ANALYTICAL METHODS FOR THE STUDY OF NITROGEN

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Deborah A. Bronk[1] and Matthew D. McCarthy[2]

[1]Department of Physical Sciences
Virginia Institute of Marine Science
The College of William and Mary
Gloucester Point, VA 23062

[2]Ocean Sciences Department
University of California
Santa Cruz, CA 95064

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I. Introduction

The study of nitrogen (N) cycling requires sensitive and accurate methods to measure the various N forms. The objective of this chapter is to review existing methods to analyze dissolved and particulate fractions of N with an emphasis on those methodologies that are most commonly used or represent the state of the art, noting that N analysis does indeed seem to be an art at times. Many techniques are temperamental at best and it is not uncommon to find small alters devoted to keeping a given mass spectrometer running. This chapter was written to stand alone as a general review of methods; more detailed descriptions of specific methods can be found in other chapters in this book. We begin with a brief note on collecting and preserving samples. The first main section of the review describes techniques to measure the concentration of N pools, followed by various approaches to chemically characterize organic N fractions. The last major section reviews techniques to measure N flux rates, including uptake, release, nitrification, denitrification, and N_2 fixation. We conclude with recommendations for future research.

This chapter was written largely from the perspective of water column studies, however, most of the methods could also be used more broadly to analyze sediments, particles, or pore water samples. The difference between a given method for these sample types is principally one of the detection limit required and matrix-specific analytical challenges. Analyzing water column samples can be difficult because concentrations can be very low. While concentrations are generally much higher in sediment pore waters, extracting the necessary volume of pore water can be difficult. With respect to rate measurements most of the techniques outlined could be applied in both the water column and the sediment, and several largely sedimentary rate measurements are specifically discussed below. Tracer approaches in sediments can be especially problematic because the substrate is so heterogeneous that getting uniform mixing of the tracer can be impossible (e.g. Middleburg et al. 1996); for a more detailed discussion of methods used in sediments see Joye and Anderson (this volume).

Before we begin, a comment on units. In general, we recommend concentrations to be presented as μmol N L^{-1} (i.e. μM, or some similar metric equivalent) and rates to be presented as μmol N L^{-1} $time^{-1}$. Historically the μg-at N unit has been used in the presentation of N

uptake rates, particularly in culture work; 1 µmol N is equal to 1 µg-at N for a substrate like NH_4^+ but is equal to 2 µg-at N in the case of a dinitrogen substrate such as urea. Despite the opinion of our esteemed colleagues (Williams 2004, Luther personal communication), we find the µg-at N unit to be useful to standardize substrate additions when measuring N flux rates using both inorganic and organic N that may have variable amounts of N per substrate.

II. Collection and storage of samples

Here we provide some very basic guidelines on collection and storage of samples. We stress, however, that each analyst should run tests to determine the optimal washing and sampling protocol for each of their own application.

Nutrient samples are generally stored in plastic bottles such as HDPE or PETG. Prior to use, all plasticware should be washed with detergent (e.g. Liquinox®), rinsed with distilled water (DW) and soaked in an acid bath (most commonly 10% HCl) for a couple hours, followed by a final rinse with copious amounts of high quality DW or equivalent. Glass bottles or vials are also often used and are the preferred choice for nutrients and organics (i.e., DON, DCAA, DFAA). Glassware has the advantage that it can be wrapped in foil and baked in a muffle oven (minimum of 450^{o}C for 2 hours) to clean; glass also has the disadvantage, however, that it can break during shipping or freezing.

To filter or not to filter – that is the question! If one is measuring dissolved compounds, it is customary to filter the sample prior to analysis. In open ocean systems, however, filtration is often not done because the amount of particulate material is small and the risk of contamination large. In coastal or estuarine systems, however, filtration is a must. Filtration of nutrient samples is generally done through a glass fiber (i.e. GF/F®) filter with a nominal pore size of 0.7 µm or a 0.2 µm – 0.45 µm polycarbonate filter (e.g. Supor® or Nuclepore®). Benefits of glass fiber filters are that they are inexpensive, have a high flow rate, and can be cleaned through precombustion (minimum of 450^{o}C for 2 hours). Polycarbonate filters should be used with care as they can introduce high blanks for some analyses. For example, Supor filters can produce high dissolved organic carbon (DOC) and DON blanks; rinsing a 47

mm Supor filter with 200 ml of DW is generally sufficient to lower the blank substantially (Carlson personal communication).

Sample storage options include acidification, refrigeration, pasteurization, or freezing (-20 or -80^{o}C). The principle behind acidification is to lower the pH sufficiently to inhibit microbial activity that could alter the concentration of the analytes; HPLC grade phosphoric acid is often used. If acidification is chosen, the analyst to should take the pH change into account prior to analysis, as many methods are pH sensitive. Refrigeration should only be considered for samples filtered through a 0.2 μm filter. Pasteurization was pursued as a storage option because of the risk that freezers could fail as well as the expense of storing large numbers of samples frozen for long periods of time (Aminot and Kérouel 1998). Though not appropriate for ammonium (NH_4^+), researchers may want to investigate its use for storage of nitrate (NO_3^-) and nitrite (NO_2^-) samples. For most samples, simple frozen storage is generally the most common method and is what we recommend, with some caveats. Care must be taken to allow space for expansion during freezing, and if using glass vials duplicate samples are highly recommended to allow for potential breakage. Also, if storing samples in a freezer, that freezer should be clean and free of meat or animal tissues and high organic sediments, particularly when storing samples for the more sensitive analyses such as DFAA.

III. Measurement of nitrogen concentrations

Nitrogen exists in the ocean at oxidation states from –3 to +5. There are three forms of inorganic N – NH_4^+, NO_3^-, and NO_2^-. Nitrate is the final oxidation product and is the dominant form of fixed N in the deep ocean. Nitrite generally occurs at very low concentrations because it is an intermediate in the processes of nitrification ($NH_4^+ \rightarrow NO_2^- \rightarrow NO_3^-$) and denitrification ($NO_3^- \rightarrow NO_2^- \rightarrow NO \rightarrow N_2O \rightarrow N_2$) and hence high concentrations seldom accumulate. Concentrations of NH_4^+ are highly variable but tend to be near the limit of detection in marine surface waters. Each of the inorganic forms has a number of manual and automated methods of analysis. We discuss the most widely used below.

A. General principles of nutrient analysis

In general, nutrient analyses are done either manually or using an autoanalyzer. The trend toward monitoring over large spatial and temporal scales and the expense of analyzing discrete samples has greatly increased the interest in sensors that can be deployed within the environment – a topic discussed at the end of this chapter. Space limits preclude the presentation of detailed protocols. An excellent general review of nutrient analysis as well as detailed method descriptions and protocols can be found in (Grasshoff et al. 1999); another excellent source of information is Sparks et al. (1996).

Many of the methods discussed below are colorimetric techniques. The basic principle behind these techniques is to add a series of reagents that will build a dye molecule containing the analyte in question. The higher the concentration of the analyte, the higher the concentration of dye that can be formed. The final step in the analysis is to measure the absorbance of the sample using a spectrophotometer at a wavelength that represents the peak absorbance for the dye in question. The conversion of absorbance to a concentration is described by the Beer-Lambert's Law.

A number of basic principles should always be followed regardless of the analysis. First, the concentration in the standard curve should bracket the concentrations of the samples being analyzed. Second, standards should be run at the same time and treated in an identical fashion to the samples being measured. Third, for many analyses, the matrix of the sample will influence the absorbance measured. For this reason, standards should be prepared in a matrix that matches the matrix of the samples. In the case of seawater samples, artificial or low nutrient seawater should be used in the preparation of the standards. In the case of estuarine samples, where the salinity can change dramatically from station to station, a salt correction is generally used. Fourth, the longer the cuvette (i.e. path length) used in the final analysis, the lower the limit of detection that can be reached. The use of long-path liquid waveguide capillary cells (e.g. meters long) has allowed great improvement in sensitivity and detection for a number of analyses (e.g. Zhang 2000).

B. Ammonium analysis

Ammonium is present at very low concentrations (0.03 to 0.5 μM) in oceanic surface waters with concentrations being orders of magnitude greater in sediment pore waters (Sharp 1983). In seawater, NH_4^+ exists as the acid base pair NH_4^+-NH_3 (ammonia); the pK_a of the pair is 9.3. As a result, NH_4^+ is the dominant species in natural waters, which generally has a pH of 8.3 or less. The methods discussed here all measure the sum of ammonium (NH_4^+), the form that dominates at the pH of seawater, and ammonia (NH_3), the volatile form that dominates under more alkaline conditions. There are many approaches to measuring NH_4^+ but we will focus on the two most widely used - phenol-hypochlorite and orthophtaldialdehyde (OPA).

The most common approach to measuring NH_4^+ is a colorimetric one that uses the Bertholot reaction where a blue color is formed by phenol and hypochlorite in the presence of NH_4^+ (Searle 1984). The reaction requires a catalyst or elevated temperatures to achieve the sensitivity needed for environmental samples. Sodium nitroprusside is commonly used as the catalyst (Solorzano 1969); salicylate can also be used as the catalyst but the limit of detection is higher (~ 0.7 μmole L^{-1}) (Bower and Holm-Hansen 1980). In either case, the reaction product is an azo dye, the absorbance of which is measured on a spectrophotometer at 630 nm.

To increase the sensitivity of the analysis, it is recommended that a 5 or 10 cm path length cell be used. The detection limit using a 10 cm cell is approximately 0.05 μM with an upper limit of 40 μM without dilution. Use of a long-path liquid waveguide capillary cell (2 m) lowered the detection limit to 5 nM with a precision of 5% in the 10 – 100 nM range (Li et al. 2005). Another approach used to decrease the limit of detection is to preconcentrate the NH_4^+ prior to analysis. In one technique the indophenol is concentrated by extraction into n-hexanol; this method has a precision of 1.9 nM at concentrations ≤ 50 nM (Brzezinski 1987). The indophenol can also be concentrated onto solid phase extraction (SPE) octadecylsilane (C18) columns (Selmer and Sorensson 1986) with the final concentration determined as outlined in (Brzezinski 1987).

There are also a number of matrix issues that the analyst should bear in mind. In seawater there is a salt effect, with lower absorbances observed in seawater versus distilled

water for the same concentration of NH_4^+. Amino acids can cause an interference with the analysis but at the concentrations of amino acids found in seawater, the interference is insignificant (Solorzano 1969). There is no interference with urea. Beware of analyzing samples with a pH higher than 11.0 because blanks become inconsistent; a pH problem is indicted if the sample has a greenish, rather than blue, color. Also, samples from low salinity environments (< 5 ‰) with high humic concentrations (see below) often have a tea color, which will interfere with the colorimetric analysis. One way to circumvent this problem is to precipitate the humics with a magnesium sulfate solution prior to analysis (Grasshoff et al. 1983). When samples cannot be run right away, another useful modification is the addition of the phenolic reagent immediately after sample collection to bind the NH_4^+. After the reagent is added, the samples can be stored in the refrigerator until analysis, circumventing the need to freeze the samples (e.g. (Cochlan et al. 2002).

The phenol-hypochlorite method has also been adapted for use in microplate readers. The advantage is very small sample size (<300 µl) and extremely high sample throughput (~1000 samples per day). The limit of detection, however, is high (~1.6 µM) making it unsuitable for water column studies, although it may be applicable for sediment pore waters or highly eutrophied systems (Baudinet and Galgani 1991).

The most common alternative to the phenol-hypochlorite method uses OPA. This technique was introduced in an automated version based on the conversion of NH_4^+ to NH_3, which is then allowed to diffuse across a membrane into a flowing stream of OPA to form a fluorescent product (Jones 1991). This final product is then analyzed fluorometrically with a detection limit of ~ 1.5 nM (Jones 1991). Advantages of the method are that it requires only a single reagent, it has a lower detection limit than the standard phenol-hypochlorite method, the salt effect is relatively small (<3% over a salinity range of 0-35 ‰) and there is no interference with primary amines. The technique has also been modified to remove the need for the gas diffusion cell, also with a reported detection limit of 1.5 nM (Kerouel and Aminot 1997); a manual version has also been published (Holmes et al. 1999).

C. Nitrate and nitrite analysis

Nitrate is often near the limit of detection in surface waters (~0.05 μM) but increasing to ~30-50 μM below the thermocline (Sharp 1983). Nitrite is an intermediate in the processes of nitrification ($NH_4^+ \rightarrow NO_2^- \rightarrow NO_3^-$) and denitrification ($NO_3^- \rightarrow NO_2^- \rightarrow NO \rightarrow N_2O \rightarrow N_2$); its presence can thus be diagnostic for these processes, but it seldom accumulates to concentrations greater than 1 μM. Concentrations of NO_3^- and NO_2^- are often reported together in the literature because their measurement is related. There are a number of methods that can measure NO_2^- directly, however, we are unaware of any method for the direct measurement of NO_3^-. To measure NO_3^-, it must first be reduced to NO_2^-, or some other more reduced form, such that the NO_2^- actually measured will include both preexisting NO_2^- plus the NO_2^- produced as a result of the reduction of NO_3^-. This combined measurement is often referred to as NO_x. The concentration of NO_3^- is then calculated as:

$$[NO_x] - [NO_2^-] = [NO_3^-] \quad (1)$$

The most common method to measure NO_2^- is based on the formation of an azo dye formed when NO_2^- reacts with two aromatic amines added sequentially (sulfanilamide and N-naphthylethylene-diamine dihydrochloride, NEDA; (Strickland and Parsons 1972). The resulting red azo dye is measured on a spectrophotometer with a linear standard curve up to 10 μM; sample concentrations greater than 3 μM should be diluted before being read. The precision of the method is ± 0.01 μM (Strickland and Parsons 1972). Application of a long-path liquid waveguide capillary cell (2 m) can lower the detection limit for NO_2^- to 1 nM for with a precision of 0.2% (Zhang 2000).

