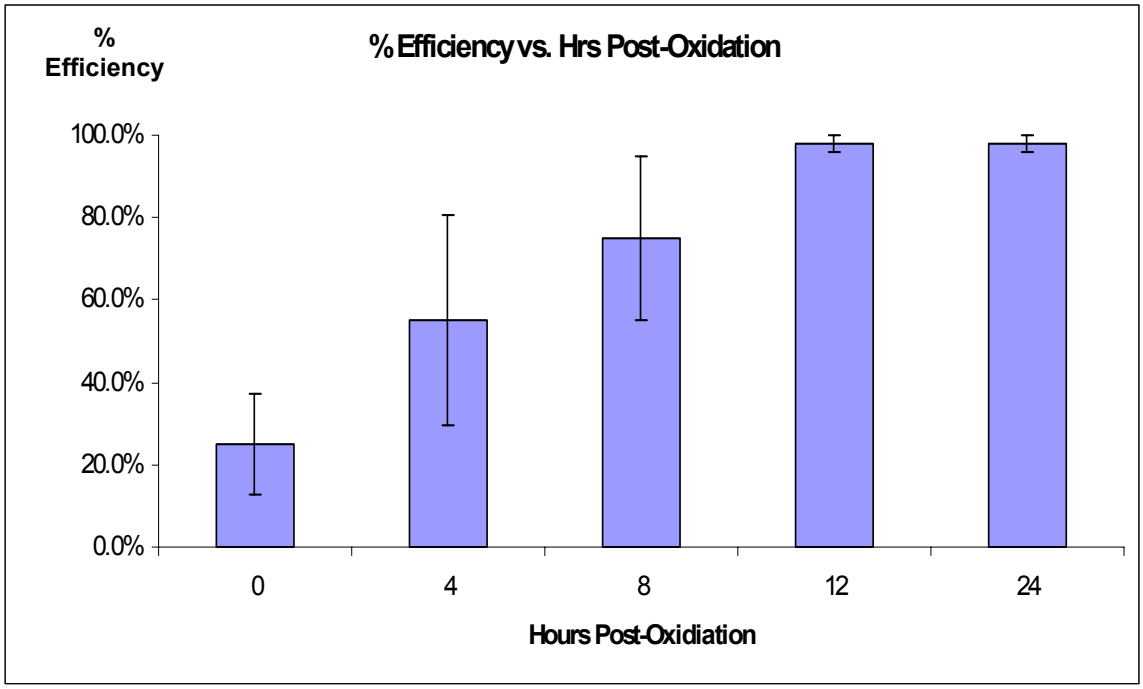

Figure 4: Comparing the % efficiency of oxidation at different times post-oxidation

This experiment showed that samples required a minimum of 12 hours, post-oxidation, to allow maximal conversion of initial products to nitrate + nitrite.

Post-Oxidized Sample Storage

Four samples were analyzed, post-UV oxidation, at designated time intervals (1 day, 7 days, 14 days and 28 days) to determine the stability of the products in the reaction matrix. 200 uL of sample were removed and injected for each IC analysis. The same bottles were subsampled for each analysis. The results are presented in Table 2.

Pre-Ox nmoles of N	Day 1 nmoles Post-Ox NO_x	Day 7 nmoles Post-Ox NO_x	Day 14 nmoles Post-Ox NO_x	Day 28 nmoles Post-Ox NO_x
0.100	0.100	0.105	0.100	0.102
0.100	0.095	0.096	0.094	0.096
0.100	0.088	0.086	0.094	0.085
0.100	0.092	0.085	0.083	0.085
Average	0.094	0.093	0.093	0.092
Standard Dev.	0.005	0.009	0.007	0.009

Table 2: Comparing the umoles of NO_x for post-oxidized photopigment standards (n=4) (5 μM photopigment, 20 uL 30% H_2O_2, and 5 mL of Milli-Q with 4 hrs. of UV Irradiation) at 4 time intervals

This experiment shows that all samples could be stored for up to a month post-oxidation without producing a significant change in [NO_x] of the sample.

Nitrate vs. Nitrite

Photopigment samples from the spinach extract (n=8) were oxidized and analyzed to see whether there was a correlation between the amount of peroxide used versus the amount of nitrate/nitrate produced. All standards were reacted with either 10 or 20 uL of H_2O_2, 5 mL of

Milli-Q water and the requisite amount of standard to produce 20 uM nitrate post-oxidation. The results of the experiment are shown in Table 3.

Amt. of H_2O_2 Added	µmoles N as $[NO_2^-]$	µmoles N as $[NO_3^-]$	% $[NO_2^-]$	% $[NO_3^-]$	µmoles N $[NO_2^-]$ + $[NO_3^-]$	% Efficiency
10	0.032	0.071	31%	69%	0.104	104%
10	0.024	0.073	25%	75%	0.096	96%
10	0.030	0.064	32%	68%	0.094	94%
10	0.026	0.062	30%	70%	0.089	89%
Average	0.028	0.068	29%	71%	0.096	96%
RSD	0.004	0.005	3%	3%	0.006	6%
20	0.007	0.094	7%	93%	0.101	101%
20	0.009	0.082	9%	91%	0.091	91%
20	0.011	0.082	12%	88%	0.094	93%
20	0.014	0.068	16%	84%	0.081	81%
Average	0.010	0.082	11%	89%	0.092	92%
RSD	0.003	0.011	4%	4%	0.008	8%

Table 3: µmoles of post-oxidized photopigment samples (5 µM photopigment, either 10 or 20 µL of 30% H_2O_2, and 5 mL of Milli-Q with 4 hrs of UV Irradiation) as either nitrite or nitrate

While the oxidation efficiencies for each reaction were similar between each matrix, the samples which contained 10 uL of H_2O_2 produced an average of 29 ± 3 % NO_2^- vs. the 11 ± 4 % NO_2^- for the samples which contained 20 uL of H_2O_2. Nitrate was the dominant end product in the presence of higher amounts of H_2O_2. However, our measure of oxidation efficiency shows that the concentration of nitrate plus nitrite seems unaffected at H_2O_2 levels greater than 10 uL.

To examine the amount of NO_x produced in different matrices, glutamic acid standards (USGS40) and photopigment extracted from diatoms were oxidized to analyze the percent of NO_2^- vs. NO_3^- produced. The results of the experiment are shown in Table 4.

Substrate	µmoles N as $[NO_2^-]$	µnoles N as $[NO_3^-]$	% $[NO_2^-]$	% $[NO_3^-]$	µmoles N $[NO_2^-] + [NO_3^-]$	% Efficiency
Glutamic Acid	0.035	0.061	37%	63%	0.096	96%
Glutamic Acid	0.037	0.061	38%	62%	0.098	98%
Glutamic Acid	0.029	0.051	37%	63%	0.080	80%
Average	0.034	0.057	37%	63%	0.091	91%
RSD	0.004	0.006	0.80%	0.80%	0.010	10%
Diatom	0.065	0.029	69%	31%	0.094	94%
Diatom	0.051	0.024	68%	32%	0.076	76%
Diatom	0.076	0.026	74%	26%	0.102	102%
Average	0.064	0.027	71%	29%	0.091	91%
RSD	0.012	0.003	3%	3%	0.014	14%

Table 4: µmoles of post-oxidized glutamic acid or diatom photopigment extracts (5 µM photopigment or 20 µM glutamic acid, 20 µL 30% H_2O_2, and 5 mL of Milli-Q with 4 hrs of UV Irradiation) as either nitrite or nitrate

A glutamic acid standard and the diatom extract were reacted with 20 uL of H_2O_2 in 5 mL of Milli-Q and the requisite amount of standard/extract to produce 20 uM nitrite/nitrate post-oxidation. While both samples were oxidized at an efficiency of >90%, the oxidation of glutamic acid standards (n = 3) produced 37 ± 0.8 % NO_2^- while the oxidation of the photopigment extracted from diatom standards (n = 3) produced 70 ± 3 % NO_2^-.

Isotopic Analysis

After pigment extraction and purification, samples from the photopigment extract (n = 5) as well as glutamic acid reference materials (USGS40 and USGS41) (n=11) were prepared for elemental analysis on the Isotope Ratio Mass Spectrometer (IRMS) with Elemental Analysis (EA) Interface. The $\delta^{15}N$ and $\delta^{13}C$ of the CO_2 and N_2 are determined by the IRMS and referenced to USGS40 and USGS 41 glutamic acid reference materials. The $\delta^{15}N$ of USGS 40 is -4.52 while the $\delta^{15}N$ of USGS 41 is 47.264. Isotopic values are reported as ‰ relative to air for $\delta^{15}N$ and Pee Dee Belamite (PDB) for $\delta^{13}C$ (Table 5).

