

A ^{15}N tracer method for the measurement of dissolved organic nitrogen release by phytoplankton

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ABSTRACT: We present a new method for separation of dissolved organic nitrogen (DON) from dissolved inorganic nitrogen (DIN) in marine samples which permits the measurement of DO^{15}N production during DI^{15}N uptake and regeneration experiments. Ion retardation resin was used to separate DI^{15}N from DO^{15}N and the isolated DO^{15}N was subsequently analyzed by mass spectrometry. Variation in DO^{15}N atom % enrichment in duplicate samples, determined with the ion retardation column method, was less than 4%. We also separated the low molecular weight (LMW) DO^{15}N from total DO^{15}N using ultrafiltration and found the ratio of these variables to be a useful index in determining what release processes were likely occurring in a given sample. Examples are presented from 2 types of experiments conducted in Chesapeake Bay waters: first, a 6 h time-course of DO^{15}N release from both $^{15}\text{NH}_4^+$ and $^{15}\text{NO}_3^-$ uptake, and second, a series of short-term (0.5 h) release measurements from $^{15}\text{NH}_4^+$ uptake over the course of a day. In the former, total DO^{15}N release rates resulting from $^{15}\text{NH}_4^+$ uptake were several-fold higher than those resulting from $^{15}\text{NO}_3^-$ uptake due to extremely low rates of incorporation of $^{15}\text{NO}_3^-$ into cellular organic material. The release of LMW DO^{15}N resulting from $^{15}\text{NH}_4^+$ uptake decreased from 78 to 21 % of total DO^{15}N release during the 6 h incubation which suggests that processes other than direct release by phytoplankton (e.g. sloppy feeding, cell lysis) likely became more important as the incubation progressed. In the latter experiment, LMW DO^{15}N release was 0 to 16 % of total DO^{15}N release, which indicates that, in this case, also, processes other than direct release by phytoplankton were dominating the flux of DON from phytoplankton.

INTRODUCTION

Dissolved organic nitrogen (DON) comprises a large fraction of the total dissolved nitrogen (TDN) in the marine environment. DON concentrations of 4 to 7 $\mu\text{g-at. N l}^{-1}$, as determined by persulfate oxidation, are common even in oceanic waters where levels of the dissolved inorganic nitrogen (DIN) species may be below the level of detection (Sharp 1983). Recent application of high temperature combustion techniques to the analysis of TDN suggests that traditional methods of analysis may underestimate the concentration of DON by factors of 2 to 4 (Suzuki et al. 1985). Suzuki et al. (1985) found the highest concentrations of DON in surface water, and that the concentration of high molecular weight DON (> 20 000 Daltons) de-

creased rapidly with depth, which is indicative of a biologically labile pool. This new information compels us to reevaluate the traditionally held concept that DON is largely refractory and therefore relatively unimportant to microbial nutrition.

Numerous studies have investigated the uptake and release of individual components of DON such as amino acids (Wheeler & Kirchman 1986, Fuhrman 1987, Fuhrman 1990), urea (McCarthy et al. 1977, Hansell & Goering 1989), and DNA (Paul et al. 1987). Studies of individual DON compounds are useful in the analysis of small DON pools which cycle rapidly. These studies, however, focus on a very small fraction of the total DON pool and probably underestimate the importance of the DON pool as a whole. The large number of

potentially labile compounds of DON argues strongly for a more comprehensive analysis of the cycling of the DON pool. We have applied ^{15}N tracer techniques to investigate the release of DON from phytoplankton utilizing inorganic nitrogen. However, in using ^{15}N in the study of DON release there is an analytical problem in separating the DIN forms of ^{15}N tracer added from the DO^{15}N released during the course of an experiment. A method to separate DO^{15}N from DI^{15}N in freshwater systems was introduced by Axler & Reuter (1986). Interference from salt in this method prompted us to develop a method more applicable to marine studies.