The two most common methods for reduction and measurement of NO_3^- are cadmium reduction and chemiluminescence. Both involve an initial step to reduce the NO_3^- to NO_2^-. Reduction with copper coated cadmium can be done manually using a reduction column or, more commonly, an autoanalyzer (Hansen and Koroleff 1999). After the reduction is completed the sample is treated like a NO_2^- sample as described above. The limit of detection of the technique is reported as 0.05 μM (Strickland and Parsons 1972); slight modifications to

the automated procedure have been reported to increase the precision of the NO_2^- and NO_3^- analysis to ± 1.24 nM and 2.87 nM respectively (Raimbault et al. 1990). Application of a long-path liquid waveguide capillary cell (2 m) can lower the detection limit for NO_3^- to 2 nM with a precision of 0.8% (Zhang 2000).

A useful alternative to cadmium columns is reduction of individual samples with spongy cadmium (Jones 1984). Spongy cadmium is made by standing zinc sticks in 20% copper sulfate overnight, during which time the cadmium precipitates on the surface of the zinc. The spongy cadmium is then added to individual seawater samples, with added ammonium chloride, and shaken for 90 min. The spongy cadmium has the advantage of having a much higher surface area than the copper granules used in cadmium columns. Advantages of the method include the ability to run a large number of samples simultaneously and stability in reductor efficiency over time. In addition, when sample concentrations vary over a wide concentration range, the spongy cadmium method can be used without the need to rerun samples using different calibrations, as is often required with autoanalyzers. This method is especially useful for sediment pore water samples that may have a residual amount of sulfide, a compound that will reduce the efficiency or completely inactivate a cadmium reduction column. The limit of detection of the spongy cadmium technique is reported as 0.033 μM (Jones 1984) but in practice limits of detection are closer to 0.1 μM, such that we reserve its use for samples from more eutrophic environments.

A common source of error in NO_3^- measurements is caused by a decrease in reductor efficiency over time, which has been reported as ~1% per 4-6 hour of autoanalyzer run-time (Garside 1993). Reduction efficiency can decrease due to a decrease in surface area, precipitation of hydroxides on the cadmium, and loss of the copper coating. It is recommended that NO_2^- and NO_3^- standards be analyzed periodically during a run and the results used to correct for the losses in reductor efficiency (Garside 1993). An efficient method to regenerate or reactivate cadmium columns, manual or in autoanalyzers, is to pass a copper-EDTA complex through the column (Otsuki 1978). This reactivation method has the advantage that it does not produce an excess of reduced colloidal copper that can clog up the column, necessitating the column being unpacked each time it is regenerated.

The other commonly used method to measure NO_3^- and NO_2^- is chemiluminescence. In this technique NO_2^- and NO_3^- are reduced to nitric oxide (NO), which is then measured on a N oxide analyzer after its chemiluminescent reaction with ozone (Garside 1982). The reduction of NO_3^- to NO_2^- is effected by injecting the sample into a heated reservoir containing an iron (II)-molybdate solution (Garside 1982). The amount of NO produced is dependent on the volume of sample injected into the machine. Volumes up to 10 ml can be injected for low concentration samples. The range of the method was reported as 0.002 to 20 μM. The method is more sensitive than the cadmium reduction methods, with a precision of ± 0.002 μM NO_3^- and ± 0.0005 μM for NO_2^- (Garside 1982). Vanadium (III) has now largely replaced the iron (II)-molybdate solution because it is a more reactive reducing agent, retains its reducing power at much lower acidities and after repeated injections of seawater (Braman and Hendrix 1989).

D. Total dissolved nitrogen analysis

The total dissolved N (TDN) pool consists of an inorganic N (NH_4^+, NO_3^-, and NO_2^-) fraction and an organic fraction. To calculate the dissolved organic N (DON) fraction, the sum of the inorganic N concentrations are subtracted from the TDN concentration, with the residual being defined as DON.

$$DON = TDN - (NH_4^+ + NO_3^-/NO_2^-) \quad (2)$$

The standard deviation of the DON concentration is calculated by propagating the error with the following equation:

$$s_{DON} = (s^2_{TDN} + s^2_{NH4+} + s^2_{NO3-/NO2-})^{1/2} \quad (3)$$

where s^2 is the variance of the three measurements (Bevington 1969). DON measurements thus have inherently large errors, since the final estimate of DON contains the combined analytical uncertainty of three analyses: TDN, NH_4^+, and combined NO_3^-/ NO_2^-. This

propagated uncertainty becomes an increasingly large problem in subsurface and deep ocean waters, where DON concentrations are small while DIN concentrations are large.

A cautionary note regarding statistics commonly used in nutrient chemistry – know the difference between Model I and Model II regressions (Laws and Archie 1981). When one is plotting a standard curve, where the parameter on the x-axis is controlled, a Model I regression should be used. When comparing methods (e.g. as was done with the DON method intercomparison) where the same parameter is measured in a number of different ways, or with most field data, a Model II regression should be used (see Laws and Archie 1981).

There are currently three methods commonly used to measure TDN concentrations: persulfate oxidation (PO, Menzel and Vaccaro 1964, Sharp 1973, Valderrama 1981); ultraviolet oxidation (UV, Armstrong et al. 1966, Armstrong and Tibbitts 1968); and high temperature oxidation (HTC, Sharp 1973, Suzuki and Sugimura 1985). A comparison of the three methods was undertaken with 29 sets of analyses done on five field samples (Sharp et al. 2002a). The coefficient of variations for the five samples ranged from 19 to 46%, with the poorest replication observed on deep ocean samples. No significant differences were found between the different methods. Kjeldahl digestion is another method used to measure TDN that uses sulfuric acid to convert DON to NO_3^-. It has high blanks and low precision and is therefore not recommended for analysis of aquatic samples (D'Elia et al. 1977).

Analysis of TDN by the PO method involves adding a potassium persulfate oxidizing reagent to a sample and then autoclaving it for 0.5 hour at 121^{o}C and 15 lb/in^2 pressure. The oxidation converts the fixed N forms to NO_3^- and then the concentration of the NO_3^- is measured with one of the techniques described above. The PO method often suffers from high blanks that are a function of the relatively large persulfate addition required. If high blanks are obtained, recrystallization of the potassium persulfate once or twice can be done to exclude N contaminants in the crystals thus lowering the blanks (Hansen and Koroleff 1999); this process, however, is time consuming. The required sample volume generally ranges from 5 to 20 ml depending on the volume required for the subsequent TDN- NO_3^- analysis. Of all the methods, the PO technique has the lowest instrumentation cost. Samples can also be

boiled if an autoclave is not available (Bronk et al. 2000). Readers are, however, cautioned against the use of Teflon or HDPE bottles. Teflon bottles are permanently misshapen during autoclaving and Teflon becomes pliable when heated repeatedly, resulting in loose caps and loss of sample. Bottles made of HDPE can be used, but must be autoclaved first because ~10% of them explode or leak during autoclaving. The safest method is to use sealed ampoules because they remain intact.

Analysis of TDN by the UV method requires an instrument that can hold samples at a fixed distance from a UV light source without allowing the samples to overheat (Armstrong et al. 1966). One common design for such an instrument was developed by Armstrong and Tibbitts (1968). Most commonly the method uses a 500-1200 W mercury vapor lamp. The sample tubes must be made of quartz to allow the passage of the UV radiation through the sample wall; hydrogen peroxide is often used as an oxidant. Oxidation times vary from 3 hours to 24 hours depending on the UV lamp; each new lamp should be tested to determine the oxidation time required (Bronk et al. 2000). The advantage of the UV technique is the small addition of H_2O_2 used, which results in generally lower blanks than the PO method. Disadvantages of the UV methods include the cost and effort of building a UV machine, and the long oxidation time (18 to 24 hours) required; automated approaches have been proposed that could increase sample throughput (Collos and Mornet 1993, Le Poupon et al. 1997).

With the HTO method, samples are combusted at high temperatures in the presence of pure oxygen. Though chemically simple, the HTO method requires great care to achieve reproducible results from day to day. In general the HTO method is not considered to have a salt effect (Walsh 1989). Advantages of the HTO method are the small sample volume required. The HTO method also has a relatively low blank (e.g. 1.22 ± 0.61 μM, Bronk et al. 2000). The main disadvantage with this method is the expense of the equipment, and the time and effort required for set-up and troubleshooting.

E. Particulate nitrogen

In marine surface waters most of the fixed N is bound in particulate N (PN) with concentrations commonly < 1 μM in oceanic waters and < 10 μM in coastal systems (Sharp

1983). To measure PN, also known as particulate organic N (PON), a field sample is first filtered, commonly through a precombusted (450°C for a minimum of 2 hours) Whatman GF/F filter (nominal pore size 0.7 μm); silver filters (Gordon and Sutcliffe 1974) and Anapore® alumina silicate filters can also be used. The filter is then dried and analyzed with a CHN (carbon hydrogen nitrogen) analyzer that combusts the filter and quantifies the resulting gases (Ehrhardt and Koeve 1999).

IV. Direct measurement of major DON components

A. Urea

Urea ($(NH_2)_2CO$) is excreted by organisms, is a product of bacterial organic matter decomposition, and is a highly labile form of N for plankton nutrition. Published concentrations in oceanic waters are relatively scarce but are quite low (< 0.5 μM; Antia et al. 1991). There are currently two methods commonly used to measure urea concentrations - the urease method, which involves enzymatic hydrolysis of urea to CO_2 and NH_3 (McCarthy 1970), and the monoxime method, which involves the direct colorimetric measurement of urea using diacetyl monoxime (Price and Harrison 1987, Mulvenna and Savidge 1992).

In the urease method, urease is used to catalyze the hydrolysis of urea to NH_4^+ and CO_2^- (McCarthy 1970). The released NH_4^+ is then measured using the phenol hypochlorite method described above. The urease method can suffer from incomplete hydrolysis and resulting underestimation of urea concentrations.

Difficulties associated with the monoxime method include precipitate formation when the sample cools and rapid loss of color (Mulvaney and Bremer 1979); the method also needs a high reaction temperature (85°C) thus requiring a heated water bath, which can limit the number of samples that can be run simultaneously. Goeyens et al. (1998) published a modified version of the monoxime method that allowed the reaction sequence to take place at room temperature albeit with a much longer reaction period - 72 hours versus 20 minutes for the high temperature method. The limit of detection was similar for the high temperature (85°C) method, 0.14 μM, and the room temperature (20°C), method, 0.10 μM (Goeyens et al.

1998). A monoxime method has also been introduced for use with an autoanalyzer (Cozzi 2004).

Comparisons of the two methods found that the urease method tends to measured lower concentrations in seawater and so recommend the monoxime method for analysis of marine samples (Price and Harrison 1987, Revilla et al. 2005). With respect to estuarine samples, the monoxime method is more accurate and less affected by salinity than the urease method (Revilla et al. 2005).

B. Humic substances

Humic substances are a broad class of organic substances operationally defined based on their solubility at different pHs and retention on hydrophobic resins (Thurman 1985, Aiken 1988). There are three operational sub-categories of humic substances: humic acids, which are soluble at a higher pH but become insoluble at a pH < 2 (isolated using XAD-8 resin); fulvic acids, which are hydrophilic acids soluble under all pH conditions (isolated using XAD-4 resin), and humin, which is insoluble at any pH (Ishiwatari 1992). For a review of humic substances in aquatic systems, see Hessen and Tranvik (1998), Benner (2002), and Aluwihare and Meador (this volume).

There are two common approaches used to quantify humic substances – measurement of fluorescence or extraction onto a resin. The use of fluorescence measurements is of limited use in N studies, however, because the N fraction of the humic substances cannot be quantified directly. In resin methods, humic substances are extracted onto a macroporous acrylic ester resin. The most commonly used resin was formerly Amberlite XAD-8, but now most labs us Supelite DAX-8. Both resins have been shown to isolate similar bulk humic fractions from natural waters and to produce solutions with similar chemical compositions (Peuravuori et al. 2002). These resins have been shown to bleed small amounts of organic molecules (Aiken 1988). Therefore, prior to the extraction of humics, the resin should be cleaned over several days via a Soxhlet extraction procedure (solvents include ether, acetonitrile, and methanol) followed by extensive rinses of hydrochloric acid (HCl), sodium hydroxide (NaOH), and DIW (Aiken 1985, Thurman 1985). When isolating humics, the

sample pH is reduced to < 2 with 6 N HCl, which protonates acid groups, prior to passage of the sample through the resin. After the sample passes through the resin, the column is rinsed with DIW to remove any residual salts. The humics are then eluted from the column with 0.2 N NaOH. Complete elution of all humic-N is difficult, however. As a result, the most accurate approach to measure humic-N concentrations is to measure the TDN concentration before and after the sample has passed through the column with the concentration of humic-N taken to be the difference, rather than to measure the concentration of TDN in the eluted humic fraction.

One should be cautious when quantifying humic-N based on the use of resin extraction, however. Recent work indicates that N associated with humics can dissociate when the pH is taken down to < 2 prior to the passage through the resin column (See and Bronk 2005). The end result being that the C:N ratio of the isolated humic substances is likely higher than it is in natural waters.