Reference #	Total Weight	Total Weight (μg)	μg N	$\delta^{15}N$	C:N Mass Ratio
Spinach Chl a 1	----------	----------	11.60	5.81	17.46
Spinach Chl a 2	----------	----------	11.66	5.20	17.40
Spinach Chl a 3	----------	----------	14.21	5.24	15.08
Spinach Chl a 4	----------	----------	11.99	5.33	17.95
Spinach Chl a 5	----------	----------	9.18	4.55	18.11
USGS 40	0.27	270.00	25.65	-4.30	4.29
USGS 40	0.28	282.00	26.79	-4.35	4.29
USGS 40	0.43	433.00	41.14	-4.40	4.29
USGS 40	0.52	516.00	49.02	-4.36	4.29
USGS 40	0.59	592.00	56.24	-4.31	4.29
USGS 40	0.77	770.00	73.15	-4.34	4.29
USGS 40	0.82	820.00	77.90	-4.31	4.29
USGS 41	0.49	487.00	46.27	47.23	4.29
USGS 41	0.59	592.00	56.24	47.42	4.29
USGS 41	0.48	479.00	45.51	47.19	4.29
USGS 41	0.74	742.00	70.49	47.23	4.29

Table 5: μg of Nitrogen and the carbon:nitrogen Ratio for Samples from 1st Photopigment Extract and Glutamic Acid Reference Materials (September 2006)

Nitrogen content (% nitrogen), carbon content (% carbon), and the carbon:nitrogen ratios of the extracts were calculated from calibration curves between the total weight (in μg) of nitrogen or carbon in each glutamic acid standard versus the N and C area from the IRMS chromatogram (Appendix C). The C:N ratio of chlorophyll a is 11.78, therefore, our extract contained carbon that was not from photopigment.

$\delta^{15}N$ Analysis of Spinach Photopigment Source Material

Two samples of a photopigment standard, extracted from spinach, were oxidized to determine the change between the $\delta^{15}N$ of the extracted photopigment and NO_x from oxidation and whether the change in the $\delta^{15}N$ of the substrate and the product is reproducible. Experiments with the first photopigment sample (n=3) and the second photopigment sample (n=4) were performed. Results of the IRMS-EA on the first photopigment standard are presented in Table 6

Sample	Chl a (μmoles/mL)	Post Ox (μmoles/mL)	% Efficiency	Chl a $\delta^{15}N$	Post Ox $\delta^{15}N$	$\delta^{15}N$ Offset
1st Chl a	0.381 ± 0.058	0.304	80%	5.2 ± 0.4	-0.7	-5.9
1st Chl a	0.381 ± 0.058	0.33	87%	5.2 ± 0.4	0.9	-4.3
1st Chl a	0.381 ± 0.058	0.331	87%	5.2 ± 0.4	2.1	-3.1
Average	0.381	0.322	84%	5.2	0.7	-4.5
Std. Dev.	0.058	0.015	4%	0.4	1.4	1.4

Table 6: μmoles/mL of Nitrogen and $\delta^{15}N$ Value for 1st Photopigment Standard and Post-Oxidized Samples Derived from 1st Spinach Extract

For the first photopigment sample (n=3), the $\delta^{15}N$ of photopigment before oxidation was 5.2 ± 0.4 ‰. Once these samples were oxidized, they were shipped to the USGS for [NO_x] and $\delta^{15}N$ analysis. Post-oxidation, the $\delta^{15}N$ of the oxidized samples (n=3) were 0.7 ± 1.4 ‰. When these values were compared to the starting material, the method produced an isotopic depletion of the $\delta^{15}N$ by 4.5 ± 1.4 ‰ relative to the bulk extract. Therefore, the samples became isotopically lighter after oxidation and denitrification

Results from IRMS-EA analysis of the second photopigment sample are in Table 7.

Sample	Chl a (µmoles/mL)	Post Ox (µmoles/mL)	% Efficiency	Chl a $\delta^{15}N$	Post Ox $\delta^{15}N$	$\delta^{15}N$ Offset
2^{nd} Chl a	0.61 ± 0.05	0.61	100%	6.7 ± 0.5	3.0	-3.7
2^{nd} Chl a	0.61 ± 0.05	0.51	84%	6.7 ± 0.5	2.6	-4.1
2^{nd} Chl a	0.61 ± 0.05	0.44	72%	6.7 ± 0.5	3.7	-3.0
2^{nd} Chl a	0.61 ± 0.05	0.50	82%	6.7 ± 0.5	2.7	-4.0
Average	N.D.	0.56	92%	N.D.	3.0	-3.7
Std. Dev.	0.05	0.06	10%	0.5	0.5	0.5

Table 7: µmoles/mL of Nitrogen and $\delta^{15}N$ Value for 2^{nd} Photopigment Standard and Post-Oxidized Samples Derived from 2^{nd} Spinach Extract

For the second sample of photopigment (n=4), the $\delta^{15}N$ of the bulk photopigment extract (pre-oxidation) was 6.7 ± 0.5 ‰. Post-oxidation, the denitrifier analysis showed that the $\delta^{15}N$ of the oxidized product was 3.0 ± 0.5 ‰. When these values were compared to the starting material, the method produced a depletion of the $\delta^{15}N$ by 3.7 ± 0.5 ‰ relative to the bulk extract. In order to properly compare the data sets from two different batches of spinach, we decided to compare the average amount of change from the $\delta^{15}N$ of the starting material to that of the final oxidized product. Considering all spinach photopigment samples (n=7) , the average change in the $\delta^{15}N$ between the spinach photopigment extract and the post-oxidized sample was a 2.96 ± 0.48 ‰ decrease from the bulk extract to post-oxidized N.

$\delta^{15}N$ Analysis of Diatom Photopigments

Since the photopigments from the diatoms was extracted from muffle-furnaced GF/D filters, there were 3 times at which the $\delta^{15}N$ values were obtained. The first data obtained was from the filter which contained the nitrogen composition of the bulk diatoms. This value would provide the $\delta^{15}N$ value for all nitrogen (e.g., photopigments, amino acids, and proteins) that was deposited on the GF/D filter. The results from the IRMS-EA analysis of the bulk starting material trapped on the filter are presented below in Table 8.

Sample	Bulk (µmoles/L)	Chl a (µmoles/mL)	Post Ox (µmoles/mL)	% Efficiency	Bulk $\delta^{15}N$	Chl a $\delta^{15}N$	Post Ox $\delta^{15}N$	$\delta^{15}N$ Offset
1st Diatom	0.50 ± 0.05	0.44 ± 0.02	0.37	83%	-0.5	-1.4 ± 0.3	-7.1	-5.7
1st Diatom	0.50 ± 0.05	0.44 ± 0.02	0.44	100%	-0.7	-1.4 ± 0.3	-6.5	-5.1
1st Diatom	0.50 ± 0.05	0.44 ± 0.02	0.43	98%	-0.3	-1.4 ± 0.3	-7.2	-5.8
1st Diatom	0.50 ± 0.05	0.44 ± 0.02	N.D.	N.D.	-0.2	-1.4 ± 0.3	N.D.	N.D.
Average	0.50	0.44	0.41	94%	-0.4	-1.4	-6.9	-5.5
Std. Dev.	0.05	0.02	0.04	9%	0.2	0.3	0.4	0.4

Table 8: µmoles/mL of Nitrogen and $\delta^{15}N$ values for Bulk Filters, Extracted Photopigments, and Post-Oxidized Samples from the 1st Culture of Diatoms by IRMS-EA Analysis

IRMS-EA analysis of four samples showed that the $\delta^{15}N$ was -0.4 ± 0.23 ‰. After extracting photopigments from the diatom filters, 2.5 mL of diatom extract were blotted onto GF/D glass fiber filters and analyzed by IRMS-EA. Extracted photopigments from the filter, had a $\delta^{15}N$ value of -1.4 ± 0.3 ‰. The resulting NO_x samples (n=3) were analyzed using the denitrifier method and the $\delta^{15}N$ value was determined to be -6.9 ± 0.4 ‰. Therefore, the average change in the $\delta^{15}N$ value of the photopigments and the post-oxidized product was -5.5 ± 0.4 ‰ (Table 8). As with the photopigment standards, the overall extraction process showed an isotopic depletion of the sample following oxidation.

A second diatom extraction was prepared and treated in the same manner. Results from the analyses are presented in Table 9.

Sample	Bulk (µmoles/L)	Chl a (µmoles/mL)	Post Ox (µmoles/mL)	% Efficiency	Bulk $\delta^{15}N$	Chl a $\delta^{15}N$	Post Ox $\delta^{15}N$	$\delta^{15}N$ Offset
2nd Diatom	0.42 ± 0.04	1.0 ± 0.2	0.886	88%	-0.7 ± 0.1	-2.7 ± 0.3	-6.2	-3.5
2nd Diatom	0.42 ± 0.04	1.0 ± 0.2	0.789	78%	-0.7 ± 0.1	-2.7 ± 0.3	-6.1	-3.4
2nd Diatom	0.42 ± 0.04	1.0 ± 0.2	0.806	80%	-0.7 ± 0.1	-2.7 ± 0.3	-6.5	-3.8
2nd Diatom	0.42 ± 0.04	N.D.	N.D.	N.D.	-0.7 ± 0.1	N.D.	N.D.	N.D.
2nd Diatom	0.42 ± 0.04	N.D.	N.D.	N.D.	-0.7 ± 0.1	N.D.	N.D.	N.D.
Average	0.42	1.0	0.827	82%	-0.7	-2.7	-6.2	-3.5
Std. Dev.	0.04	0.2	0.052	5%	0.1	0.3	0.2	0.2

Table 9: µmoles/mL of Nitrogen and $\delta^{15}N$ values for Bulk Filters, Extracted Photopigments, and Post-Oxidized Samples from the 2nd Culture of Diatoms by IRMS-EA Analysis

IRMS-EA analysis from the samples (n=5) showed that the $\delta^{15}N$ of the bulk samples was -0.7 ± 0.1 ‰. After extracting the photopigments from the diatom filters, 2.5 mL of diatom extract were blotted onto GF/D glass fiber filters and analyzed on the IRMS-EA. The $\delta^{15}N$ value of the extracted photopigments was determined to be -2.7 ± 0.3 ‰. The $\delta^{15}N$ value of the post-oxidized product was -6.2 ± 0.2 ‰. Therefore, the isotopic offset in the $\delta^{15}N$ value of the bulk diatom N and the post-oxidized product was -3.5 ± 0.2 ‰ (Table 9). As with the spinach photopigment standards, the overall extraction process showed an isotopic depletion of the sample following oxidation.