Conceptual overview

The technique we describe allows us, in a single ^{15}N tracer experiment, to measure the rate of uptake of DIN and the subsequent release of DO^{15}N by phytoplankton. We are therefore only measuring DO^{15}N which is released from phytoplankton or other cells which have become labeled with ^{15}N during the course of the incubation. These pathways include DON release by phytoplankton from direct release, cell lysis, viral infection (Procter & Fuhrman 1990, Suttle et al. 1990), or sloppy feeding by heterotrophs. In addition, we assume that the source of the released DON is the intracellular pools of phytoplankton. We routinely use precombusted Whatman GF/F filters to collect the particulate material at the end of an experiment to determine the rate of DI^{15}N uptake. In the determination of DO^{15}N release rates, we collect a second aliquot of the particulate material in parallel and measure the amount of ^{15}N incorporation into intracellular DON. The measured ^{15}N atom % enrichment of the phytoplankton intracellular DON pool reflects the current nutritional status of the phytoplankton community (Glibert & McCarthy 1984) as well as its preexposure history. It is important to underscore the fact that this method traces the release of DON that was derived from uptake processes; it does not measure total DON flux.

Some bacteria are also collected on the GF/F filters we use, and bacteria have been shown to compete with phytoplankton for the uptake of NH_4^+ . Bacteria, however, do not possess large intracellular DON pools as do many phytoplankton species. Most of the inorganic nitrogen which is taken up by bacteria is rapidly shunted into structural material which would be precipitated out during our protocol for extracting intracellular DON.

Low molecular weight (LMW; < 10 000 Daltons) DON release rates can be determined by combining ultrafiltration with the ion retardation column method we describe. Measuring both LMW and total DON

release gives insight into different mechanisms of release. For example, if LMW DON release represents a large percentage of total DON release, the dominant mechanism is probably direct release by phytoplankton as exudates from actively growing phytoplankton are often dominated by LMW compounds (Mague et al. 1980).

With the protocols we outline, the method does not measure other sources of DON release such as zooplankton excretion. When such processes occur, the ^{15}N label in the extracellular DON becomes isotopically diluted, and the result is an underestimate of DON release. Note that tracing the release of DON from an ^{15}N -labeled organic form of nitrogen, such as urea, is possible, but more problematic as separation of the added ^{15}N -labeled urea from the DON pool would be required.

MATERIALS AND METHODS

Incubation and filtration. Experiments were performed on unfiltered water, in polycarbonate bottles, incubated with $^{15}\text{NH}_4^+$ or $^{15}\text{NO}_3^-$, generally added at a concentration of 10 % of the ambient nutrient concentration. At the end of the incubation period (0.5 to 6 h) the sample was split into 3 subsamples and gently vacuum-filtered (< 50 mm Hg). One subsample was filtered through a 0.2 μm Nuclepore filter and the filtrate was retained (Fig. 1; discussed below). The second subsample was filtered through a precombusted (500 $^\circ\text{C}$, 2 h) Whatman GF/F filter and was used to determine the ^{15}N atom % enrichment of the intracellular DON pool as outlined below. The final subsample was filtered through a precombusted GF/F filter, dried, and sealed in an ampoule for mass spectrometric analysis to determine the DIN uptake rate (Table 1). The GF/F filtrate was used to measure the isotope dilution in NH_4^+ uptake experiments and the concentrations of DON, NO_3^- , NH_4^+ , and total dissolved primary amine (DPA) were measured (Table 1).

Nutrient samples (DON , NO_3^- , and NH_4^+) and filtrates were stored in acid-washed polyethylene bottles. Total DPA samples were filtered through 0.2 μm Nuclepore filters into muffled (500 $^\circ\text{C}$ for 1 h) glass scintillation vials with acid washed solid polyethylene caps. A glycine standard was used in the DPA measurements and the DPA values were corrected for fluorescence due to NH_4^+ (Table 1). All DPA concentrations are presented as glycine equivalents.

DON isolation. Filtrate from the 0.2 μm Nuclepore filter was used in the DON isolation. We used 0.2 μm filtrate instead of the GF/F filtrate to prevent any ^{15}N which may have been taken up by bacteria from appearing in the DON fraction. The concentrations of