C. Amino Acids

Amino acids are the largest component of total DON identifiable at the molecular level, and a wealth of information is available from both molecular-level and enantiomeric analyses. The fact that amino acids are one of the few compound classes that can be readily quantified in unconcentrated seawater has resulted in a broad literature on marine amino acids evolving for nearly 30 years, using methods that have changed relatively little.

Dissolved amino acids are commonly divided into two pools that must be analyzed separately: dissolved free amino acids (DFAA) exist as individual monomers in solution, while dissolved "combined" amino acids (DCAA) are defined operationally as additional amino acid liberated by acid hydrolysis. DCAA are thus presumably present mostly as polypeptides, a supposition supported in at least the high molecular weight (HMW) fraction by ^{15}N-nuclear magnetic resonance (NMR) spectroscopy data (discussed below). The operational nature of the DFAA vs. DCAA definitions means that amino acids liberated from difficult matrixes (e.g. humic substances) also could make up a part of DCAA. "Total hydrolysable" amino acid (THAA) is another term commonly used to denote both pools

together, when the sample is hydrolyzed but DFAA are not independently determined. Because the DFAA pool is typically much smaller than DCAA, THAA values are often assumed to be similar to DCAA.

Amino acids are polar and chemically diverse molecules, with side chains including a single proton, aliphatic chains, acids, bases, and aromatic rings. Methods used to determine the 15-20 most common protein and non-protein amino acids generally use derivatives to aid in separation, detection, or both. Because molecular-level amino acid analyses are routinely made in biomedical research, a variety of automated amino acid analyzers are commercially available. These typically consist of liquid chromatography coupled to automated-fluorescent derivatization, detector, and software optimized for common protein amino acids. Automated analyzers offer easy and rapid analysis of biological materials or purified proteins within a well known matrix, and have also been used successfully in ocean research (Ittekkot 1982, Lee and Bada 1977). Most recent investigators, however, have opted to set up their own amino acid methods using liquid or gas chromatographic systems tailored to their specific goals. In principle the only analytical difference separating DCAA and DFAA is the presence or absence of hydrolysis. In practice, however, the extremely low detection limits needed for DFAA limits the range of chromatography and detection options that can be used, while hydrolysis itself is problematic for some amino acids. For these reasons, DFAA and DCAA analysis are treated individually below.

1. Free amino acid methods

While DFAA make up a minor part of the total THAA pool (1-10%) they are nevertheless extremely important as substrates for microbial growth (e.g. Keil and Kirchman 1991a) and important components of rapidly cycling DON. Because of extremely low concentrations, use of fluorescent derivatives that allow pmol – fmol detection levels are necessary in order to make measurements directly in seawater. Reverse-phase HPLC separations after derivatization with OPA have remained the most widely used approach in ocean sciences since the early 1980's (e.g. Lindroth and Mopper 1979, Parsons et al. 1984, Jones et al. 1981). Compound-specific DFAA measurements are commonly made directly in

filtered seawater using automated pre-column OPA derivatization/injection coupled with reverse-phase chromatography and florescence detection (e.g. Coffin 1989, Keil and Kirchman 1991a, Cherrier and Bauer 2004). For coastal samples, interference by NH_4^+ can be a problem, and corrections may be necessary (Tada et al. 1998). As long as NH_4^+ levels are not high, total DFAA can also be estimated by a bulk fluorometric approach (Parsons et al. 1984), because DFAA concentrations are often equal to concentration of total dissolved primary amines (e.g. Kirchman et al. 1989, Delmas et al. 1990).

In practice, many difficulties of routine DFAA analysis in natural ocean water, in particular subsurface waters, stem from the extremely low concentrations present. Great care must be taken to avoid contamination and to monitor procedural blanks. Assuming no special chromatographic problems exist, detection limits can also be greatly affected by the sensitively of the florescent detector used. Simply replacing an old lamp may help substantially, and new generations of florescence detectors (e.g. offered by Shimadzu, Agilent, Varian, etc.) claim substantial improvement in power and optics, such that labs upgrading from older detectors have reported marked increases in sensitivity. It is also possible to use column pre-concentration to effectively increase sensitivity (Lee and Bada 1975). This approach could be extremely useful for investigation of minor DFAA components, such as D-enantiomers of DFAA (Lee and Bada 1975, 1977). However, because most of the common DFAA components can be measured directly, pre-concentration approaches have not been widely used.

2. Combined amino acids

a. Hydrolysis methods

Molecular-level analysis of THAA is dependent on hydrolytic cleavage of peptide bonds. Standard acid hydrolytic conditions developed for pure proteins remain in common use, typically employing 6N HCl at 100-110 °C for 20-24 hrs; the reaction is done under a N_2 atmosphere to minimize oxidative destruction of sensitive side-chains (e.g. Lee and Bada 1977, Henrichs et al. 1984, Parsons et al. 1984, Dauwe et al. 1999, Van Mooy et al. 2002,

Ziegler and Fogel 2003). Several amino acids with acid-sensitive side-chains are, however, destroyed or severely degraded under these conditions. Asparagine and glutamine are quantitatively converted to aspartic acid and glutamic acid, while the side-chains of tryptophan, and cysteine are substantially degraded. In order to effectively measure acid-sensitive amino acids, base hydrolysis can be used (Wu and Tanoue 2002). A number of ancillary approaches have also been devised to protect sensitive side chains from degradation, for example by adding sacrificial compounds or organic acids (Liu and Chang 1971). However, because the affected amino acids represent only a minor molar percent of the total amino acid pool, such measures (let alone performing separate base and acid hydrolyses) have rarely been used for oceanic THAA analyses.

Alternatives to the standard acid hydrolytic conditions have also been examined for marine samples. Elevated temperatures can reduce reaction time, and addition of multiple internal standards can more effectively track losses related to specific side-chain functionality (Cowie and Hedges 1992a). Gas phase hydrolysis (Keil and Kirchman 1991b) is also an alternative to standard aqueous hydrolysis that results in much reduced analysis time, and may have an important impact on THAA quantitation in some samples. Keil and Kirchman (1991b) observed substantial increases in amino acid yield for a range of oceanic samples using a vapor-phase HCL/TFA mixture at 156°C. The highest increases were for open ocean DOM, suggesting that THAA in DON may have been underestimated by up to 300%. More recently, Jørgensen and Jensen (1997) demonstrated a vapor-phase microwave method using only HCl, which reduced hydrolysis time to ~ 20 min. while also increasing THAA yields relative to standard aqueous phase protocols. Because of the potential for shortened reaction times and increased recovery of some problem amino acids such as cysteine, vapor-phase has become common for protein hydrolysis in some biochemistry applications (e.g. Strydoma et al. 1993) and have also been used to analyze marine samples (e.g. Cherrier and Bauer 2004).

b. Derivatization and chromatography

Once individual amino acids are liberated in dissolved form, they are typically separated by either high pressure liquid chromatography (HPLC) or gas chromatography (GC) for

molecular-level quantitation. Both GC and HPLC have been used effectively, with each having strengths and limitations. As with DFAA, reverse-phase HPLC separations after derivatization with OPA (Lindroth and Mopper 1979) has become perhaps the most widely used method (e.g. Cowie and Hedges 1992b, Keil and Kirchman 1993, Hubberten et al. 1994, McCarthy et al. 1996, Dauwe and Middelburg 1998, Ingalls et al. 2003, Lee et al. 2000, Van Mooy et al. 2002, Yamashita and Tanoue 2003). Because hydrolyzed amino acids are relatively stable at room temperature, large sample sets can be derivatized and chromatographed automatically using HPLC auto-injectors equipped with sample pre-treatment capability. However, some amino acids are either not derivatized at all by OPA (e.g. proline, secondary amines in general), or typically have poor quantitation and high variability (e.g. cysteine). 9-fluorenylmethyloxycarbonyl chloride (FMOC) is a common agent for derivatizing secondary amines, and can be used in combination with OPA to both detect proline as well as increase sensitivity for several other amino acids (Godel et al. 1992). In practice, however, again because these amino acids are minor components of TDAA, such enhancements to basic HPLC methods have rarely been used for ocean samples. One inherent drawback to HPLC methods is reduced chromatographic resolution relative to GC. Liquid chromatography-mass spectrometry (LC-MS) methods offer the sensitivity as well as molecular specificity to identify even unusual amino acids without needing to fully resolve components (Whitehead and Hedges 2002); however the necessity to desalt samples for electrospray MS applications diminishes its direct applicability for marine DON .

GC methods offer an attractive alternative to HPLC for applications where higher detection limits are acceptable (e.g. McCarthy et al. 1998, Harvey and Mannino 2001, Ziegler and Fogel 2003). A wide variety of volatile amino acid derivatives can be made by sequential esterification and acylation of the carboxyl and amino terminal ends respectively (see Knapp 1979 for a long list). These derivatives are readily separated by common GC phases, making GC a good method for quantification, isotopic, and enantiomeric amino acid analyses. Advantages of GC-based analysis include greatly increased chromatographic resolution, the relative ease and reproducibility of GC systems, as well the option for unambiguous peak and mixture identification using common bench-top GC-MS instruments available in most organic

laboratories. Drawbacks are the relatively labor intensive wet chemistry used to make needed derivatives and the lower sensitivity of common GC detectors. The need to make derivatives by hand (and thus in small batches) greatly limits sample throughput, and larger samples are required relative to HPLC-florescent detection methods. However, using N and phosphorous selective detectors (NPD), or selective ion monitoring with GC-MS, can increase sensitivity about an order of magnitude vs. standard flame ionization detectors (FID), while at the same time producing extremely clean and selective traces composed essentially uniquely of the compounds of interest. However, even with these approaches sensitivity is generally too low for unconcentrated seawater analysis, and the enormous salt content (relative to ON) can cause practical problems in performing derivatization reactions on dried seawater samples. These factors make GC techniques most useful for concentrated DON isolates, and in particular for investigation of enantiomeric (D/L), non-protein or minor amino acids where complex traces are anticipated, or MS compound verification is important.

3. Chiral (D/L) amino acids

Enantiomeric amino acid analysis has recently been of significant interest as an indicator of bacterial DON sources (Amon et al. 2001, Fitznar 1999, McCarthy et al. 1998). Using GC, amino acid enantiomers can be easily resolved by substituting a chiral column for a non-chiral phase (e.g. McCarthy et al. 1998). The same derivatives applicable for other GC techniques can be used, with the two most common being triflouro and pentaflouro anhydrydes (TFA, PFA). While one derivative is not necessarily preferred over others, the availability of different derivatization agents can be useful for resolving minor peaks such as many D-AA in complex traces (such as often result from ocean DON). If a particular peak of interest is not well resolved or its identity is unclear, running the same sample derivatized with a different acylation agent may well resolve it.

For direct analysis of chiral amino acids in total seawater, a diasteriomeric-derivative HPLC approach has recently been described (Fitznar et al. 1999). Florescent diasteriomeric derivatives are made that can then be separated using a standard (and thus relatively inexpensive) reverse-phase HPLC column. This allows chiral amino acids to be measured in

unconcentrated seawater samples with a protocol similar to standard OPA-based amino acid analysis (Dittmar et al. 2001). Obtaining good resolution of the doubled number of HPLC peaks can be somewhat challenging, however the diasteriomeric method has a neat advantage: since the derivatization reagent is chiral, two separate diasteriomers can be made for each compound. Each sample can thus be run using each reagent (D and L) in turn, resulting in unique separations which generally allow peaks that overlap in one trace to be resolved in the other (Fitznar et al. 1999). While an increase in work, this approach also represents a powerful check on peak identification, allowing even minor D-AA peaks to be identified.

D. Total Protein/Peptide

1. Colorimetric methods for bulk protein

As mentioned above, the large majority of recoverable amino acids are in combined form. This indicates that peptides, and possibly proteins, are important DON constituents. Operational measurements of the amount of total "protein" in the DON can be made by a wide variety of fluorometric and colorimetric assays. A partial list of those that have been applied to marine samples include coomassie blue (e.g. Setchell 1981, Mayer et al. 1986, Nunn et al. 2003), Lowry's method (Clayton et al. 1988), the fluorescamine assay (Garfield et al. 1979), the bicinchoninic acid assay (Nguyen and Harvey 1994), and the CBQCA (3-4-carboxybenzoyl-quinoline-2-carboxaldehyde) assay (Nunn et al. 2003). A number of the more common methods of protein analysis used in biochemical research, including a discussion of issues affecting their quantitative application, have recently been reviewed by Sapan et al. (1999).

Total protein assays have the advantage of being relatively straightforward compared to molecular-level analyses. Methods with fluorescence-based detection are also highly sensitive, and thus amenable directly to DON. Quantitative interpretation for environmental mixtures such as seawater, however, may be problematic for some samples. Most methods react with specific moieties (e.g. coomassie blue binds to lysine and arginine) and thus results obtained can depend on protein composition, size distribution, and even conformation (Sapan

et al. 1999), making the careful choice of calibration standards important. In addition, common components of natural samples, such as humic materials (e.g. Mayer et al. 1986), carbohydrates (Sapan et al. 1999), or ammonia may interfere with quantitation. Overall, colorimetric methods can be very useful as quick, likely semi-quantitative estimates of total protein or peptide. However, potential biases inherent in the mechanism of a specific method should be considered before one is chosen, and application of newer molecular assays (e.g. CBQCA) should be carefully examined in terms of natural sample matrix (Nunn et al. 2003).