Applying the Approach to Natural Samples

To test the efficacy of the method with natural samples, 5 dilutions of cultured diatoms and estuarine waters containing high amounts of detritial organic nitrogen were prepared. Estuary water was collected from Hewlett's Tidal Creek, NC. The sample matrices are as follows: 1) Creek Water (CW, 8L) from the Hewlett's Creek Watershed, 2) Thessalonia Weisfloggi Diatom Culture (DC, 2L), 3) A 4:4 mixture (8L) of CW:DC, 4) A 6:2 (8L) mixture of CW:DC, and 5) A 7:1 (8L) mixture of CW:DC.

Each mixture was filtered through GF/D glass fiber filters using a Geopump. The results from the IRMS-EA analysis are presented in Table 10.

Reference #	µmoles N	Observed $\delta^{15}N$	Predicted $\delta^{15}N$
HC	0.272	4.79	4.79*
7:1	0.291	4.15	3.64
6:2	0.311	2.78	2.49
4:4	0.339	-0.92	0.19
Diatom	0.372	-4.25	-4.25*

Table 10: IRMS-EA analysis of GF/D filter bulk nitrogen extracts from mixed media solutions.

(* Indicates that predicted values = observed values)

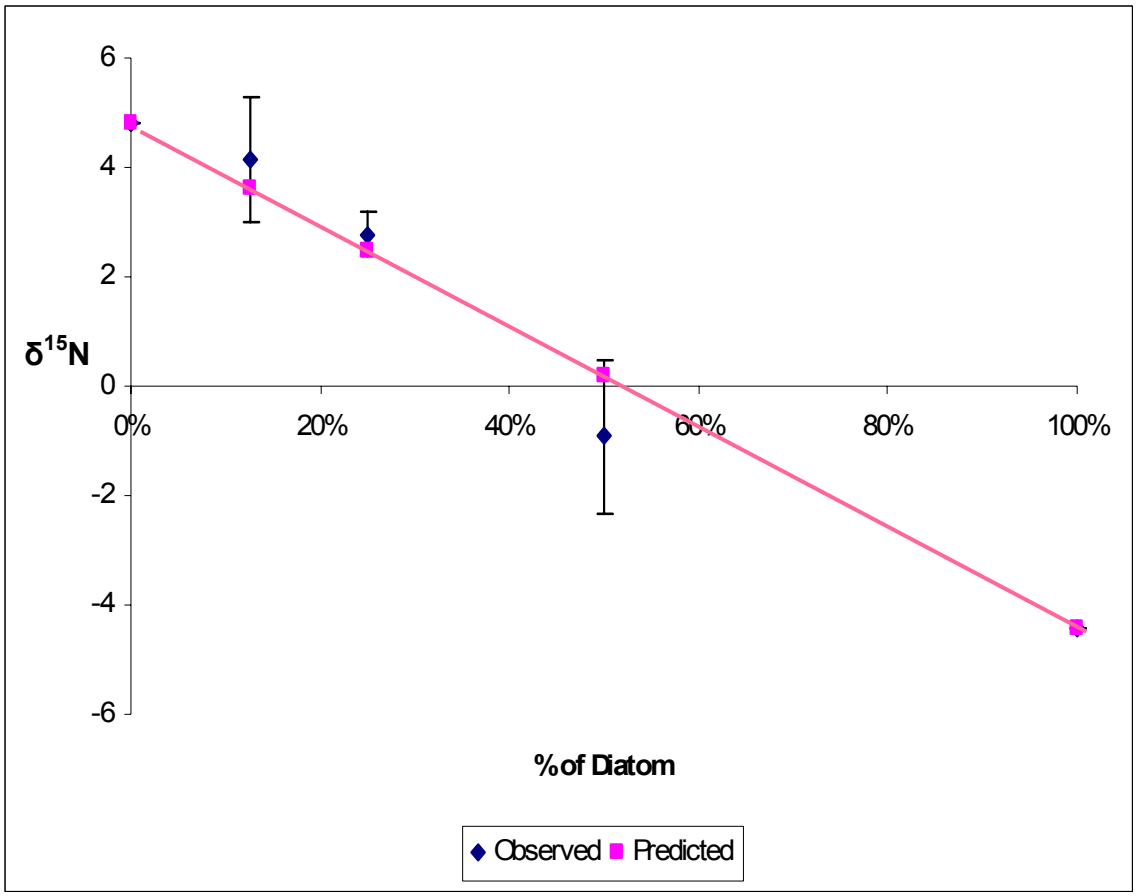

Figure 5: Mixture curve for mixed media solutions with actual and predicted $\delta^{15}N$ values

(The red line shows the expected $\delta^{15}N$ values for each of the matrices.)

IRMS-EA analysis showed that the diatom solution was isotopically lighter than the creek water solution by approximately 9 ‰. Therefore, the higher the ratio of diatoms in a mixture, the isotopically lighter the predicted $\delta^{15}N$. $\delta^{15}N$ values for the extracted photopigments and the post-oxidized product are listed in Table 11.

Sample	Bulk (μmoles/L)	Chl a (μmoles/mL)	Post Ox (μmoles/mL)	% Efficiency	Observed Bulk $\delta^{15}N$	Chl a $\delta^{15}N$	Post Ox $\delta^{15}N$
HC	0.034	0.046	0.011	24%	5 ± 1	N/A	-7.2 ± 0.6
7:1	0.053	0.074	0.012	17%	4 ± 1	2 ± 2	-10 ± 5
6:2	0.058	0.073	0.017	24%	2.8 ± 0.4	1 ± 3	-15 ± 2
4:4	0.051	0.078	0.024	30%	-0.9 ± 3	-3 ± 1	-14 ± 5
Diatom	0.051	0.016	0.014	89%	-4.4 ± 0.4	-2.64 ± 0.09	-14.6 ± 0.1

Table 11: μmoles/mL N and $\delta^{15}N$ analyses of the bulk samples, extracted photopigments, and the post-oxidized samples

After extracting the photopigments from the diatom filters, 2.5 mL of diatom extract were blotted onto GF/D glass fiber filters and analyzed by IRMS-EA. Once these samples were oxidized, the samples were shipped to the USGS for [NO_x] and $\delta^{15}N$ analysis. Upon oxidizing the photopigment, the resulting NO_x samples (n=3 for all other than the HC) were analyzed using the denitrifier method. The results from the denitrifier analysis are shown on Figure 6.