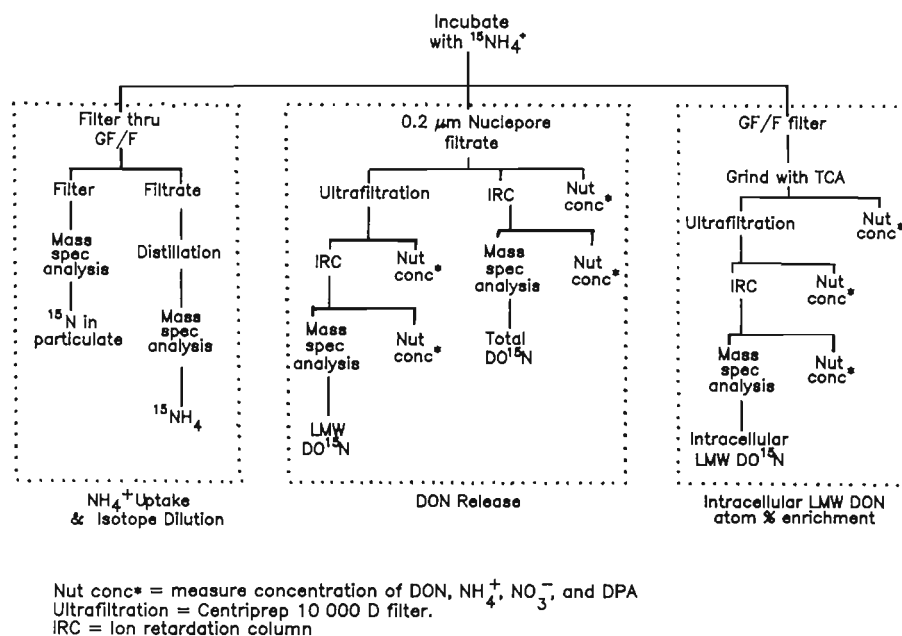


Fig. 1. Flow diagram of the protocol used to determine $^{15}\text{NH}_4^+$ uptake and isotope dilution, the atom % enrichment of the total dissolved organic nitrogen (DON) and low molecular weight (LMW) DON pool, and the atom % enrichment of the intracellular LMW DON pool with the ion retardation column method

DON, NO_3^- , NH_4^+ , and DPA were determined for the Nuclepore filtrate (Table 1), and the remaining Nuclepore filtrate was separated into 2 subsamples used to determine the release of total DON and LMW DON as described below (Fig. 1). The filtrate may be frozen at this point for later analysis.

A subsample (9 ml) of 0.2 μm Nuclepore filtrate was passed through an ion retardation column to remove inorganic ions. The ion retardation column (BioRad #737-2520; 2.5 cm \times 20 cm) contained 40 g of resin (BioRad #142-7834; AG 11 A8, 50-100 mesh size) which retards the passage of charged ions. A needle

(B-D Precision Glide 22G1) was attached to the bottom of each column to allow a slow and constant flow rate (about 2.5 ml min^{-1}). To force the sample through the resin, sufficient distilled water (DW) was passed through the resin to collect the desired amount of eluate.

When samples from $^{15}\text{NH}_4^+$ incubations were processed, a total of 40 ml of eluate was collected. When $^{15}\text{NO}_3^-$ samples were processed, only 30 ml of eluate were collected (discussed below). When the concentration of DON in the sample was low ($< 25 \mu\text{g-at. N l}^{-1}$) we combined the eluates from up to 3 columns for mass

Table 1. Methods used for nutrient, biomass, and rate measurements

Measurement	Technique	Source
NH_4^+	Phenol hypochlorite	Solarzano (1969)
NO_3^- , NO_2^- , urea	Colorimetric analysis	Parsons et al. (1984)
DON	Persulfate oxidation	Valderrama (1981)
DPA	Fluorometric <i>o</i> -phthaldehyde derivatization	Parsons et al. (1984)
DFAA	Reverse-phase HPLC	Lindroth & Mopper (1979)
DCAA	Acid hydrolysis and reverse-phase HPLC	Keil & Kirchman (1991)
NH_4^+ uptake and release	^{15}N isotope tracer and isotope dilution	Glibert et al. (1982)
NO_3^- uptake	^{15}N isotope tracer	Dugdale & Goering (1967)
Chl <i>a</i>	Fluorometric analysis	Parsons et al. (1984)
Particulate N	Elemental analyzer	

spectrometric analysis.

The column eluate was collected in a 125 ml acid-washed Erlenmeyer flask (Flask 1). The eluate was basic when it left the column and was neutralized to a pH of 7 to 8 with 1M HCl. A 20 ml aliquot of the eluate from Flask 1 was then placed into a 50 ml Erlenmeyer flask (Flask 2) and weighed. The eluates in both flasks were boiled down to < 2 ml. After boiling, the solution in Flask 1 was spotted onto a precombusted GF/F filter, dried, and sealed in an ampoule under vacuum for mass spectrometric analysis (Fiedler & Proksch 1975). DW was added to Flask 2, to reconstitute the solution to its original weight, and the concentrations of DON, NO_3^- , NH_4^+ , and DPA were measured (Table 1). The DON concentration in the eluate from Flask 2 was used in the release rate calculations and to determine whether carrier nitrogen must be added to the sample after boiling to provide sufficient mass for mass spectrometric analysis (1 $\mu\text{g-at. N}$).