2. Specific protein analysis

Isolation and sequencing of specific proteins in the DON pool has recently begun to reveal a wealth of specific source information, as well as suggesting mechanisms for DON preservation (see Tanoue 2000 for review of literature through 1990's). Proteins can be concentrated from large volumes of seawater using ultrafiltration with a nominal >10kD size cutoff (discussed below, Powell and Timperman 2005). Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) has also been successfully used in both one and two dimensions to isolate discrete proteins from DON (e.g. Tanoue 1995, Jones et al. 2004, Yamada and Tanoue in review). This approach allows estimation of both the number and MW distribution of apparently intact proteins in DON. Total proteinaceous material isolated with this approach has been estimated to be up 10-30% of total DCAA, corresponding to 2-12% of total DON (Tanoue 2000). Recovery, however, is strongly dependent on the exact protocol used for extracting proteins from seawater (Tanoue 1995), and it is likely that significant work remains to be done to constrain quantitative estimates. The presence of humic materials and salts can also significantly limit gel quality (Jones et al. 2004). Because of the difficulty of isolating entire proteins, as well as the fact that most proteinaceous material likely exists as smaller, partially degraded peptide units, approaches such as immunological techniques to probe smaller structures (e.g. Orellana et al. 2003) may also be extremely valuable.

While methods for isolating discrete proteins are analytically challenging, when coupled with N-terminal amino acid sequencing (e.g. Tanoue et al. 1995), immunochemical

approaches (e.g. Suzuki et al. 1997), or newer mass spectrometric techniques (Powell et al. 2005), specific proteins can be identified, potentially offering a wealth of new information about DON sources. Recently, glycoproteins have also been detected in DON (Yamada and Tanoue 2003), and 2-D electrophoresis suggests that highly varied glycosolated residues may even dominate the protein distribution of some ocean regions (Yamada and Tanoue in review). These results suggest that a method for analyzing glyoproteins in DON may be an important future research target.

E. Amino sugars

Amino sugars are abundant compounds in plankton and bacteria, and thus constitute one of the largest potential DON sources. Biochemical "interrogation" studies have shown rapid turnover of common amino sugars, such as N-Acetyl glucoseamine (e.g. Kirchman and White 1999, Riemann and Azam 2002), indicating that amino sugars are key components of a rapidly cycling DON pool. Amino sugars are also major components of structural or cell-wall polymers in prokaryotes such as bacterial peptidoglycan, lipopolysaccharides, and archaeal pseudopeptidoglycan (Brock et al. 1994). These structures are thought to be important sources for preserved DON (e.g. McCarthy et al. 1998, Tanoue et al. 1995, Boon et al. 1998). NMR-based analyses of HMW-DON has also suggested that amino sugars could be a major component of this material (McCarthy et al. 1997, Aluwihare et al. 2005).

Common hexoseamines (glucoseamine and galactoseamine) can be quantified using modifications of standard amino acid HPLC protocols (Dauwe and Middelburg 1998), or by HPLC after a more mild sugar-specific hydrolysis and subsequent desalting (Ittekkot et al. 1984a and b). In addition, a range of GC and GC-MS based methods are available that can quantify amino sugars after formation of volatile derivatives (Neeser and Schweizer 1984, Whiton et al. 1985). GC-MS methods have the substantial advantage of unambiguous peak identification, but again making derivatives can be time consuming and result in losses. In addition, even in sediments and sinking particles the relatively small yields of amino sugars obtained by such methods (Dauwe and Middelburg 1998, Ittekkot et al. 1984a) suggest that they would not be suitable for direct DON analysis, although they can be used for

concentrated samples (Aluwihare et al. 2002). Overall, despite the potential importance of amino sugars to DON composition and cycling, relatively little research has directly targeted this compound class, most likely because of the lack of common methods for direct measurements.

The problem of sensitivity has recently been addressed with a high-performance anion-exchange chromatography - pulsed amperometric detection (HPLC-PAD) approach (Borch and Kirchman 1997), specifically modified for amino sugars in seawater (Kaiser and Benner 2000). A similar method has also been published for amino sugars (as well as neutral sugars) in unconcentrated freshwaters (Cheng and Kaplan 2003). While seawater samples must still be desalted via resin chromatography, the HPLC-PAD method of Kaiser and Benner (2000) allows fmol detection levels without derivatization, and it is readily coupled to automated sample injection. After optimizing hydrolysis conditions, near quantitative yields were reported for amino sugars spiked in natural DOM, and good recoveries were reported from a variety of natural materials (Kaiser and Benner 2000). HPLC-PAD thus makes amino sugar molecular-level analysis possible on unconcentrated seawater samples, and also allows direct quantitative comparison with results from HMW DON and ocean particles. The first comprehensive set of such measurements has provided important new source information, however, absolute contribution of amino sugar N to the total DON pool was found to be small (2-5%, Benner and Kaiser 2003).

Recently several innovative, if less specific, analytical approaches have also been applied to concentrated DON samples, providing information about total amino sugar content and character in isolated DON. Direct temperature-resolved mass spectrometry (DT-MS) coupled with chemical ionization (NH_3) can be used to examine thermal sugar degradation products in solid materials (Boon et al. 1998). Though not strictly quantitative, both DT-MS as well as Pyrolysis-MS (Lomax et al. 1991) provide information on relative abundance of types of amino sugars, while at the same time possibly accessing material protected from direct acid hydrolysis (Minor et al. 2001). These methods have indicated that both oceanic and estuarine HMW DOM has an important contribution from N-acetylated amino sugars (Boon et al. 1998, Simjouw et al. 2005). Another recent approach has been to apply differential (i.e. weak vs.

strong) acid hydrolysis in concert with ^{15}N-NMR analysis (Aluwihare et al. 2005). This experiment exploits the relative ease of hydrolyzing acetyl groups from N-acetyl sugars, combined with the clear ^{15}N-NMR distinction between amides versus amines. An estimate of total amino sugar in a sample can be obtained by comparing NMR spectra before and after weak hydrolysis. In HMW DON fraction this method suggests that amino sugars comprise up to half of surface DON, but substantially lower amounts in subsurface waters (Aluwihare et al. 2005). It should be noted that the quantities of unidentified N-CHO suggested by this approach (at least for HMW DON) are about an order of magnitude higher than direct measurements made on similar materials using molecular-level methods discussed above (Benner and Kaiser 2003). The gap between direct analysis and NMR-based estimates presents a major analytical conundrum for understanding the abundance and cycling of amino sugars in DON, and solving it is an important goal for future research.

F. Nucleic acids

DNA and RNA are major N-rich biochemical components of bacteria and viruses, and thus represent important potential sources to the DON pool. Recent methods have found oceanic nucleic acid concentrations in the low $\mu g\ L^{-1}$ range (Karl and Bailiff 1989), suggesting that intact nucleic acids correspond to only a few percent of the total DON pool, despite the fact that rapid turnover suggests they are key N recycling compounds (e.g. Antia et al. 1991, Jørgensen et al. 1993).

Methods to directly measure concentration of dissolved DNA (dDNA) and dissolved RNA (dRNA) typically involve an initial concentration or precipitation step, followed by detection and quantification by a florescent stain or dye. Common approaches have included ethanol precipitation followed by Hoechst 33258 staining (e.g. DeFlaun et al. 1986) that detects DNA only. Both DNA and RNA can be simultaneously measured using CTAB precipitation followed by reaction with 3-5 diaminobenzoic acid (Karl and Bailiff 1989). Filtration and ultracentrifugation approaches for initial concentration steps have also been used (Paul et al. 1991), and have been combined with immunological detection (Jiang and Paul 1995). Recent improvements have substantially decreased sample volumes and

processing times required for dDNA analysis. Use of tetrasodium EDTA coupled with centrifugal concentration and SBYR Green-I florescent dye requires only ~15ml of oligotrophic seawater and several hours processing time per sample (Brum et al. 2004). A similar SBYR Green-I method has also been adapted for freshwater dDNA (Matsui et al. 2004).

One complication for estimating the importance of nucleic acids as a source of *total* DON is, however, that common methods developed with biological processes in mind target relatively "intact" dDNA or dRNA, and may be less sensitive to partially degraded, bound, or otherwise altered material. The initial precipitation steps and dyes used for detection are commonly specific for molecule conformation or size. The SBYR Green-I dye (on which newer methods are based), for example, is a double stranded stain and is at least an order of magnitude less effective with single stranded molecules (Matsui et al. 2004), while the EDTA step used for precipitation is most effective for material larger than 20 base pairs (Brum et al. 2004). In addition, the operationally defined total "dissolved" DNA and RNA includes not only material truly in solution (and thus amenable to precipitation), but also material within viruses and that tightly associated with colloids, humics or detritus (e.g. Jiang and Paul 1995, Brum et al. 2004). DNA present in an unknown "bound" form has been estimated in some marine studies at about half of that present in "free" pool (Jiang and Paul 1995). It is thus difficult to estimate what contribution degraded or altered nucleic acids may make to more refractory DON using current compound-specific methods, although NMR data has hinted that such material could be an important source of unsaturated N observed in deep ocean HMW material (McCarthy et al. 1997).

V. Bulk organic N characterization

The compounds that current methods can directly analyze likely make up the bulk of the rapidly cycling DON pool. However, the large majority of the total DON, especially in the subsurface ocean, still cannot be identified at the molecular-level. This presents us with a familiar analytical quandary. Without additional clues about the chemical nature of the majority of unidentified material, it is impossible to know where the methodological

challenges lie: is it in the nature of the compounds themselves, i.e. novel or substantially altered molecular structures for which new methods must be devised? Or are there limitations inherent in current methods, perhaps related to the natural seawater/DON matrix, which are the key barriers? To get beyond this circular problem, an enormous variety of spectroscopic and organic chemical methods exist that could be applied to organic mixtures and supply new information about the nature of "bulk" DON, including the distribution of compound classes, functional groups, isotopic ratios, etc. However, the bugbear is in the natural matrix - very few such techniques can be performed directly on aqueous samples. Low natural DON concentrations, coupled with vastly larger salt content, rule out many organic techniques that require a reasonably pure (non-diluted by mineral or salt) and fairly large (mgs or more) sample. Finally, it is almost a truism for detailed organic analysis that "more is better" - the larger the sample, the greater the analytical possibilities become. For these reasons, a method that could quantitatively isolate a salt-free sample of the total dissolved material from seawater is needed (Bronk 2002). To date no such method has been developed, however, a great deal of information continues to be gained from a number of partial DON isolation techniques.

A. Organic isolation techniques

1. Ultrafiltration

Tangential flow ultrafiltration (UF), also referred to as "cross flow filtration," has become a widely used method since the early 1990's for isolating and desalting large quantities of DOM from seawater (e.g. Benner et al. 1992, Amon and Benner 1994, Guo et al. 1996, Aluwihare et al. 1997, McCarthy et al. 1996, Benner et al. 1997, McCarthy et al. 1998, Guo 2000, Kolowith et al. 2001). Ultrafiltration is a size-based technique in which the sample is constantly recirculated across a membrane, progressively concentrating components that do not pass the membrane pores. Most seawater studies targeting total DOM have used polysulfone membranes with a nominal size cutoff of 1000 D, mainly because this is the smallest cutoff commonly manufactured that does not begin to retain larger seawater salts,

thus allowing the filtration to proceed at a workable rate (e.g. Benner et al. 1992, Benner et al. 1997). Dissolved material isolated by UF has been referred to in several different ways in the literature. In papers targeting the composition or cycling of the entire DOM pool, it has typically been abbreviated as UDOM (ultrafiltered DOM); this designation is functionally based, and thus avoids implicit assumptions about the nature of material isolated. Another body of literature uses "colloidal" OM to describe the same material, because the nominal >1000 D membrane cutoff also corresponds to a nominal size range for colloidal material, despite the fact that coupled UF and desalting likely destroys any natural non-covalent aggregates. It is also important to bear in mind that while in principle UF membranes retain only the HMW components exceeding the membrane pore size, in practice UF retains material based both on size as well as *charge* and other interactions between compounds and the membrane surface. One consequence of this more complicated mode of retention is that UF actually retains both high and low MW material during filtration (Guo 2000). Final DOM recoveries thus depend on a concentration factor (i.e. total volume processed/final volume), and heavily on desalting, during which much of the originally retained DOM passes the membrane as ionic strength decreases (e.g. Benner et al. 1997, Guo 2000). After desalting, UF with 1000 D membranes typically recovers ~30% of surface DOM and about ~20% from deeper waters. While relatively modest in terms of absolute recovery, because concentration takes place in a natural seawater matrix without chemical treatments or use of selective resins, UF isolates a more representative sample of total dissolved material than had been possible using previous chemically-selective methods (Benner et al. 1997, Kaiser et al. 2003). In addition, because UF systems can be operated in a continuous flow mode, extremely large volumes of water can be processed. This allows isolation of large amounts (grams are possible) of nearly pure DOM, making UF extremely useful for sample-intensive techniques.

A key issue with UF-based studies is how representative UDOM is of the total dissolved pool. For most bulk properties of DOM (e.g. total C:N ratio and stable and radiocarbon isotopes) material isolated by UF has been found to be similar to the bulk pool (e.g. McCarthy et al. 1996, Benner et al. 1997,Voparil and McCarthy in preparation). At the same time UDOM also displays small offsets in some of its bulk properties, consistent with size-

reactivity continuum observations which have suggested that HMW material is generally less degraded than LMW DOM (Amon and Benner 1994). For example, ^{14}C content of UDOM in the subsurface Pacific indicates ages of 4-5000 years (Loh et al. 2004, Voparil and McCarthy in preparation), which are similar to commonly reported values of 4-6000 for total DOC, and clearly unrelated to modern ^{14}C values typical of POC. At the same time, comparison of measured UDOM ^{14}C values to literature ^{14}C values for total DOC (although not taken from the same location) has also suggested that UDOM is somewhat younger than the total DOC at the any given depth horizon (Loh et al. 2004).