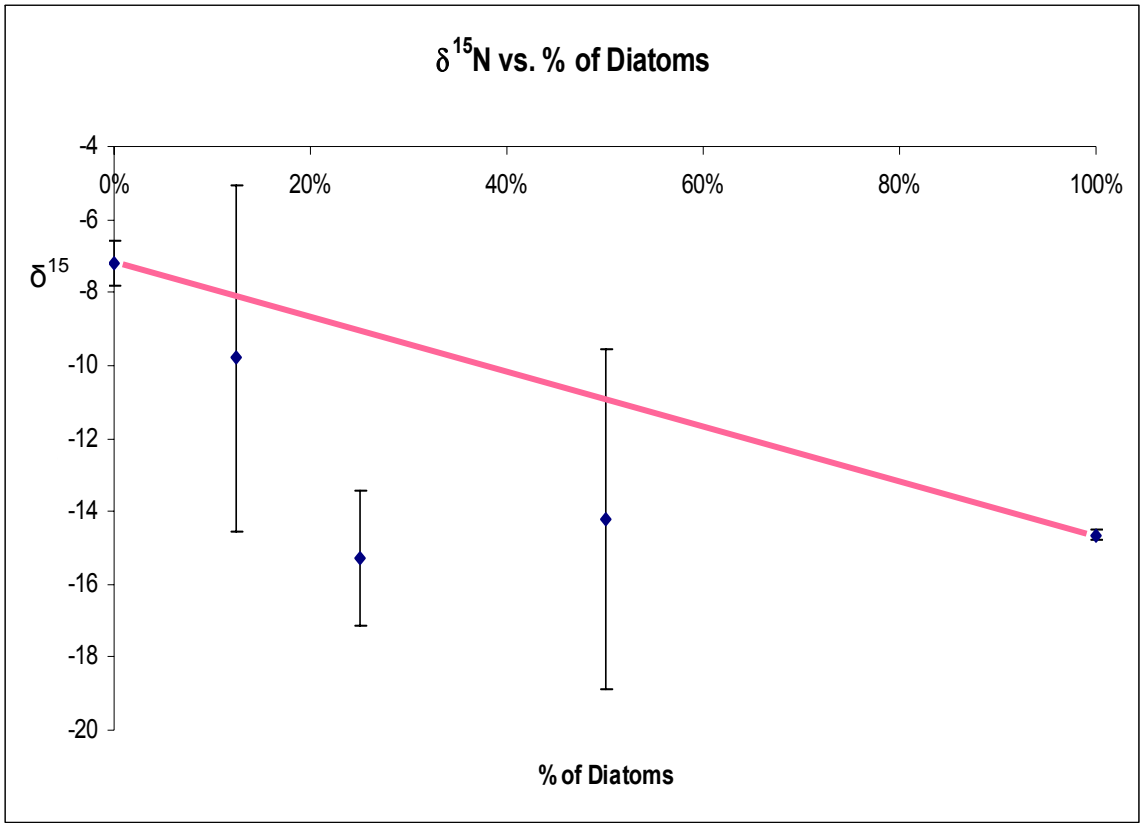

Figure 6: $\delta^{15}N$ values, via the denitrifier method, of post-oxidized photopigments from mixed natural samples. (The red line displays the expected values for the post-oxidized products of the creek water solution and the mixed natural samples.)

While the samples from the diatom matrix were consistent and highly reproducible, the extracts that were derived from natural samples were highly variable and the $\delta^{15}N$ values were not bracketed by the homogeneous samples. The highly variable results are due to the poor oxidation efficiency of the samples.

DISCUSSION

Pigment Extraction/Separation

Our analysis demonstrated that liquid-liquid separation coupled with flash column chromatography was relatively effective at achieving pigment separation. In this study, the purity of the pigments was verified via HPLC, therefore, pigment collection via HPLC was deemed unnecessary. This was determined by the presence of only 2 peaks, chlorophyll a and phaeophytin a, in the chromatogram as seen in Figure 7.

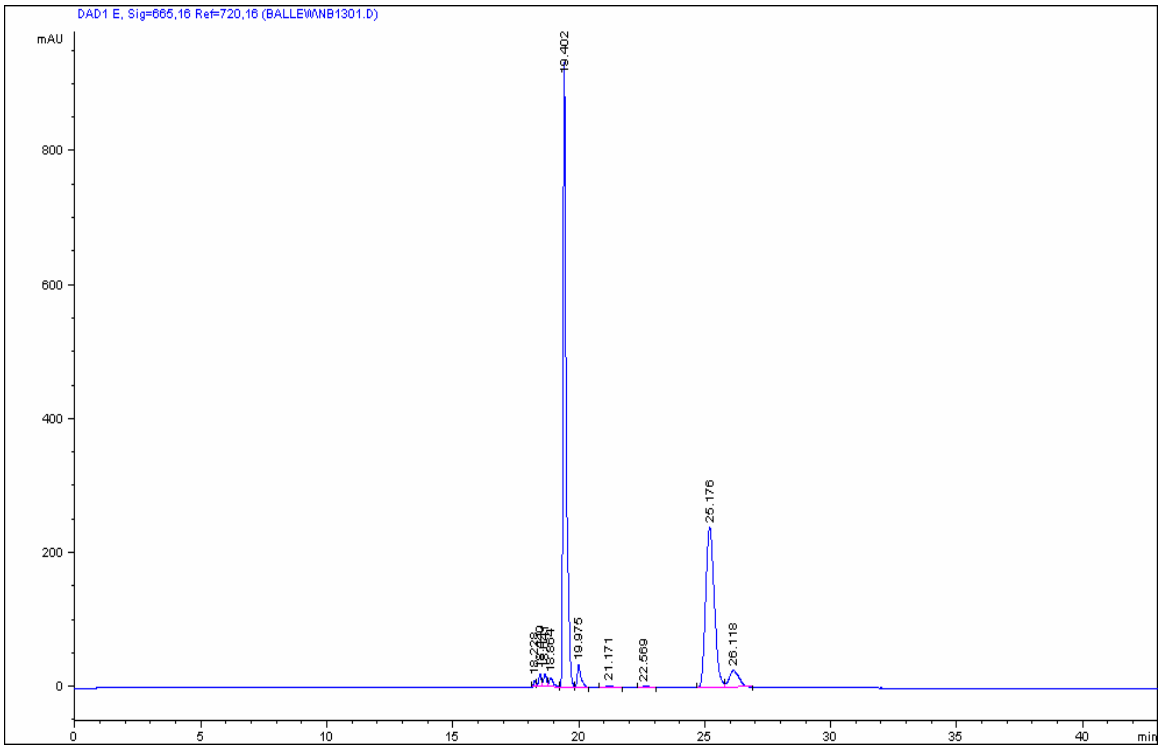

Figure 7: HPLC of Diatom Extract at 663 nm

The PDA spectra of the peak at 19.51 minutes (Figure 8a) and the peak at 25.24 minutes (Figure 8b) are those of chlorophyll a and phaeophytin a respectively. The difference between their spectra is the presence of 3 small bumps in the 500 nm range which are distinctive for phaeophytin a.

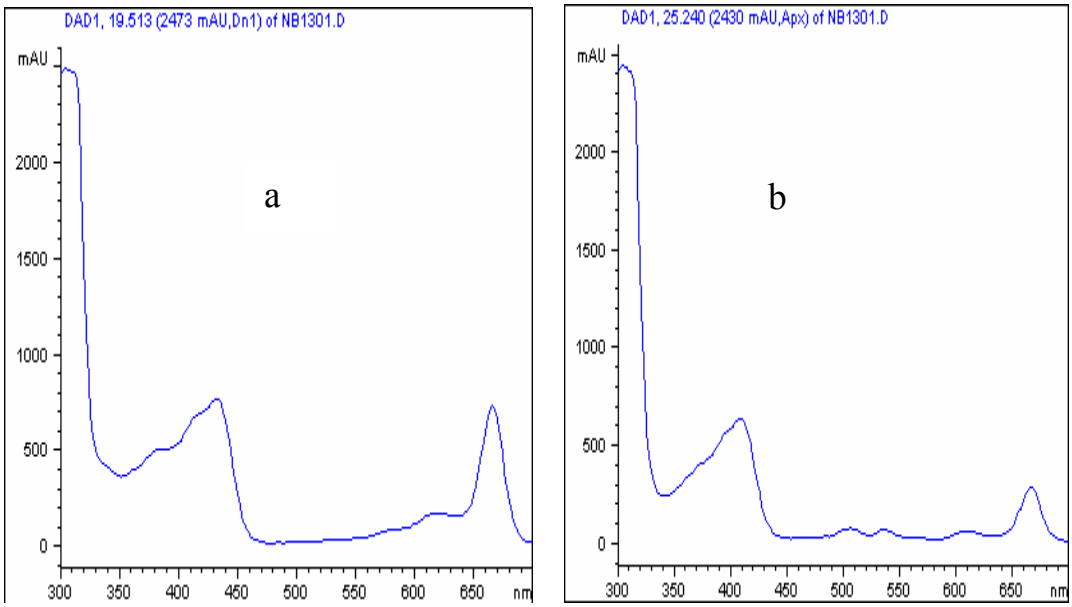

Figure 8: PDA Spectra of peaks at a) 19.51 and b) 25.24 minutes

This extraction technique differs from that of Sachs (Sachs et al., 2000); who utilized an approach which coupled phase separation via sonication with both normal-phase and reversed-phase HPLC. This method first required the ultrasonic extraction of SPOM with solvents, followed by two liquid-liquid separations. Then a normal-phase analytical SiO_2 column and a reversed-phase HPLC separation preparative C_{18} column were used to complete the separation. This process was used to determine extract composition, extract purity, and from which the samples were collected for $\delta^{15}N$ analysis. However, this process is very time consuming because the HPLC/EA approach required a large amount of sample (2 umoles of N), which required multiple concurrent runs on the HPLC since the photopigment samples were collected from the HPLC. Our combined column chromatography/HPLC approach allowed for optimizing sample throughput and collection of pigment mass while maintaining relative confidence in pigment purity.

However, a problem with our extraction was noticed during IRMS-EA analysis of our extracts. Regardless of pigment source, it was observed that the carbon:nitrogen (C:N) mass

ratio was larger than the known C:N ratio for photopigment of 11.78. Our analyses provided a C:N ratio of 15-20 for terrestrial (spinach) samples and the ratio was much larger with marine (diatom and creek water) samples. These observations are in agreement with Bidigare (Bidigare et al., 1991) whose research stated that algal culture extracts are lipid-rich. Therefore, the increased C:N ratio leads one to believe that carbon sources, most likely lipids, were co-extracted with our photopigments.

Lipids, such as triglycerides, diglycerides, and free fatty acids, are carbon dense, nitrogen poor, non-polar and are undetectable using a photo diode array detector since they absorb UV light at similar wavelengths to that of the mobile phase. Stained TLC plates from our extracts showed faint spots with R_f values and staining characteristics which are consistent with diglycerides. Since these co-extracted compounds are N-poor, they should not impact the $\delta^{15}N$ value for our photopigment extracts. However, the co-extraction of carbon containing compounds can diminish oxidation efficiency. Further optimization of the photopigment extraction may be necessary to apply this method in lipid-rich systems. Since column chromatography collection is much quicker than HPLC collection, the use of another column chromatography solvent could improve the separation of lipids from the photopigments.

Oxidation/Analysis

Two oxidation methods, persulfate oxidation and UV/H_2O_2, were compared to determine which method would be more effective at converting photopigment to NO_x. Both persulfate and UV/H_2O_2 were utilized by Bronk (Bronk et al., 2000) in her study which determined the optimal oxidation method to use for total dissolved nitrogen analysis. In her study, urea, ammonium,

glycine, and other nitrogen standards were oxidized. However, the compounds used in Bronk's study were polar and water soluble.

In our studies, the persulfate method produced poor percent conversions and green globular particulates were observed post-oxidation. It is suspected that photopigment was unable to react with aqueous persulfate due to poor solubility of the non polar photopigment in the very polar water. Therefore, the photopigment extract was never able to break into the hydration shell formed by the intermolecular forces between the aqueous persulfate and the water molecules.

While Bronk's study showed inconsistent oxidations with the UV/H_2O_2 approach, the UV/H_2O_2 approach produced high oxidation efficiencies, as calculated by combining the mass of nitrate and nitrite and dividing that value by the mass of organic nitrogen pre-oxidation. This oxidation method involves the production of $OH^{\bullet}$ radicals which are strong oxidants and oxidize organic matter with a high efficiency.

One difference between our product analysis and that of Bronk was that her N analysis utilized spongy cadmium (to reduce NO_3^- to NO_2^-) and colorometric analysis to determine the production of total NO_x while we used ion chromatography to determine $[NO_2^-]$ and $[NO_3^-]$. Spongy cadmium approach only measures the amount of NO_2^- and NO_3^- post-reduction. However, our post-oxidation product analysis allowed for the determination of all products formed in the reaction, NO_2^-, NO_3^- and unreacted peroxide.

It was assumed that the oxidation of photopigment would only produce nitrate, which is the thermodynamically stable end product. However, our analysis showed that both nitrate and nitrite were produced in the reaction. Our studies showed that the NO_x produced in the reaction was stable for up to a month post-oxidation within our matrix.

While the exact pathway for the oxidation of organic nitrogen is unknown, it is widely assumed that gaseous intermediates are formed in the reaction. Our research showed that, when the reaction vials had holes in the caps, the oxidation efficiencies were incredibly poor. It is assumed that the most likely gaseous intermediate is NO_2. Once the gaseous intermediates are formed, there can be forward or reverse reactions which occur that can cause substantial isotopic and substrate fractionation. This fractionation can produce variable products and variable oxidation efficiencies.

While nitrate and nitrite can be used indiscriminately by the denitrifier method, nitrite is a lesser oxidized sample of nitrogen. It was observed that the ratio of nitrate:nitrite varied with the amount of peroxide used as well as the type of starting material. Increasing the amount of peroxide increased the nitrate:nitrite ratio.

With respect to optimization of the method, the experiments demonstrated that there was a direct correlation between the amount of peroxide and the amount of NO_x in the blanks. (Figure 2) Therefore, the less peroxide used to perform the reaction, the lower the background signal. Secondly, it was determined that the optimum time for UV irradiation was 4 hours. (Figure 1) Increased exposure made no difference to the oxidation efficiency of the reaction. Thirdly, these tests determined that the optimum amount of peroxide to perform the reaction was 20 μL. (Figure 2) This amount was chosen because the reaction was unsuccessful without using peroxide and as the amount of added peroxide was increased above 20 μL, the amount of nitrate background increased without increasing the oxidation efficiency of the reaction. It was necessary that the samples were allowed to settle for 12 hours, post-oxidation, to allow maximal conversion of initial products to nitrate + nitrite.

Finally, it was observed that the source of H_2O_2 affected the blank as well. 30% H_2O_2 from two suppliers, Fisher and VWR, were used to determine the amount of NO_x produced. Hydrogen peroxide from VWR provided a substantially lower blank, approximately 0.05 uM vs. 2.5 uM NO_x when using 20 μL of peroxide, without impacting oxidation efficiency. (Figure 3) While neither type of peroxide contained nitrogen stabilizers, such as the commonly used acetamide, the peroxide purchased through VWR provided a much lower background with respect to N blank.

Isotopic Analyses

In order to analyze the $\delta^{15}N$ isotopic offsets between the bulk source materials (spinach and diatoms), extracted photopigments, and post-oxidized NO_x products, subsamples from each interval were analyzed on the IRMS-EA. In a previous approach, (Sachs et al., 1999), the isotopic offset between the bulk source material and the purified photopigments was analyzed, whereas the isotopic offsets seen at each part of the extraction/oxidation were analyzed in our study. Therefore, our method can be used to determine the isotoptic offset seen from the extraction of the photopigments from the bulk source material as well as the isotopic offset seen during the oxidation. Furthermore, this method utilized the denitrifier method which converted NO_x products into N_2O in an attempt to see if the post-oxidized products would have a similar $\delta^{15}N$ value to the extracted photopigments.

The first source material analyzed in this method was organic spinach. This source material was used in previous photopigment extraction studies, (Gokman, et.al 2002, Schwartz, et.al 1981). The concentration of photopigment (ug of chl a/g of spinach) is higher in spinach than other green leafy vegetables, so it served as a preferred source of photopigments. For this

project, the $\delta^{15}N$ of the bulk spinach source was not analyzed. However, the $\delta^{15}N$ of the extracted photopigment from two spinach samples was determined to be 5.2 ± 0.5 ‰ for the 1st spinach extract and 6.7 ± 0.5 ‰ for the 2nd spinach extract.

The other homogeneous source material analyzed in this study was marine (*T. Weisfloggi*) diatom cultures. Unlike the spinach samples, bulk isotopic values were established for this compound. In a previous study, (Sachs et al., 1999), isotopic depletion of 5.06 ± 1.13 ‰ between the extracted photopigments and the bulk sample of 8 types of cultured marine phytoplankton was reported. In another study (Kennicutt, et al., 1992) 7 different types of plants and algae were examined. Observed $\Delta\delta^{15}N$ between the extracted photopigments and the bulk material in the range of -0.85 to 6.35 ‰.

For this project, two separate diatom cultures were grown. The bulk $\delta^{15}N$ for the 1st diatom culture was determined to be -0.4 ± 0.2 ‰. The bulk $\delta^{15}N$ for the 2nd diatom culture was determined to be -1.9 ± 1.0 ‰. In both the 1st and the 2nd diatom cultures an isotopic depletion between the bulk N and the extracted photopigments was observed. The 1st diatom culture showed an isotopic depletion of 1.0 ‰ between the extracted photopigments and the bulk N while the 2nd diatom culture showed an isotopic depletion of 2.0 ‰.

It is known that most of the nitrogen in marine phytoplankton is contained in proteins with smaller amounts of photopigments, amino acids, and amino sugars. Some diatoms have been found to contain significant quantities of chitin, a polymer of the amino sugar *N*-acetyl-D-glucosamine, such that amino sugar nitrogen may amount to 15–20% of total cellular nitrogen in some instances (Smucker and Dawson, 1986). In our extraction, differences in polarity between the non-polar photopigments and the polar amino acids and amino sugars are used to separate the

photopigments and the other sources of N. Therefore, the photopigment extract should not contain alternate sources of organic N.

Photopigment Oxidiation with the Denitrifier Method

The denitrifier analysis of the post-oxidized products was performed at the USGS's Stable Isotope Lab in Reston, Virginia. This technique quantitatively converts both nitrate and nitrite to N_2O. $\delta^{15}N$ of the samples is calculated from the distribution of mass/charge ratios (m/z) of 44, 45, and 46 after accounting for $^{18}O/^{16}O$ composition in the N_2O.

Once the extracted photopigments from spinach were oxidized via UV/H_2O_2, $\delta^{15}N$ of the samples were determined via the denitrifier method. The 1st batch of photopigment was oxidized at 93 ± 9% efficiency and produced an isotopic depletion of 5.5 ± 0.4 ‰ while the 2nd batch of photopigment from spinach was oxidized at 92 ± 10% with an isotopic depletion of 4.2 ± 0.5 ‰. Upon comparing the isotopic depletion with the oxidation efficiency, the P-test value was 0.25, which indicates that there is not a significant correlation between isotopic depletion and oxidation efficiency. This results from this oxidation shows that the UV/H_2O_2 oxidation produces an isotopic depletion of the sample, which is in agreement for reactions that do not have 100% oxidation efficiency.