For DON studies, ON mass balances for UF have not been directly determined, however, UF typically recovers material with C:N of ~ 14 in surface waters and 17-22 in the deep ocean (Benner et al. 1997). Comparison to literature C:N ratios (DOC:DON data) from different locations can be problematic, in part because the large uncertainty that can be involved in subsurface DON estimates discussed above propagate into the DOC:DON ratio, however, broad compilations (Bronk 2002, Hopkinson and Vallino 2004) suggest that C:N ratios of UDOM are slightly enriched in C, and thus UF may slightly under sample total DON. Molecular-level comparisons for amino acids, however, are encouraging. These indicate that THAA content and molar composition is similar between UDON and total DON (McCarthy et al. 1996), as are its enantiomeric (D vs. L) ratios (Amon et al. 2001, Dittmar et al. 2001). Amino sugar comparisons between LMW and HMW DOM have not been directly made, however, carbohydrates are generally more abundant in the higher MW DOM in upper ocean waters (e.g. Benner et al. 1992, Pakulski and Benner 1994, Skoog and Benner 1997). Recent work has also suggested that amino sugars are far more abundant in surface vs. deep water UDOM (Aluwihare et al. 2005). It is thus possible that UF might over-sample amino sugars relative to total DON, especially in surface ocean.

Overall, UF has numerous practical advantages for isolating large quantities of DON needed for detailed chemical study, and available data suggests its composition does not depart substantially from the bulk oceanic DON pool. In particular amino acid data indicates that for the largest DON compound class it is possible to compare at the molecular level, UF is a good way to examine the total pool. However, uncertainty remains, especially when

departing from bulk composition and considering specific N-containing compounds that cannot be analyzed directly in seawater - caution should always be used in extrapolating results to the bulk DON pool. Additional molecular-level and isotopic data comparing UDON with total DON will be extremely valuable, in particular comparison between $\delta^{15}N$ of UDON vs. total DON as new methods for measuring $\delta^{15}N$ of total DON evolve.

2. Ion retardation resins

Perhaps the easiest method for isolating DON is through the use of ion retardation resin (e.g. BioRad AG 11 A8), which retards the flow of charged particles. The resin quantitatively removes salts including NH_4^+, NO_3^-, and NO_2^-, allowing DON to be isolated in the eluate (Bronk and Glibert 1991, 1993a, Hu and Smith 1998, Nagao and Miyazaki 1999). Unfortunately, we know of no current distributor of ion retardation resin that is suitable for use with aquatic samples. DOW Chemical, the company that manufactured the resin marketed by BioRad and other distributors, changed the manufacturing process of the resin sometime in the mid 1990s such that the resin now retains variable amounts of DON. This DON retention is believed to be due to an accumulation of an organic film on the resin beads during manufacturing (BioRad personal communication). To overcome this problem, AG 11 A8 resin can be manufactured and purified by buying another resin (Dowex anion exchange resin, BioRad AG1-X8) and then chemically altering it to produce AG 11 A8 as described in Hatch et al. (1957). The homemade resin, however, tends to breakdown rapidly, a problem not previously encountered with the resin produced by BioRad.

3. Hydrophobic resin isolations

Another approach that could potentially be used for DON isolation is chemically selective resins. As discussed above, hydrophobic resin isolation has long been used in both terrestrial (Thurman and Malcolm 1981, Ertel et al. 1986) and marine waters (e.g. Stuermer and Harvey 1974, Wilson et al. 1983) to study dissolved humics. Thus as a bulk isolation technique, resins are appropriate mainly for the humic component of DON, as described above for XAD-type isolations. A somewhat different spin on this theme is the use of solid-

phase extraction (SPE) with C-18 columns, which has recently become widely used as a method to isolate hydrophobic DOM components for analyses that require desalting (e.g. Kaiser et al. 2003, Kim et al. 2003b, Koch et al. 2005, Schwede-Thomas et al. 2005). SPE has also been used in combination with UF in estuarine waters to increase the amount of total DOM recovered to near 70% (Simjouw et al. 2005). Analysis of SPE-isolated material indicates, however, that SPE preferentially recovers the humic-like fraction (as would be expected), at the expense of polar biopolymers such as sugars and amino acids (Simjouw et al. 2005). This is consistent with observations that SPE can strongly discriminate against total N, isolating material with significantly elevated C:N ratios (Koch et al. 2005, See and Bronk 2005). To overcome such resin-specific issues, it has been reported that a variety of different resins with a range of polarities and retention characteristics can be used in series to isolate nearly the entire DOM pool as discrete fractions, from both fresh and saline waters (Leenheer et al. 2004). Such multi-resin experiments might be useful in the design of investigations targeting a specific fraction of the DON pool, however, their relatively labor-intensive nature and concerns about blank contamination makes them less attractive for routine DON study.

4. Dialysis

Dialysis of discrete water samples is another approach that could potentially be used to isolate smaller samples of DON. Dialysis can be used effectively to isolate HMW DOM material (Heissenburger and Herndl 1994), and with small membrane pore sizes could potentially be used to purify discrete DON samples. In freshwaters, dialysis has been used to remove inorganic N as a way to increase the accuracy of total DON measures, however, losses due to passage of LMW DON through the membrane and adsorption were concerns (Lee and Westerhoff 2005). Dialysis has also been used in freshwaters for isolating DON for $\delta^{15}N$ measurement (Feuerstein et al. 1997). To date, however, dialysis has not been extensively investigated for seawater DON. Contamination and adsorption issues, as well as the relatively small amounts of material that can be collected, make dialysis most promising for compound-specific investigations. Other potential problems with the dialysis approach are the large amount of time needed to process samples (100 and 200+ hours for fresh and salt

water samples respectively), the lack of complete removal of salts and inorganic N, and the risk of bacterial contamination that could chemically alter the final dialyzed product.

B. NMR spectroscopy

Nuclear magnetic resonance spectroscopy has proven to be one of the most powerful techniques for characterizing complex natural geochemical samples because of its ability to non-destructively probe the chemical character of a range of organic nuclei that include most building blocks of organic matter (^{13}C, ^{15}N, ^{1}H, and ^{31}P). Basic techniques, theory and applications have been reviewed in a variety of articles and texts (e.g. Wilson 1987, Sanders and Hunter 1988, Preston 1996). NMR spectra for geochemical samples such as DOM primarily differ from those of pure compounds in having broad and overlapping spectral lines, reflecting the fact that natural mixtures contain multiple variations within structural families. While specific compounds can thus rarely be identified, integration of spectral regions provides a broad view of the composition of the entire sample, in contrast to very narrow but detailed information available from molecular-level analyses. Coupled with sample isolation methods both solid and liquid-state NMR techniques have been used to study oceanic DOM (e.g. Stuermer and Payne 1976, Benner et al. 1992, Hedges et al. 1992, McCarthy et al. 1996, Aluwihare et al. 1997, Clark et al. 1998, Repeta et al. 2002), as well as to target specifically nitrogenous material (McCarthy et al. 1997, Aluwihare et al. 2005).

Solid-state experiments use a dry sample that is packed into a rotor and spun at high frequency inside the spectrometer's magnetic field. This approach is termed "cross polarization magic angle spinning" (CPMAS), and is the standard protocol for solids. It relies on transfer of magnetization from protons to C (or other nuclei) in order to achieve rapid analyses with reasonably narrow spectral lines. Cody et al. (2002) provides one recent overview of solid-state NMR techniques in geochemical samples. With CPMAS essentially the full range of material in a mixture can be examined, including very HMW or insoluble components, making it particularly valuable for materials that have undergone major diagenetic alteration. CPMAS also has the advantage (especially significant for N studies) of concentrating the maximum amount of sample inside the magnetic field. The most common

solid-state experiments on DOM isolates have been ^{13}C-CPMAS (e.g. Benner et al. 1992, Hedges et al. 1992, McCarthy et al. 1993), however ^{15}N (McCarthy et al. 1997) and ^{31}P (Clark et al. 1998) experiments can also be readily performed.

Liquid-state experiments are the more familiar variety used for organic structural elucidation. The sample is dissolved in a specialized solvent that is as free as possible of the nucleus of interest (e.g. if a ^{1}H-NMR liquid experiment is to be performed using an aqueous solvent, D_2O is used). Liquid state experiments inherently produce much sharper spectral lines, and thus have potential to provide more detailed structural information. In practice, however, very complex mixtures such as natural DOM produce complex overlapping resonances resulting in spectra that can closely resemble the broad lines of their solid state counterparts (e.g. Repeta et al. 2002). ^{1}H-NMR experiments can be conducted rapidly on marine DOM, revealing the major distribution of those structural types that are soluble (e.g. Aluwihare et al. 1997, 2002). In addition, liquid state NMR offers the possibility of a wider variety of elaborate experimental possibilities, including multi-dimensional experiments targeting linkages between specific nuclei (e.g. Hertkorn et al. submitted, Kaiser et al. 2003, Kim et al. 2003a).

For the nitrogenous component of DOM, as indicated above, ^{15}N nuclei can be examined directly (e.g. McCarthy et al. 1997, Aluwihare et al. 2005). Even with sample concentration, however, the relatively low natural abundance of ^{15}N makes solid-state experiments the method of choice. Experiments with ^{15}N-CPMAS have been valuable in revealing the major functional distribution of N in HMW DON (McCarthy et al. 1997), and when combined with variable hydrolysis methods have been used to probe the relative amounts of amino-sugar and peptide amide bonds (Aluwihare et al. 2005). However, the fairly limited range of common N biochemical functionality makes the information potential of ^{15}N-NMR more limited than that for either C or H. ^{15}N spectra for DON study are thus most valuable when used in combination with other approaches mentioned above. Multi-dimension experiments to directly examine the linkages between N and C are also possible, however, the low abundance of ^{15}N makes precludes their use on natural samples with current technology. Such techniques might, however, be employed in the future in mesocosms with DON production

and degradation studies. Solid-state double cross-polarization experiments between C and N have been conducted with degrading plant remains, but required isotopically enriched samples in both ^{13}C and ^{15}N (Knicker 2002). A great deal more solid-state ^{15}N work has been described in the terrestrial and sedimentary literature (e.g. see Knicker 2004 for a review), which might be applied to future work with isolated DON material, and particularly the more humic fraction from the deep ocean.

VI. Basic principles for the measurement of nitrogen flux rates

A. Bioassays

There are two main approaches used to determine uptake rates – bioassays and tracer methods. In the bioassay approach the concentration of a given substrate is monitored over time (e.g. Seitzinger et al. 2002). The advantage of bioassays are they are relatively easy and require only that suitable incubation conditions be maintained and the ability to measure the concentration of the substrate of interest. There are, however, drawbacks and limitations to the use of bioassays. First, they only provide net uptake rates. If there is substantial substrate regeneration, such as one would expect in the case of NH_4^+, the uptake rate will be underestimated or even unmeasurable if the rate of regeneration is greater than the rate of uptake. Second, there is a fine line between incubating a sample long enough so that there is a detectable change in concentration versus incubating so long that bottle effects skew the results. Care must also be taken to maintain the sample under as close to *in situ* conditions as possible.

B. Stable isotopes

1. Basic principles

The other approach used to measure flux rates is the application of isotopic tracers. In the case of N, there are two isotopes that have been used – radioactive ^{13}N and stable ^{15}N. Unfortunately ^{13}N has a very short half-life (~10 minutes) such that easy access to a nuclear reactor is required (e.g. Fuhrman et al. 1988, Suttle et al. 1990). The isotope ^{15}N, however, is

widely used to trace N flow in the water column and sediment both in short-term, small volume incubations (e.g. Dugdale and Goering 1967) and long-term, whole-system enrichments (e.g. Tobias et al. 2003); isotope tracer methods are reviewed in detail in Glibert and Capone (1993 and Lipschultz, this volume).

The use of isotopic tracers is based on following the flow of a labeled compound from one pool (the source pool) to another (the target pool) (Sheppard 1962). The principles described below are the same regardless of whether one is measuring rates in the water column or sediment. What changes are the protocols used to isolate the different fractions prior to isotopic analysis. The basic equation to calculate the flux rate from one pool to another is:

$$\text{Rate} = \frac{\text{Atom \% (R) of the Target Pool}}{\text{Atom \% (R) of the Source Pool X Time}} \text{ X [Target Pool]} \qquad (4)$$

where atom %, often referred to as R in the literature, is the ratio of ^{15}N:$^{15}N + ^{14}N$ in a pool, time is the length of incubation, and the brackets denote concentrations (see Lipschultz, this volume, for nuances in the use of this equation).

Advantages of the isotope tracer method include higher sensitivity, shorter incubation times, ability to measure gross uptake (e.g. uptake plus subsequent release), and the ability to measure uptake in the presence of high rates of regeneration.

2. Isolation of pools

Prior to isotopic analysis, either for natural abundance measurements or at the end of tracer incubations, the relevant N pools must be isolated. Here we review the isolation of the most important N fractions: PN, NH_4^+, NO_3^-, NO_2, DON and urea.

a. NH_4^+

There are several methods available to isolate NH_4^+ including distillation, SPE, wet chemical, and direct diffusion. In distillation the pH of the sample is raised to convert soluble NH_4^+ to volatile NH_3, generally with NaOH or MgO (e.g. Gilbert et al. 1982). The sample is then heated, preferably under vacuum, condensed in a cool condenser, with the condensate ultimately trapped in a mildly acidic solution that converts the isolated NH_3 back to soluble NH_4^+. The acidified NH_4^+ is then evaporated and spotted on a filter (e.g. Harrison 1978) or captured on a molecular sieve (e.g. Velinsky 1989, Hoch et al. 1992). One cautionary note about isolating NH_4^+ with zeolite molecular sieves: while it is a quick efficient method for freshwater, it is not usable with brackish or seawaters (Lipschultz 1984 in Glibert and Capone 1993).