The photopigments extracted from diatoms provided similar results. The oxidiation of photopigments from the 1st culture of diatoms produced an oxidation efficiency of 94 ± 9% and produced an isotopic depletion of 5.5 ± 0.4 ‰ while the 2nd culture of diatoms were oxidized at 82 ± 5% efficiency and produced an isotopic depletion of -3.5 ± 0.2‰. Upon comparing the isotopic depletion with the oxidation efficiency, the P-test value was 0.91, which indicates that

there is a significant correlation between isotopic depletion and oxidation efficiency for this matrix.

Within isotopic reaction studies, a common relationship that is observed is Rayleigh fractionation. Rayleigh fractionation is an exponential relation that describes the partitioning of isotopes between two reservoirs as if one reservoir decreases in size. Typical Rayleigh fractionation shows that the product is isotopically lighter than the reactant, until all of the reactant is converted to product. At that point, the $\delta^{15}N$ of the product is equal to the $\delta^{15}N$. Within the scope of this project, the reactant is organic N in the form of photopigment and the product would be post-oxidized NO_x. For any reaction that obeys Rayleigh fractionation, the lower the oxidation efficiency, the more isotopically depleted the post-oxidized $\delta^{15}N$. The relationship between oxidation efficiency and the $\delta^{15}N$ should be expected if Rayleigh fractionation occurs during the reaction. Furthermore, Rayleigh fractionation can be used to describe an isotope fractionation process if: (1) material is continuously removed from a mixed system containing molecules of two or more isotopic species, (2) the fractionation accompanying the removal process at any instance is described by the fractionation factor, α, and (3) α does not change during the process.

The oxidation of photopigment consistently produced lighter products, which is in agreement with the theory of isotopic fractionation. As the method was applied, the product became isotopically depleted since the lighter isotope is preferentially reacted relative to the heavier isotope. However, it was observed that, as the oxidation efficiency of the reaction improved, the products were not always isotopically lighter. This is in disagreement with the theory of Rayleigh fractionation.

An example of this disagreement is seen with the second spinach sample. A graph of the five post-oxidized samples, shown in Figure 8, shows the relationship between oxidation efficiency and the isotopic offset between the pre-oxidized starting material and the post oxidized product. The graph shows that the reactants which had a lower oxidiation efficiency have a lower isotopic offset while the reactions with the highest oxidiation efficiency have the largest isotopic offset. If a reaction agrees with Rayleigh fractionation, reactions with higher oxidation efficiencies should produce products with a $\delta^{15}N$ values which are closer to that of the starting material. This is not true for our samples.

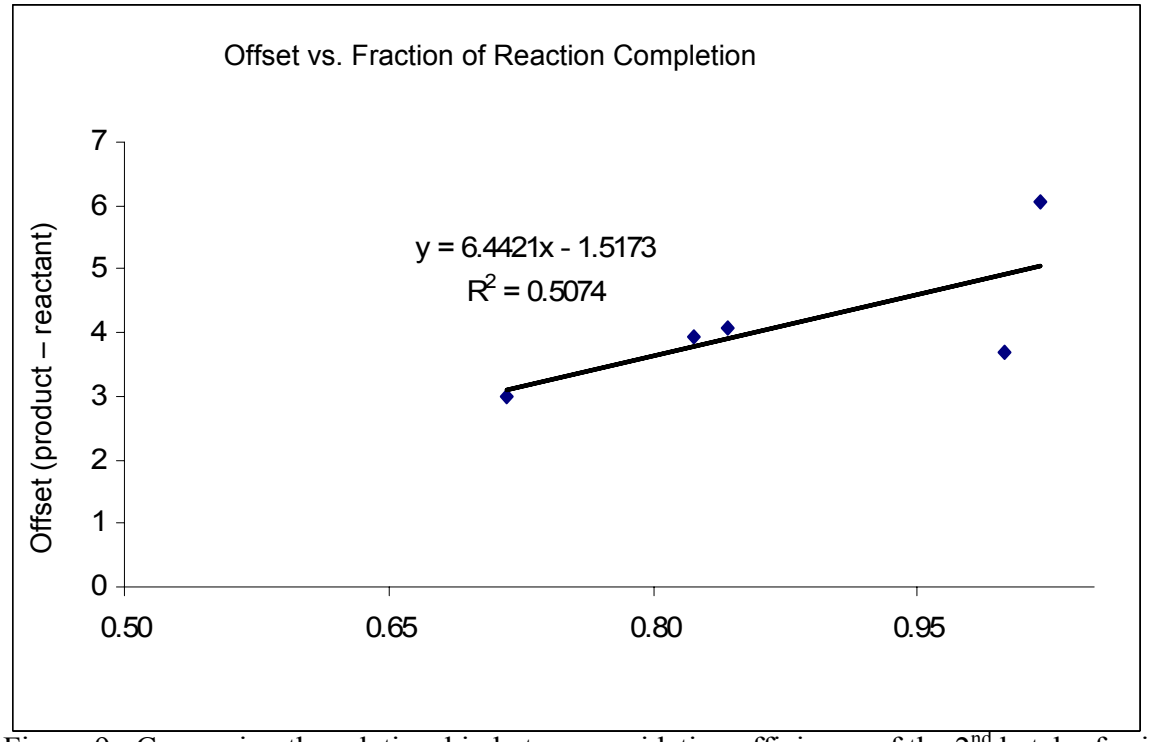

Figure 9: Comparing the relationship between oxidation efficiency of the 2nd batch of spinach samples and the isotopic offset between the pre-oxidized substrate and the post-oxidized product

As stated earlier, when photopigment is converted to NO_x via UV/H_2O_2 oxidation, the reaction proceeds through a complex pathway of reversible equilibria reactions and irreversible unidirectional kinetic reactions. Each type of reaction exhibits isotopic fractionation and thus has numerous points at which to violate the single step unidirectional reaction assumption underlying Rayleigh fractionation. Rayleigh fractionation is also thrown off if the organic N extract, due to co-extraction, contains different $\delta^{15}N$ components that oxidize preferentially.

Our hypothesis is that throughout the course of the conversion of photopigment to nitrate, numerous gaseous intermediates are produced. As stated earlier, the conversion of organic N into NO_x undergoes a series of reactions which experience fractionation. Graham's law of diffusion demonstrates that lighter isotopes are preferentially favored relative to the heavier

isotopes. Furthermore, when the NO_x gases are reconverted to nitrate and nitrite, the lighter isotope, ^{14}N, will be preferentially favored. Typically, the products which are not successfully converted to nitrate/nitrate will be that of the heavier isotope, ^{15}N. Therefore, reactions which have lower oxidation efficiencies will be isotopically heavier since the heavier isotopes will not be lost as NO_x gas.

Natural Samples

The testing of mixed matrices, samples which contained both terrestrial and marine samples, provided valuable information about the practicality of our method. The bulk $\delta^{15}N$ values that were obtained for the 3 solutions containing both SPOM and diatom samples were in agreement with the predicted $\delta^{15}N$ values for the mixtures (Figure 5). However, this was not the case for post-oxidized samples. While the $\delta^{15}N$ offset for samples which contained only marine or terrestrial extracts produced a consistent $\delta^{15}N$ offset post-oxidation, with an $\delta^{15}N$ isotopic depletion of -10‰, the mixed samples produced highly variable $\delta^{15}N$ that were not within the brackets of the terrestrial and marine samples (Table 11).

IRMS-EA analysis showed that our samples contained a very high carbon:nitrogen ratio. The excessive amount of carbon in the sample likely swamped the oxidation capacity of the matrix, which contributed to poor oxidation efficiencies. TLC analysis demonstrated that the natural samples contained a large amount of non-polar lipids. As stated earlier, these lipids do not contain nitrogen; however, they contain a large amount of carbon. Since the peroxide is not substrate specific, both the lipids and the photopigments will be oxidized. Therefore, if the amount of lipids in the sample can be decreased, the oxidation efficiency will increase accordingly.

CONCLUSIONS

A new method for the isotopic analysis of photopigments has been developed. Photopigment purification was accomplished using a column chromatography/HPLC approach. While this method displayed problems with the co-extraction of non-polar lipids, these compounds dodid not contain nitrogen nor did they directly impact the isotopic analysis of nitrogen.

The photopigments were converted to nitrate and nitrite via UV and Oxidation with 30% H_2O_2. Optimum conditions for the reaction of photopigments were 4 hours of UV irradiation in a UV digestion chamber with 20 uL of 30% H_2O_2 and a 12 hour equilibrium time post-reaction. Under these conditions, consistent oxidation efficiencies (determined by adding the amount of nitrate and nitrite in the sample divided by the amount of nitrogen in the pre-oxidized sample) were seen for the photopigments at a concentration of 5 uM. Depending on the type of 30% H_2O_2 used in the reaction, the blanks were less than 10% of the total signal. While the conversion of the photopigments via UV oxidation produced both nitrate and nitrate, both products were suitable for isotopic analysis.

An Isotope Ratio Mass Spectrometer with an Elemental Analysis Interface (IRMS-EA) was used to determine the $\delta^{15}N$ value for the pre-oxidized samples. Post-oxidized samples were sent to the US Geological Survey (IRMS) to perform isotopic analysis of post-oxidized samples. Analyses of bulk samples, extracted photopigments, and post-oxidation NO_x products were performed to determine whether there was a consistent $\Delta\delta^{15}N$ between the pre-oxidized substrate and the post-oxidized product.