In SPE the NH_4^+ in the sample is converted to indophenol following a variation of the phenol/hypochlorite method described above for measuring NH_4^+ concentrations. The indophenol solution is passed through an octadecyl C18 SPE column that binds the indophenol to the resin. The indophenol is then eluted with methanol. The recovery of NH_4^+ with this method is generally low, less than 30% (Selmer and Sorensson 1986). Alternately the indophenol can be extracted into dichloromethane (Dudek et al 1986).

There are a number of wet chemical approaches used to isolate NH_4 including precipitation with mercuric chloride (Fisher and Morrisey 1985). This method, however, is problematic with marine samples and generates particularly nasty waste.

Ammonium has also been isolated using direct diffusion. In this method the pH is raised and an acidified glass fiber filter is suspended above the sample and the bottle is sealed. The sample is then placed in a heated oven (e.g. Slawyk and Raimbault 1995) or placed on a shaker table. The increase in pH will produce volatile NH_3, which is then captured on the acidified filter. Another variation of this approach places the acidified filter into a Teflon envelope. Any method that requires a change in pH, such as diffusion or distillation, should be used with care, however, because of the risk of hydrolyzing amino groups from organic compounds (Brenner 1965). This will result in transfer of label from the DON pool into the NH_4^+ pool that is difficult to detect. Another potential artifact to consider is that incomplete

isolation can lead to isotopic fractionation; this is especially problematic with distillation and diffusion methods. Finally, whatever method is selected it is important to quantify the method blank and to correct the final atom % enrichment accordingly (see section below).

b. NO_3^- and NO_2^-

Isolation procedures for NO_3^- generally involve converting the NO_3^- to some other form. Nitrate can be converted to NH_4^+ with DeVarda's Alloy (e.g. Bronk and Ward 1999), zinc dust (e.g. Gardner et al. 1995) or titanium III chloride ($TiCI_3$, Cresser 1977) and then the NH_4^+ is isolated using any of the suite of NH_4^+ isolation methods described above.

Nitrate can also be converted to NO_2^- using cadmium reduction (columns or spongy cadmium) as described above. Once the NO_3^- is in the form of NO_2^-, the NO_2^- can be isolated via organic extraction (e.g. Olson 1981) or with SPE after the NO_2^- is converted to an azo dye (Kator et al. 1992). Nitrate can also be isolated by conversion to N_2O through the use of a genetically engineered denitrifier (Sigman et al. 2001). The bacteria will denitrify NO_3^- in a sample to N_2O but lack nitrous oxide reductase and so cannot take the reaction to completion and form N_2. The N_2O produced can then be analyzed on a mass spectrometer. A more detailed discussion of these methods is presented in Lipschultz (this volume).

c. DON

At present, there are three basic approaches used to isolate DON for tracer experiments: wet chemical isolation, ion retardation (described above) and dialysis (described above). The suitability of a method is judged by its ability to remove all of the DIN forms (NH_4^+, NO_3^-, and NO_2^-) and to isolate the DON pool with high efficiency. Ultrafiltration is not a suitable isolation method for use in determining DON release rates because it only isolates the larger MW fraction and thus allows the LMW moieties, which are likely important short-term release products, to be lost.

There are several variations of the wet chemical approach, first introduced by Axler and Rueter (1986), but all involve removing inorganic N species from solution so that the remaining DON can be analyzed. In this approach, NH_4^+ is removed by raising the pH

slightly, thus effecting a change from soluble protonated NH_4^+, to the more volatile NH_3 via diffusion in a heated oven (Slawyk and Raimbault 1995) or vacuum distillation (Bronk and Ward 1999). A high-speed concentrator (e.g. SpeedVac) is particularly useful for NH_4^+ removal because the process can take place at room temperature or lower thus decreasing the possibility of acid hydrolysis (Bronk unpubl. data). Nitrate in the sample can then be converted to NH_3 with DeVarda's alloy, with the NH_3 again removed through volatilization (Slawyk and Raimbault 1995, Bronk and Ward 1999) or any of the other approaches outlined above. Both of these techniques can suffer from the artifact of losing labile DON as a result of base hydrolysis (Bronk and Ward 2000). The problem in the isolation is likely the lengthy diffusion step undertaken to remove NH_4^+ and NO_3^- and NO_2^- from solution. A number of other protocols have been used with varying success to remove NO_3^- and NO_2^- including vanadium (VIII, Cox 1980, Garside 1982), titanium (TiIII, Cresser 1977, Cox 1980, Bronk et al. unpubl. data), and other DeVarda's alloy approaches (Page et al. 1982). The breakdown and loss of DON is always a danger due to the rigorous reducing conditions needed to remove NO_3^-. Some researchers even use heated diffusion as a mechanism to remove labile DON before isolation of NO_3^- (Sigman et al. 1997). For a review of potential artifacts during wet chemical DON isolation and the different types of DON release rates and their calculation protocols see Bronk and Ward (2000) or Lipschultz (this volume).

d. Urea

Urea can be isolated for isotopic analyses by using urease to breakdown urea to NH_4^+ as described above (e.g. Lomas et al 2002). Before urease is added to the sample, however, the preexisting NH_4^+ must be removed, ideally with distillation. Rysgaard and Risgard-Peterson (1997) remove the preexisting NH_4^+ with high efficiency using cation exchange.

e. Particulate Nitrogen

Particulate N is isolated by filtration with a filter capable of withstanding the combustion needed to convert the N to N_2 gas so that the sample can be analyzed isotopically. The most commonly used filter is the glass fiber (e.g. GF/F) with a nominal pore size of 0.7 μm. Many other commonly used filters, such as polycarbonate or cellulose filters, produce a large

amount of CO_2 upon combustion, which will rupture ampoules or combustion tubes in mass spectrometers. One problem with using glass fiber filters, however, is that they retain a variable amount of detritus and bacteria, in the addition to phytoplankton. Some separation of bacteria and phytoplankton can be achieved using size fractionation but any filter used must withstand the combustion required with mass spectrometric analysis. Other suitable filters include those made of silver or alumina silicate (e.g. Anopore®). Silver filters are available in a range of pore sizes (0.2 to 5.0 μm) but they are fiendishly expensive with notoriously slow filtration rates. The alumina silicate filters come in 0.2 μm sizes, suitable for isolating the bacterial fraction, however, they too have very low flow rates as well as the annoying characteristic of being very brittle. If one wants to collect the bacterial fraction, it is often quicker to preconcentrate over a 0.2 μm filter with a higher flow rate, such as a Supor® membrane, and then filter the concentrated sample onto a silver or Anopore® filter as a final step. Regardless of the filter type used, all should be precombusted (450^{o}C for a minimum of 2 hours) to remove contaminant N; be sure to remove the plastic ring on Anopores® prior to combustion.

Flow cytometry can also be used to separate phytoplankton from bacteria and detritus (Lipshultz 1995). Another technique being used to remove detritus from particulate samples is centrifugation with colloidal silica (Hamilton et al. 2005). With this technique the sample is mixed with the colloidal silica and then centrifuged. Detritus and living cells are separated based on density differences; detritus tends to be heavier.

3. Isotopic sample analysis

Isotopic analysis is based on the separation of isotopes on the basis of small but significant differences in mass (Lipschultz this volume). This can be done using an emission spectrometer or mass spectrometer. With both types of instruments the sample must be converted to a gas prior to analysis. Emission spectrometers operate based on the principle that an N_2 gas molecule comprised of a ^{15}N and ^{14}N will fluorescence at a different frequency then a molecular comprised of two ^{14}Ns or two ^{15}Ns (reviewed in Preston 1993). Isotope ratio mass spectrometers separate and quantify the different N_2 gas molecules based on their

behavior when they are accelerated through a magnetic field (reviewed in Mulvaney 1993). Emission spectrometers seem to have fallen out of favor in the quest for bigger and better instruments. They do have advantages, however. Emission spectrometers are much cheaper to purchase and maintain, they require smaller masses of N for analysis than many mass spectrometers, and there is no possibility for carry over from sample to sample because each sample is sealed in an ampoule prior to analysis.

VII. Natural abundance measurements

The stable isotope ^{15}N is useful as tracer because it has the chemical characteristics of ^{14}N – almost! There is a kinetic isotope effect that causes the rate of reaction of molecules containing ^{15}N to be slightly different than those containing ^{14}N (reviewed in Shearer and Kohl 1993). In chemical reactions that involve phase changes (i.e. evaporation) or are catalyzed by enzymes this kinetic isotope effect will result in a slight discrimination against the heavier isotope, a process known as fractionation. For example, if one were to boil a solution containing NH_3, of a known enrichment, the NH_3 that evaporated initially would have a slightly higher proportion of ^{14}N and the pool of NH_3 remaining in solution would become progressively more enriched with ^{15}N. These slight differences in ^{15}N natural abundance levels can be exploited to determine nutrient sources (e.g. Peterson 1999, Tobias et al. 2001), define trophic relationships or to decipher large-biogeochemical changes (e.g. Altabet et al. 2002). For a more thorough coverage of natural abundance methods and applications see Lajtha and Michener (1994), Fry (2006) and Montoya (this volume).

Natural abundance measurements are made against a standard, atmospheric N_2 in the case of ^{15}N measurements, and are expressed in del (δ) units:

$$\delta^{15}N = \frac{R_{sample} - R_{standard}}{R_{standard}} \text{ X } 1000‰ \quad (5)$$

R is defined in equation 4 above; data is described as the per mil ^{15}N excess (‰ ^{15}N). Prior to isotopic analysis, the various inorganic and organic N fractions must be isolated as described in the sections above.

VIII. Measuring rates of nitrogen uptake and release

A. Uptake rates

1. Substrate additions

A large suite of substrates are now available with ^{15}N label (NH_4^+, NO_3, NO_2, urea, amino acid mixtures, and individual DFAA). Many of these organic forms are also available with dual labels (^{15}N and ^{13}C) such that the flux of both C and N can be followed simultaneously. Similarly, ^{13}C-labeled sodium bicarbonate can be added to ^{15}N incubations such that an estimate of primary production and N uptake can be derived from the same sample (Slawyk et al. 1977, Dauchez et al. 1995, Legendre and Gosselin 1997).

In addition to commercially available tracers, methods exist for making a number of other labeled substrates. In the case of DON uptake studies, one option it to let the resident plankton community make the DON tracer for you by adding ^{15}N-labeled NH_4^+ or NO_3^- and then incubating (Veuger et al. 2004). During the course of the incubation the labeled substrate is incorporated and some fraction is released as $DO^{15}N$. This $DO^{15}N$ can then be isolated and added back to a new water sample to quantify DON uptake (Bronk and Glibert 1992b, Bronk et al. 2004). Labeled humic substrates can be made by growing *Spartina alterniflora*, a common marsh plant, with ^{15}N-labeled NH_4^+ in the sediment for several months (See and Bronk 2005, See et al. in press). The *Spartina* is then cut, dried, ground in a mill, and then allowed to spin in the dark in recently collected coastal seawater. The resident bacteria in the seawater "humify" the *Spartina* particles generating humic substances. These labeled humics can then be isolated from solution using macroporous resins, as described above, and then used to quantify humic-N uptake (See 2003, See et al. in press). The humics can also be dually labeled with ^{13}C by enclosing the *Spartina* plant in a bag and volatizing ^{13}C-labeled sodium bicarbonate with acid (See et al. in press).

2. Incubation conditions

Three things must be considered when incubating samples – light, temperature, and time. To measure *in situ* (in place) rates, ideally, all conditions during the incubation would the same as those where the sample was collected – or as close to it as possible. With respect to light, the light field should mimic the location and depth where the samples were collected. This can be accomplished by neutral density screens or, better yet, neutral density film (e.g. Cochlan and Bronk 2003). Temperature is also important to consider, particularly when measuring rates (think Q_{10}). If vertical profiles are done, cells deeper in the water column are generally much cooler than those at the surface and must be incubated accordingly. Temperature controlled water baths are ideal; in a pinch a cool and ice packs can do the job. One way to avoid issues of light and temperature is to do *in situ* incubations where samples are tethered on a line at the depths where they were collected (e.g. Smith et al. 2000). With respect to length of incubation time, generally speaking, the shorter the better. The shorter the incubation time, the lower the likelihood and severity of bottle effects (e.g. Venrick et al. 1977). The desire for short incubations must be balanced, however, with the need for incubations to be long enough to see measurable changes in the parameter of interest. One common exception to the shorter is better rule occurs when daily integrated rates are needed, in which case, 24 hour incubations are used.

3. Calculations

Traditionally, N uptake rates have been based on the accumulation of ^{15}N-label in cells, defined as a net uptake rake (Bronk et al. 1994), and are calculated based on the tracer principles described above with the extracellular substrate pool (e.g. NH_4^+) as the source pool and PN as the target pool:

$$\text{Uptake Rate} = \frac{\text{Atom \% (R) PN}}{\text{Atom \% (R) } NH_4^+ \text{ X Time}} \qquad (6)$$

Uptake rates can be presented as specific uptake (often designated as V), with units of per time, or absolute uptake (often designated as rho, ρ), with units of mass N per volume per time. Specific uptake rates are useful when comparing size fractions or systems that may differ substantially in biomass as they are more a measure of the physiological process of uptake.

During the course of the incubation, two processes can occur that impact uptake rates: isotope dilution and DON release. In the case of isotope dilution, unlabeled substrate is released during the course of the incubation, diluting the atom % enrichment of the source pool. In the case of NH_4^+ this occurs as a result of bacterial remineralization and zooplankton excretion (e.g. Glibert et al. 1982). Failure to account for isotope dilution in the case of NH_4^+ uptake can result in large underestimates in NH_4^+ uptake rates. In the case of NO_3^- and NO_2^- uptake, isotope dilution can occur as a result of nitrification (e.g. Ward et al. 1989). Most studies do not take into account isotope dilution of the NO_3^- pool, with some notable exceptions (Lipschultz 2001). Evidence suggests, however, that nitrification can be an important process at the base of the euphotic zone and so NO_3^- isotope dilution should be quantified (Ward et al. 1989). Methods to isolate NH_4^+ and NO_3^- for isotopic analysis are described above (also see Lipschultz this volume). Isotope dilution could also affect uptake rates of organic substrates such as urea, which can have high rates of regeneration (Bronk et al. 1998); though this correction is not commonly done due to the labor intensive methods involved. To correct uptake rates for isotope dilution an exponential average is calculated based on the initial and final atom % enrichment of the source pool (e.g. Glibert et al. 1982).

In addition to isotope dilution of the DIN pools, DON can be released during the course of the experiment. The DON pool can be isolated so that the amount of ^{15}N released to the pool can be quantified. This recently released N was taken up by the cells and so should be included in the uptake calculation. This rate is referred to as a gross uptake rate, ρ_G. The difference between gross and the traditionally measured net uptake rates results from the release of ^{15}N-label to the DON pool ^{15}N. When ^{15}N-labeled DON is released to the extra cellular DON pool, it is no longer in the pool and so is not included in the traditional

calculation of N uptake (Bronk et al. 1994, Bronk and Ward 2000). The following equation is used to calculate a gross N uptake rate (ρ_G):

$$\rho_G = \frac{(PN \times PN\ at\%xs) + (DON \times DON\ at\%xs)}{DIN\ at\%xs \times Time} \quad (7)$$

where PN and DON at%xs are the ^{15}N atom % enrichments of the PN and DON pools minus the atom % of an atmospheric N standard, DIN at%xs is the atom % excess enrichment of the dissolved inorganic nitrogen (DIN) pool, and time is the period of incubation (Bronk et al. 1998).

B. Release rates

1. NH_4^+ regeneration

Here we describe methods to measure three release rates: NH_4^+ regeneration, urea regeneration and DON release. Rates of NH_4^+ regeneration can be measured using the principle of isotope dilution. To measure regeneration a tracer addition (<10% of the ambient concentration) is added to a water sample. At the end of the incubation the NH_4^+ pool is isolated using one of the methods outlined above. The assumption is made that any NH_4^+ released during the incubation is $^{14}NH_4^+$. This will dilute the $^{14}NH_4^+$ and $^{15}NH_4^+$ present at the start of the experiment. The regeneration rate is then measured by following the flow of the unlabeled NH_4^+ form into the labeled NH_4^+ source pool (Blackburn 1978, Caperon et al. 1979). To measure rates using the principles of isotope dilution one must measure the change in isotopic composition of a pool over time and then solve the following equations:

$$P_t = P_0 + (d - u)\ t \quad (8)$$

$$\ln (R_t - R_a) = \ln (R_0 - R_a) - [d/(d - u)]\ [\ln P_f/P_0] \quad (9)$$

where P_t and P_0 are the ambient concentrations of NH_4^+ at the end and beginning of the incubation respectively, *u* is the absolute uptake rate, *d* is the regeneration rate, t is the incubation time, R_t and R_0 are the atom % enrichments of PN at the end and beginning of the incubation and R_a is the atom % of ^{15}N in the atmospheric standard.

2. Urea regeneration

Rates of urea regeneration can be calculated in an analogous fashion to that used for NH_4^+. The urea pool can be isolated using the method outlined above. The atom % of the isolated urea pool is measured at the end of the experiment and the regeneration rate is calculated using equation 9 above replacing NH_4^+ with urea. Urea uptake and regeneration can also be measured simultaneously by performing parallel incubations with comparable concentrations of ^{14}C and ^{15}N-labeled urea. The ^{14}C-labeled samples are used for the determination of isotope dilution and the rate of urea regeneration (Hansell and Goering 1989).

3. DON release

There are two general ways to calculate a DON release rate. The first is to measure the passage of ^{15}N from an intracellular organic nitrogen (ON) pool to the extracellular DON pool (Bronk and Glibert 1991, 1993a). This rate is specified the intracellular pool (IP) DON release rate to indicate that intracellular pools are used in its calculation. The isolation of the intracellular DON pool also allows the calculation of a transformation rate, which is a measure of the transformation of NH_4^+ to organic N intracellularly (Bronk 1999).

An easier less labor intensive means of measuring DON release rates was later introduced by Bronk et al. (1994) that was based solely on the change in the ^{15}N atom % enrichment of the extracellular DON pool and did not require isolation and measurement of the intracellular ON pool (Bronk et al. 1994). This DON release rate is calculated as the difference between the gross N uptake rate (ρ_G) and the traditionally determined net N uptake rate (ρ) defined above.

$$\text{DON release} = \rho_G - \rho \qquad (10)$$

The DON release rate above, referred to as the extracellular pool (EP) rate, is equivalent to the rate termed ρ_{DIN} by Slawyk et al. (1998). We suggest that the more common EP DON release rate should be taken as "the DON release rate" (Bronk et al. 1994, Bronk and Ward 2000). When the transfer of N from the intracellular to the extracellular DON pool is being measured, that rate should be specified an IP DON release rate as originally described (Bronk 1999).

Though in general the IP and EP DON release rates are similar, short-term variations in the atom % enrichment of the intracellular ON pool can cause some variations between them (Bronk 1999, Mulholland et. al. 2005). The IP DON release rate is also very labor intensive and requires a host of assumptions and therefore is less robust than the EP DON release method. The IP and EP distinctions are only necessary when both types of rates are being discussed (Bronk and Ward 2000).

There are three potential artifacts that must be considered when measuring DON release. First, is the breakage of cells during filtration (e.g. Goldman and Dennett 1985). If cells break releasing ^{15}N into the DON pool the rate of DON release will be overestimated. Second, is the potential for stressing cells during collection or incubation. Every effort should be made to maintain samples at *in situ* light and temperature conditions at all times and to avoid any additional environmental stresses. Third, underestimation of DON release can also occur due to loss of DON during the isolation process. Any method that includes heating of the sample under basic conditions has the potential to cause base hydrolysis and loss of recently released DON and thus should be carefully evaluated before use on a given set of samples (Slawyk and Raimbault 1995, Bronk and Ward 1999). Loss of DON would be expected to vary depending on the composition of the DON pool, which could explain the different DON recovery efficiencies reported using various methods. These include 91.2 to 98.9% recovery for waters off the coast of France (Slawyk and Raimbault 1995), generally less than 80% in Japanese coastal waters (Hasegawa et al. 2000), and 42.7 ± 8.8% in waters from two rivers in Georgia and the South Atlantic Bight (Bronk unpubl. data).

IX. Measuring other nitrogen transformations

A. Nitrification

Nitrification is the process that converts NH_4^+ to NO_2^- and then NO_3^- (see Ward this volume). There are four main methods for measuring nitrification – bioassays, incubation with $^{14}CO_2$ with N-serve inhibition, incubation with $^{15}NH_4^+$, and NO_3^- isotope dilution (see method comparison by Enoksson 1986). We offer a brief review here but direct the reader to Ward (this volume) for a more detailed discussion of the nitrification process and its measures.

The bioassay approach involves incubating a sample in the dark and then measuring the change in NO_2^- and NO_3^- concentrations over time after the addition of NH_4^+. This is the simplest method but it suffers from all the problems of bioassays noted in earlier sections, most notably the requirement of long incubation times and the simultaneous uptake of the recently produced NO_2^- and NO_3^- resulting in an underestimated or unmeasurable rate. Some studies have supplemented this approach with the application of inhibitors. For example, acetylene and N-serve have been used to inhibit NH_4^+ oxidation and chlorate has been used to inhibit NO_2^- oxidation (Billen 1976, Bianchi et al. 1997). If NO_2^- oxidation is inhibited by the addition of chlorate, for example, any change in NO_2^- concentration can be attributed to NH_4^+ oxidation.

One of the most sensitive ways to measure nitrification is to combine the inhibitor N-serve with ^{14}C incubation (Enoksson 1986). This approach exploits the chemolithoautotrophic nature of nitrifiers whereby they incorporate $^{14}CO_2^-$ while oxidizing N. Fixation of $^{14}CO_2^-$ is measured in the presence and absence of the N-serve (see Ward this volume for nuances of N-serve use) with the difference being attributed to nitrifiers. To get from the $^{14}CO_2^-$ fixation rate to a nitrification rate requires the application of a conversion factor, which can be problematic (e.g. Glover 1985).

In the $^{15}NH_4^+$ method, the appearance of ^{15}N label in the NO_2^- and NO_3^- pools is measured overtime (e.g. Olson 1981). The advantage of this technique is that it is a direct, unambiguous measure of the process. One disadvantage is that the increase in NH_4^+

concentration caused by the tracer addition can result in enhanced NH_4^+ oxidation rates (Helder and De Vries 1983). When small additions of NH_4^+ are used the isotope dilution of the NH_4^+ pool must be monitored over time. Another tracer approach that has been used is to quantify NO_3^- isotope dilution over time as described above. This is an analogous approach to the much more commonly applied NH_4^+ isotope dilution approach (Glibert et al. 1982).

B. Denitrification

Denitrification is the reduction of $NO_3^- \rightarrow NO_2^- \rightarrow NO \rightarrow N_2O \rightarrow N_2$ gas that is mediated by anaerobic bacteria most generally in microbial mats and sediments. There are a number of methods to measure denitrification: acetylene inhibition, isotope pairing, changes in N_2 fluxes, and changes in the N_2 to argon (Ar) ratio. Each of the techniques has their pros and cons and none is clearly superior under all conditions (see reviews by Cornwell et al. 1999 and Devol this volume).

In the acetylene inhibition technique, acetylene is added to a water sample, which inhibits the reduction of N_2O to N_2 (Sorensen 1978). The accumulation of N_2O is then measured using gas chromatography and an electron capture detector and the denitrification rate is taken to be equal to the total N_2O flux. One potential problem is incomplete inhibition of N_2O reduction to N_2 particularly in the presence of hydrogen sulfide, a compound commonly found under anaerobic conditions. Another potential problem with the technique is that acetylene also inhibits nitrification, a process that often supplies the NO_3^- and NO_2^- substrates for denitrification. To inhibit nitrification is to inhibit denitrification if it is at all substrate limited (Hynes and Knowles 1978).

The isotope pairing approach was developed to quantify what fraction of measured denitrification was fueled by substrates in the water column, versus coupled nitrification-denitrification (Nielsen 1992). In this approach $^{15}NO_3^-$ is added to water overlying sediments. The denitrification rate is calculated from the rate of formation of $^{29}N_2$ and $^{30}N_2$ (Rysgaard et al. 1993). N_2 gas formed from denitrification in the water column will incorporate $^{15}NO_3^-$ while denitrified NO_3^- from coupled nitrification-denitrification will incorporate $^{14}NO_3^-$.

The most direct approach to estimate denitrification is the direct measurement of N_2 fluxes. Concentrations of dissolved N_2 gas are very high in natural waters, however, such that any direct measurement of N_2 gas flux must be done against a huge background. One way to get around this is to incubate cores with N_2-free water to remove the high N_2 gas background. Seitzinger (1987) used this approach in long-term incubations where N_2-free water overlying the sediment was changed daily for 10 days. After the 10^{th} day, the sediment was incubated for 24 hours, and the change in N_2 gas concentration in the overlying water (measured with gas chromatography) was taken to be the denitrification rate. One potential problem with this technique is the long incubation time needed and resulting depletion of labile organic substrates (e.g. Boynton et al. 1995).

The newest approach to measuring denitrification uses a mass spectrometer that can rapidly measure the N_2/Ar ratio in water using membrane inlet mass spectrometry (MIMS, Kana et al. 1994, 1998). In this approach, sediment cores are taken and incubated under continuous flow conditions. The N_2/Ar ratio is then measured at the inlet and outlet of the core over time. Advantages of the technique are the short incubation times needed (usually less than 12 hours), and the high throughput and small sample size (~5 mls) of MIMS analysis. Multiple intercomparisons of the various methods have been done. In general the direct measure of NO_2^- gas fluxes gives higher denitrification rates than the acetylene inhibition or isotope pairing techniques (Cornwell et al. 1999).

C. N_2 fixation

In the process of N_2 fixation dissolved N_2 gas is taken up and converted to NH_4^+ and ultimately biomass. Nitrogenase, the enzyme that catalyzes the fixation of N_2, is deactivated by oxygen so care must be taken not to introduce oxygen during the measurement. There are two commonly used methods to measure N_2 fixation - $^{15}N_2$ incorporation and acetylene reduction. Using the tracer approach, $^{15}N_2$ gas is injected into a gas tight bottle containing the water sample (e.g. Montoya et al. 1996, Mulholland et al. 2005). At the end of the incubation (generally 1-12 hours) the sample is filtered and analyzed mass spectrometrically in a fashion analogous to the ^{15}N uptake samples described above. During the course of the incubation

$DO^{15}N$ release can occur such that the rate measured using the tracer approach is a net uptake rate (Mulholland et al. 2006).