Our experiments showed that homogeneous samples (photopigment extracted from spinach and diatoms) produced consistent and reproducible $\delta^{15}N$ isotopic offsets whereas natural samples

produced highly variable isotopic offsets. This was seen due to the large carbon:nitrogen ratio of the sample, which diminished the oxidation efficiency of the sample. Further studies will be performed to optimize the extraction of photopigments from natural samples to reduce the co-extraction of lipids and other carbon dense compounds.

APPENDICES

Appendix A. Artificial sea water (ASW) was prepared using the following recipe

Compound	g/L
NaCl	24.5
KBr	0.1
KCl	0.7
H_3BO_3	0.03
Na_2SO_4	4.09
$NaHCO_3$	0.2
$CaCl_2 \cdot 6H_2O$	1.545
$MgCl_2 \cdot 6H_2O$	11.1

Compounds used in ASW preparation

Solution	mL/L
.3g/50 mL NaF	0.5
1.7 g/50mL $SrCl_2$	0.5

Aqueous Solutions used to prepare Artificial Sea Water

Compound	mL/L
Vitamins	0.5
Trace Metals	1
5 g/L $Na_2H_3PO_4$	1
30 g/L $NaSiO_4$	1
30 uM N stock	0.4

Solutions used to prepare ASW post-autoclave

Appendix C: Calibration Curves for Ion Chromatography

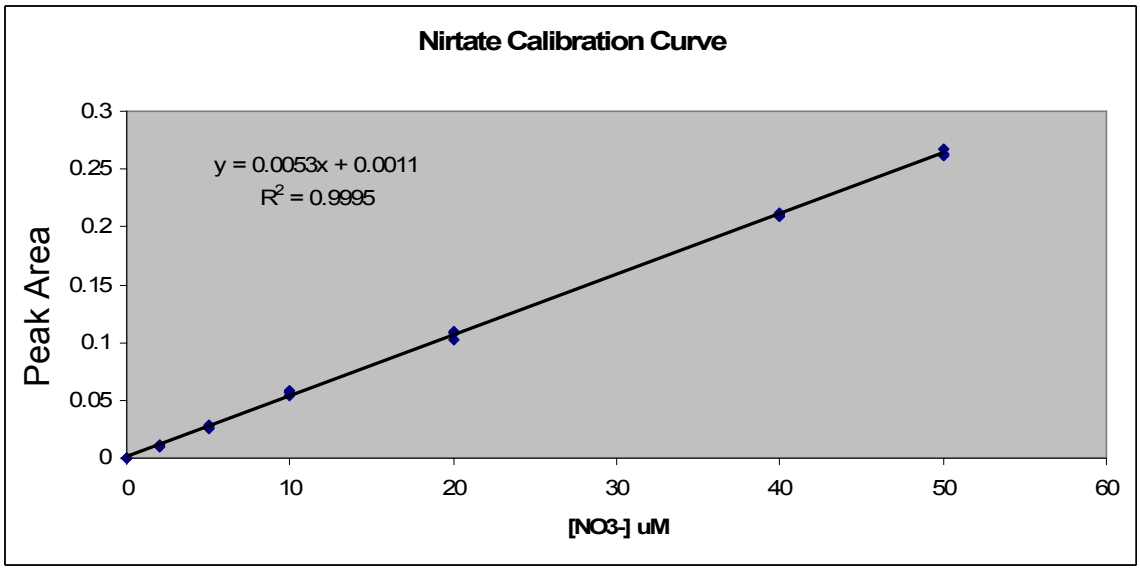

Figure 10: Nitrate Calibration Curve for Ion Chromatography

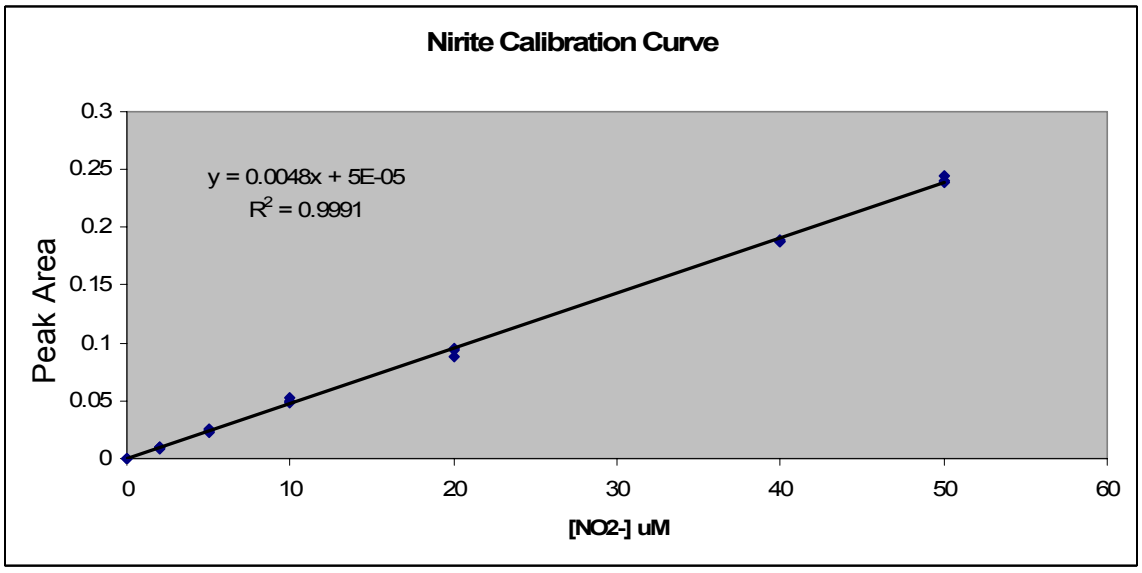

Figure 11: Nitrite Calibration Curve for Ion Chromatography

Appendix D: IRMS-EA Calibration Curve

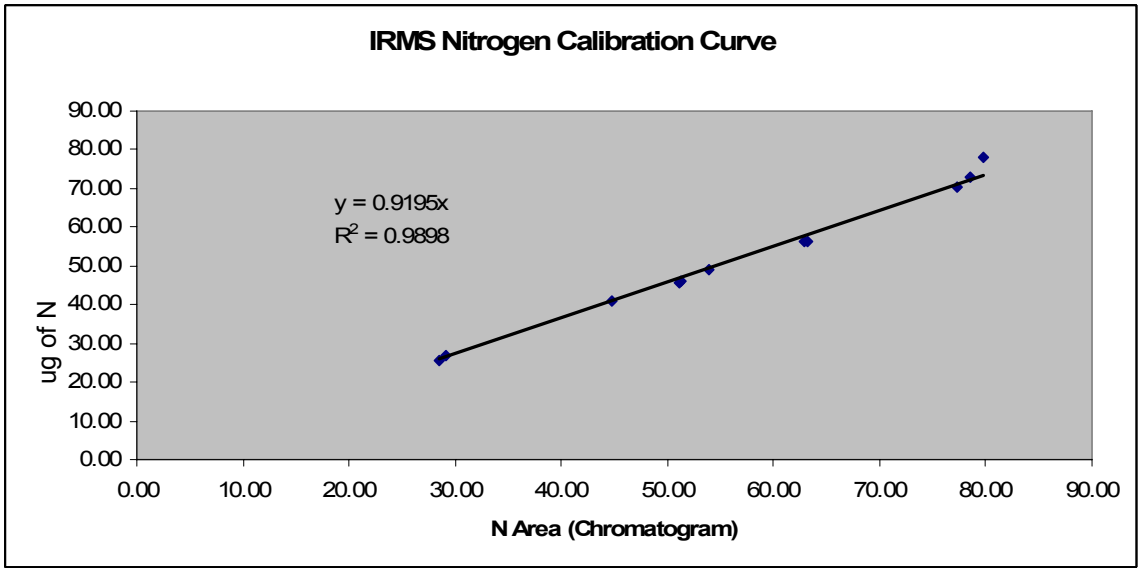

Figure 12: Nitrogen Calibration curve for IRMS-EA Analysis of USGS 40 and USGS 41 Standards

Appendix E: Basic Principles to Stable Isotopes

When performing isotopic studies, there are important concepts that should be understood prior to an analysis. The two common isotopes of nitrogen are nitrogen-14 (^{14}N) and nitrogen-15 (^{15}N). At natural abundance, the ratio of ^{14}N to ^{15}N is 99.634:0.336. Since the nitrogen-15 isotope is much rarer, it provides a smaller background and all studies involve the ratio of ^{15}N to ^{14}N in a sample. Stable isotope ratios are expressed the δ (del) notation. To calculate the del ^{15}N value of a substance, the following equation is used:

$\delta\ ^{15}N = (R_s\text{-}R_r / R_r) \times 1000$; $R_s = {}^{15}N/{}^{14}N$ of the sample

$R_r = {}^{15}N/{}^{14}N$ of the reference

The $\delta^{15}N$ values are reported as parts per thousand, although they are commonly referred to as per mille. Values are listed with ‰ as the unit. A positive δ value means that the isotopic ratio of the sample is higher than that of the standard; a negative δ value indicates that the isotopic ratio of the sample is lower than that of the standard. The designated standard for nitrogen isotopic analysis is air, which has a value of 0.

Another important principle involving isotopic reactions is the principle of fractionation. The theory behind isotopic fractionation is that isotopes of an element have slightly different chemical and physical properties because of their mass differences. Usually, chemical reactions preferentially use the lighter isotope (^{14}N) over the heavy isotope (^{15}N). Therefore, any compound produced in a chemical reaction is isotopically lighter than the starting material. The equation to determine the fractionation factor (α) is as follows:

$$\alpha = R_{reactant}/R_{product}$$

R = ratio of $^{15}N/^{14}N$ isotopes

The fractionation factor (α) is dependent many variables, although temperature is the most important variable. As the temperature of a reaction increases, the fractionation factor will decrease. Therefore, at higher temperatures, there should not be any preference for the ^{14}N versus the ^{15}N isotope.

Two types of chemical reactions which exhibit fractionation are equilibrium reactions and unidirectional reactions. Equilibrium fractionation reactions exhibit equilibrium fractionation and unidirectional reactions exhibit kinetic fractionation. The products of a kinetic fractionation are always lighter than the reactants since lighter isotopes react faster than heavier isotopes and the substrate can only be converted to product. However, this is not the case for equilibrium fractionation reactions. Equilibrium fractionation reactions can produce products that are heavier or lighter than the starting material.

REFERENCES

Altabet, M.A., Murray, D.W., and Prell, W.L. (1999). Climatically linked oscillations in the Arabian Sea denitrification over the past 1 m.y.: Implications for the marine N cycle. Paleoceanogr. 14: 732-743.

Bidigare, R.R., Kennicutt II, M.C., Keeney-Kennicutt, W.L., and Macko, S.A., (1991). Isolation and purification of chloropylls a and b for the determination of stable carbon and nitrogen isotope compositions. Analytical Chemistry 63, 130-133.

Boschker, H.T.S., S.C. Nold, P. Wellsbury, D. Bos, W. de Graaf, R. Pel, R.J. Parkes, and T.E. Cappenberg. (1998). Direct linking of microbial populations to specific biogeochemical processes by ^{13}C-labelling of biomarkers. Nature 392: 801-804

Bronk, D.A., Lomas. M.W., Glibert, P.M., Shukert, K.J., and M.P. Sanderson. (2000). Total dissolved nitrogen analysis: comparisons between persulfate, UV and high temperature oxidation methods. Mar. Chem. 69: 163-178.

Canuel, E.A. (2001). Relations between river flow, primary production and fatty acid composition of particulate organic matter in San Francisco and Chesapeake Bays: A multivariate approach. Organic Geochemistry, 32: 563-583.

Currin, C.A., S.Y. Newell, and H.S. Paerl. (1995). The role of standing dead Spartina alterniflora and benthic microalgae in salt marsh food webs: considerations based on multiple stable isotope analysis. Mar. Ecol. Progr. Ser. 121: 99-116.

Eadie, B.J. McKee, B., Lansing, M.B., Robbins, J.A., Metz, S., and Trefry, J.H. (1994). Records of nutrient-enhanced coastal ocean productivity in sediments from the Louisiana continental shelf. Estuaries. 17: 754-765.

Galloway, J.N., Aber, J.D., Erisman, J.W., Seitzinger, S.P., Howarth, R.W., Cowling, E.B. and Cosby, B.J. (2003). The nitrogen cascade. BioScience 53:341-356.

Gokmen, V., Bahçeci, S., and Acar, J. (2002). Liquid Chromatographic method for the determination of photopigments, cartenoids, and their derivatives in fresh and processed vegetables. J. Liq. Chrom. and Rel. Technol., 25(8), 1201–1213

Higginson, M.J., Maxwell, J.R., and M.A. Altabet. (2003). Nitrogen isotope and chlorine paleoproductivity records from the Northern South China Sea: Remote vs local forcing of millennial and orbital-scale variability.

Howarth, R.W., Boyer, E.W., Pabich, W.J. and J.N. Galloway. (2002). Nitrogen use in the United States from 1961-2000 and potential future trends. Ambio. 31: 88-96.

Howarth, R.W., G. Billen, D. P. Swaney, A. Townsend, N. Jaworski, K. Lajtha, J. A. Downing, R. Elmgren, N. Caraco, T. Jordan, F. Berendse, J. Freney, V. Kudeyarov, P. Murdoch, and Zhu Zhao-liang. (1996). Riverine Inputs of N to the North Atlantic Ocean: Fluxes and Human Influences. Biogeochemistry 35:75-139.

Kennicutt II M. C., Bidigare R. R., Macko S. A., and Keeney-Kennicutt W. L. (1992). The Stable Isotopic Composition of Photosynthetic Pigments and Related Biochemicals. *Chem. Geol.* 101, 235–245.

Lehman, M.F., Bernasconi, S.M., Barbieri, A., and J.A. McKenzie. (2002). Preservation of organic matter and alteration of its carbon and nitrogen isotopic composition during simulated and in situ early sedimentary diagenesis. Geochimica et. Cosmochimica Acta. 66: 3573-3584.

Montoya, J.P. (1994). Nitrogen isotopic fractionation in the modern ocean: Implications for the sedimentary record. In Carbon Cycling in the Glacial Ocean: Constraints on the Ocean's Role in Global Change (eds. R. Zahn, M.A. Kaminski, L. Labeyrie, and T.F. Pederson), pp. 259-279. Springer Verlag.

McClelland, J.W., and J.P. Montoya. (2002). Trophic relationships and the nitrogen isotopic composition of amino acids in plankton. Ecology. 83: 2173-2180.

McClelland, J.W., Valiela, I., and R.H. Michener. (1997). Nitrogen-stable isotope signatures in estuarine food webs: A record of increasing urbanization in coastal watersheds. Limnol. Oceanogr. 42: 930-937.

Nixon, S.W. (1997). Prehistoric nutrient inputs and productivity in Narragansett Bay. Estuaries. 20: 253-261.

Nixon, S.W. (1995). Coastal marine eutrophication: A definition, social causes, and future concerns. Ophelia 41: 199-219

Pinckney, J.L., Richardson, T.L., Millie, D.F., and H.W. Paerl. (2001). Application of photopigment biomarkers for quantifying microalgal community composition and in situ growth rates. Organic Geochemistry. 32: 585-595.

Pride, C., Thunell, R. Sigman, D., Keigwin, L., Altabet, M., and E. Tappa. (1999). Nitrogen isotopic variation in the Gulf of California since the last deglaciation: response to global climate change. Paleoceanogr. 14: 397-409.

Robinson, R.S., Brunette, B.G., and D.M. Sigman. (2004). Revisiting nutrient utilization in the glacial Antarctic: Evidence from a new method for diatom-bound N isotopic analysis. Paleoceanogr. 19: PA3001

Sachs, J.P., Repeta, D. J., and R. Goericke. (1999). Nitrogen and carbon isotopic ratios of photopigment from marine phytoplankton. Geochimica et. Cosmochimica Acta. 63: 1431-1441.

Sachs, J.P. and Repeta, D.J. (2000). The purification of chlorins from marine particles and sediments for nitrogen and carbon isotopic analysis. Organic Geochemistry. 31: 317-329

Schwartz, S.J., Woo, S.L., and von Elbe, J.H. (1981). High-Performance Liquid Chromatography of Photopigments and Their Derivatives in Fresh and Processed Spinach. J. Agric. Food Chem. 1981, 29, 533-535

Sigman, D.M., Altabet, M.A., Francois, R. McCorkle, D.C., and J.F. Gaillard. (1999). The isotopic composition of diatom-bound nitrogen in Southern Ocean sediments. Paleoceanogr.14: 118-134.

Sigman, D.M., Casciotti, K.L., Andreani, M., Barford, C., Galanter, M., and J.K. Bohlke. (2001). A bacterial method for the nitrogen isotopic analysis of nitrate in seawater and freshwater. Anal. Chem. 73: 4145-4153.

Smucker R. A. and Dawson R. (1986). Products of Photosynthesis by Marine Phytoplankton: Chitin in TCA "Protein" Precipitates. J. Exp. Mar. Biol. Ecol. 104, 143–152.

Tobias, C.R., M. Cieri, B.J. Peterson, L.A. Deegan, J. Vallino, and J. Hughes. (2003a). Processing watershed-derived nitrogen in a well-flushed New England estuary. Limnol. Oceanogr. 48: 1766-1778.

Tobias, C.R., A. Giblin, J. McClelland, J. Tucker, and B. Peterson. (2003b). Sediment DIN fluxes and preferential recycling of benthic microalgal nitrogen in a shallow macrotidal estuary. Mar. Ecol. Progr. Ser. 257: 25-36

Tobias, C.R., S.A. Macko, I.C. Anderson, E.A. Canuel, and J.W. Harvey. (2001). Tracking the fate of a high concentration groundwater nitrate plume through a fringing marsh: A combined groundwater tracer and in situ isotope enrichment study. Limnol. Oceanogr. 46: 1977-1989.