The acetylene reduction approach is based on the ability of nitrogenase to reduce substrates with triple N bonds. During the measurement, acetylene gas (HC≡CH) is reduced to ethylene ($H_2C=CH_2$) in a theoretical molar ratio of 3:1 relative to N_2 gas (N≡N). To estimate N_2 fixation with this approach, a water sample is sealed in a gas-tight container and acetylene is added (Capone 1993, Montoya et al. 1996). At the end of the incubation, the concentration of acetylene and ethylene is measured using flame ionization gas chromatography. The rate of N_2 fixation is then calculated using a conversion factor to convert acetylene reduction to N_2 gas fixation. Release processes do not affect the acetylene reduction method such that the rate measured approximates a gross N_2 fixation rate.

Of the two approaches, the acetylene reduction technique is much simpler and less labor intensive. It is considerably easier to take a gas chromatograph to sea so that rate measurements can be made at a field site. The acetylene reduction method is also more sensitive and reproducible than the tracer approach, and requires smaller sample volumes. The advantage of the tracer approach, however, is that no conversion factor is needed. The use of the 3:1 conversion factor has been debated for some time. A recent study compared rates of N_2 fixation in the Gulf of Mexico over a three-year period and found that the ratio of acetylene reduction to $^{15}N_2$ fixation was seldom 3:1 (Mulholland et al. 2006). A review of the literature found that it varied from 0.9 (Capone et al. 2005) to a high of 22 (Mulholland and Bernhardt 2005). Mulholland et al. (2004) suggest that the difference between N_2 fixation rates measured with acetylene reduction (gross uptake) versus those measured with $^{15}N_2$ gas (net uptake) could be exploited to estimate N release during the incubation (i.e. gross – net, Mulholland et al. 2004).

X. Recommendations for the future

In this review we have tried to cover a broad range of topics relating to the analysis of both organic and inorganic N in the sea. Necessarily, the treatment of each has had to be relatively brief, but we hope as a whole it will provide the reader a useful reference to the

current state of the art at the beginning of 21st century. We close below with a series of notes regarding the important limitations of current methods, promising ideas for ongoing research, and new areas that are rapidly evolving.

A. Reference materials

The increased focus on building large global datasets of nutrient and organic concentrations has highlighted the wide disparity that can exist in results measured in different labs (Hopkinson et al. 1993). These discrepancies were especially problematic for the measurement of DOC (Sharp et al. 1993, 2002a) and DON (Sharp et al. 2002b). Two of the most important steps in achieving consistency between laboratories have been the development of suitable reference material (Sharp et al. 2002a) and agreement on standard procedures (e.g. Knap et al. 1997). Community standards for DOC analysis are now widely used (Hansell 2005) and standard humic substances are available from the International Humic Substances Society (http://www.ihss.gatech.edu), which can be used for chemical characterization and NMR studies. There are, however, currently no corresponding community-wide set of reference materials commonly used for most of the other analyses discussed here. A widely available set of different sample types (including sediments, plankton, or different waters) for which values would be commonly reported would be an invaluable asset for standardizing results from specific organic and other analyses. An push to identify, produce, and promote the standard use of such materials could be one of the most important of analytical efforts.

B. Improving DON isolation methods

A "holy grail" for DON (and DOM in general) remains a rapid and portable method to quantitatively isolate and desalt a large dissolved sample (Bronk 2002). Such a method would not only allow a wealth of diverse techniques to be brought to bear on the largest unknown fraction of DON, but would allow direct isotopic measurements and compound-specific mass balances. Examples of some approaches currently being explored to improve DON recovery on both small and large scales include ion-retardation resins (Bronk, unpubl. data),

electrodialysis (Ingall et al. unpubl. data), and use of nano-filtration membranes coupled to standard ultrafiltration approaches (McCarthy et al. unpubl. data).

Until new methods are perfected, however, improvements might be made by altering existing isolations, and by paying closer attention to the relationship between isolated material and total DON pool. For example, as discussed above UF isolates ~ 20-30% of DOM after desalting, the material retained by UF membranes *before* desalting is typically much larger (Guo 2000, McCarthy unpubl. data). For analyses that do not require desalting (e.g. wet hydrolysis protocols) substantial increases in the fraction of total DON recovered might be achieved by simply eliminating desalting steps, while carefully monitoring losses associated with increasing concentration factor. Confidence in any DON isolation method could also be increased by routinely examining the relationship between isolated material and the bulk DON. Careful DON mass balance should be measured for any isolated fraction, and direct comparisons should be made where possible with specific compound classes examined.

C. Hobbled by hydrolysis? Chipping away at the black box.

The initial step in molecular-level analysis of DON's most abundant organic compound classes is typically acid hydrolysis, necessary to break polymeric biomolecules into monomers for separation and detection. While the subsequent derivatization and chromatography steps can be shown to be quantitative (it is only on the basis of these post-hydrolysis steps that molecular-level methods are usually termed "quantitative" in the first place), the efficiency of the key initial hydrolysis remains largely a black box - overall efficiency is not known, extent of side reactions are not well understood, and compound-specific losses are difficult to quantify. Data from the HMW-DOM literature suggests that this hydrolysis step itself may be extremely problematic, and could account for a large part of the gap between total DON and the small fraction we can identify at the molecular-level. Comparisons between ^{15}N-NMR data for HMW-DOM, and molecular-level recoveries discussed above just do not add up. They suggest that either very large amounts of mysteriously unknown classes of nitrogenous molecules dominate detrital ON, or more likely, that while hydrolysis-based analyses can be optimized for pure compounds or fresh biological

samples, efficiency in detrital natural mixtures is far lower. Some possible explanations include secondary condensation reactions (e.g. Allard et al. 1998) or the physical environment of biopolymers(e.g. Knicker and Hatcher 1997, Nagata and Kirchman 1997, Nguyen and Harvey 2001), which might act to limit efficiency of standard aqueous methods.

Since hydrolytic cleavage will likely remain central for molecular-level analyses of the main nitrogenous biopolymers, investigating hydrolysis methodology may be among the most important, if the least glamorous, of analytical endeavors. A series of extraction and hydrolysis method comparisons has recently been published for sugars in marine samples (Panagiotopoulos and Sempere 2005). A similar systematic study specifically aimed at DON components has not been done, but would be extremely valuable. In particular, approaches designed to minimize melanoidan formation during hydrolysis (e.g. Allard et al. 1998) should be investigated, and vapor-phase hydrolysis conditions might also be re-examined. The reasons for the substantial increases in vapor-phase amino acid yields noted in some samples, but not others (e.g. Keil and Kirchman 1991b, Jørgensen and Jensen 1997) have not been resolved, but might be consistent with increased penetration of shielded or encapsulated OM. Gas-phase hydrolysis might conceivably also reduce subsequent aqueous side reactions forming melanoidan-like products.

While improvements in hydrolysis conditions may be made, devising a perfect hydrolysis is not likely, which makes understanding the extent and especially the *selectivity* of losses all the more important. If hydrolytic inefficiency or secondary loss is a main cause for low molecular-level recovery, then representativeness of recovered monomers *relative to original material* will be central to data interpretation. If loss within a compound class is non-specific, then molecular-level data, while possibly accounting directly for a modest fraction of ON, could in fact be showing us a fairly accurate compositional view of total material. The contrary situation clearly has very unpleasant implications for information that can ultimately be obtained via hydrolysis-based analyses.

D. Compound-specific isotopes: new molecular level tools for cycling and origin.

The instruments to make rapid, compound-specific isotope measurements have provided one of the most important new set of organic geochemical tools in the last 20 years for tracing OM source and process. The large majority of this work has been done with non-polar compound classes using GC coupled to isotope ratio mass spectrometry (GC-IRMS). Compound-specific isotopic analysis of amino acids, and to a lesser extent sugars, are becoming common and represent a powerful new approach to understanding source and transformation of ON in the environment. Differences between diagnostic amino acids can thus reveal a new level of information about ON including original autotrophic $\delta^{15}N$ and number of subsequent trophic transfers (McClelland and Montoya 2002), food webs of origin (Corra et al. 2005), and potentially extent of microbial heterotrophic processing (Ziegler and Fogel 2003, McCarthy et al. 2004, McCarthy et al. in review).

As continuous flow IRMS instruments become common, it is likely that these measurements will greatly extend information from standard molecular molecular % data of N biopolymers. While most early work has been done on either non-marine samples or biota, compound specific $\delta^{13}C$– (McCarthy et al. 2004) and $\delta^{15}N$–amino acid (McCarthy et al. in review) have been made in HMW-DOM. Similar GC-IRMS techniques have also been developed for sugars (Derrien et al. 2003, Macko et al. 1998), although they have not yet been explored for amino sugars or other DON components. Obtaining enough sample would be a challenge here also, however, compound-specific analyses of amino sugars might be capable of addressing source issues as well as provide an independent means to assess the relative contribution of these compounds to the total DON.

In addition to stable isotopes, since the mid-1990's molecular-level radiocarbon (^{14}C) measurement has become a frontier in compound-specific isotopic measurement (Eglinton et al. 1996). Compound-specific ^{14}C provides another independent dimension of source information, and in many cases a way to directly investigate cycling rates of compound-classes of interest (Repeta and Aluwihare 2006, Aluwihare et al. 2002, Eglinton et al. 1997, Pearson and Eglinton 2000). For the major DON component compound-class or compound-specific ^{14}C offers, for the first time, the ability to directly estimate relative cycling rates of

major DON components, independent of bulk DOC. Compound class-level isolations and ^{14}C measurement of total amino acids can be made using modifications of standard hydrolysis and cleanup protocols (Wang et al. 1998). This approach has been applied to dissolved amino acids in both surface and deep ocean (Loh et al. 2004, Voparil and McCarthy in prep.). It is likely that compound-specific ^{14}C methods for collecting and purifying individual major nitrogenous compounds would significantly improve data resolution.

E. Higher order of information: Proteomics in DON

At the other end of the molecular size spectrum, rapidly advancing mass spectrometry techniques for determining protein sequence are being coupled with advances in genomics, creating new tools to decipher information locked in DON's largest surviving biomolecules. Proteomics employs the rapidly evolving field of protein mass spectrometry to examine the range of proteins expressed by organisms (e.g. Aebersold and Mann 2003, Tyson et al. 2004, Venter et al. 2004). For study of DON these constitute new tools for understanding both the phylogeny of major groups contributing to the DON pool, as well identifying active enzymes that may be important in DON cycling. Using ultrafiltration to isolate the protein size fraction, proteomics has just begun to be applied to DON (Powell et al. 2005) and has already suggested that a broader range of dissolved protein types may exist than had been previously identified. Proteomics approaches should be highly complimentary to the growing literature using N-terminal sequence approaches discussed above (Tanoue 2000).

F. DON Geomolecules? Determining the nature of "humic" DON.

Despite the fact that the majority of accumulating DON reservoir is likely composed of relatively intact biomolecules, solid state NMR of HMW-DON also suggests an increasing contribution of unsaturated N in the mid-water and deep ocean (McCarthy et al. 1996). New methods in ultra-high resolution mass-spectrometry are making significant breakthroughs in understanding dissolved humic materials, and might be applied to provide new insight into the non-polar humic-like fraction of DON. Electrospray ionization (EI) coupled to Fourier transform cyclotron resonance mass spectrometry (FTCR MS) is a rapidly evolving ultra-high

resolution MS approach that can for the first time resolve the entire suite of individual compounds in dissolved humic materials (see Kujawinski et al. 2002 for a good overall introduction, and Marshall et al. 1988 for a full review). It has been shown that every individual compound can be resolved for natural humic and fulvic samples with mass accuracy up to 0.001D (Kujawinski et al. 2002, Stenson et al. 2003), suggesting it is possible to generate molecular formulas for all components across the range of MW falling within ocean DOM (Koch et al. 2005), and presumably to focus on compounds containing N. Drawbacks include the fact that ESI is not generally a quantitative technique, and for ocean DOM it is necessary to obtain both concentrated and desalted samples.

While perhaps not quantitative in an absolute sense, the exquisitely detailed molecular information of EI-FTIR-MS promises an important increase in our knowledge of the most murky of DOM components. The few studies that have so far been done on ocean waters use some form of solid-phase extraction with acidified samples to simultaneously achieve both ends (Koch et al. 2005, Kujawinski et al. 2004), while at the same time likely removing much of the DON (Koch et al. 2005). For investigating the humic fraction of DON, this selectivity may be an advantage. SPE isolates the smaller MW components which make up majority of ocean DOM, and also likely account for the most refractory components (Amon and Benner 1994). EI-FTIR-MS has also only begun to be exploited for dissolved organics, and its applicability specifically to the N-containing component has not yet been examined.

G. Rates - who is doing what

Currently the bulk of all N uptake rates ever measured have used some form of glass fiber filter. As a result the rates represent uptake by the autotrophic as well as a variable fraction of the bacterial community. This has broad reaching implications when one considers the use of such commonly calculated parameters as the f-ratio, the ratio of new to total (new plus regenerated) production (Eppley and Peterson 1979). The f-ratio is estimated using ^{15}N-based uptake rates of NO_3^- uptake (new production) to NO_3^- plus NH_4^+ uptake (regenerated production). Now consider that a significant fraction of the NH_4^+ uptake measured is not fueling primary producers at all but is instead being taken up by bacteria caught on the GF/F

filter. Flow cytometry, when combined with other techniques, has a great potential for expanding our understanding of competitive interactions because microbial groups can be distinguished optically, sorted, and then collected for specific analyses. Consequently, incubations can include intact communities, maintaining ecological integrity, while rate processes can be evaluated on a group-specific basis. Another advantage of using flow cytometry is that detritus, an often large but inactive component of the PC and PN pools, can be removed or sorted out of samples so as not to bias estimates of cellular C and N in the active pools.

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