The ion retardation resin was regenerated after each sample by passing 20 ml of 1M NaOH, then 500 ml of DW, through the column. The 1M NaOH was passed through the column while the needle was attached to maintain a slow flow rate. The needle was removed before the DW was added to produce a better flushing of the resin. To collect column blanks, 9 ml of 1M NaOH were added to the column and 40 ml of eluate were collected. We are presently experimenting with another regeneration protocol using hydrochloric acid, sodium hydroxide, and sodium chloride in an attempt to generate a more neutral environment within the column and to prevent the need for the eluate neutralization step prior to boiling.

LMW DON separation. To separate LMW DON from total DON we used a Centriprep ultrafilter (Amicon #4305; 10 000 Daltons cutoff). Although the Centriprep ultrafilter had a molecular weight cutoff of 10 000 Daltons, in practice it retained much smaller molecules. A subsample (24 ml) of 0.2 μm Nuclepore filtrate was passed through a Centriprep ultrafilter (Fig. 1). A fraction (15 ml) of the ultrafiltrate was removed and the concentrations of DON, NO_3^- , NH_4^+ , and DPA were measured (Table 1). The remainder of the Centriprep ultrafiltrate was passed through an ion retardation column following the protocol outlined for total DON.

Analysis of intracellular DON pool. The intracellular DON pool was isolated by grinding one of the GF/F filters (Fig. 1) with 0.37M trichloroacetic acid (TCA) in a tissue homogenizing tube, as outlined in Clayton et al. (1988). The resulting homogenate was neutralized, passed through a Centriprep ultrafilter, ion retardation resin, and finally sealed in an ampoule under vacuum for mass spectrometric analysis as outlined above. Intracellular pool filters were sealed in Vycor ampoules

and CaO was added to remove CO_2 and H_2O produced during ampoule combustion (Kendall & Grim 1990).

Mass spectrometric analysis. A Nuclide 3" 60° Sector analyzer with dual mass collection was used to analyze all samples for ^{15}N analysis. Tank N_2 gas (UN 1066) was used as the atmospheric nitrogen standard, and standards were run approximately every 7 samples. Precision of analyses in the range of < 1 to 10 atom % enrichment was better than ± 0.001 atom % (Glibert et al. 1991).

Calculation of rates. Before a DON release rate could be determined, the ^{15}N atom % enrichment of both the extracellular and intracellular DON was calculated. These values must be corrected for the small amount of NH_4^+ or NO_3^- that was not removed by the resin, and for the addition of any carrier nitrogen added to the eluate, according to the following equation:

DON atom % enrichment =

$$\frac{(\sum \text{mass})(\text{at}\% \text{ A}) - \text{B}(\text{at}\% \text{ B}) - \text{C}(\text{at}\% \text{ C}) - \text{D}(\text{at}\% \text{ D})}{\text{mass DON}} \quad (1)$$

where at% A was the measured ^{15}N atom % enrichment of the column eluate; and at% B, C, and D were the ^{15}N atom % enrichment of the NH_4^+ pool (see below), the NO_3^- pool, and the N_2 standard (i.e. atom % normal as measured on our mass spectrometer) respectively. B and C represent the masses of NH_4^+ and NO_3^- respectively in the column eluate; D was the mass of the carrier addition; and $\sum \text{mass}$ was the total mass of nitrogen in the final sample which equals $\text{B} + \text{C} + \text{D} + \text{mass of DON}$ as measured analytically.

In addition, the calculation of intracellular DO^{15}N enrichment is affected by the common problem in ^{15}N tracer studies of distinguishing between phytoplankton and heterotrophic and/or detrital nitrogen. If we assume that all the extractable intracellular DON is attributable to phytoplankton we overestimate the concentration of phytoplankton intracellular DON, and in turn, underestimate the enrichment of ^{15}N in this pool. A rough approximation of the heterotrophic contribution to this pool can be obtained from the particulate nitrogen (PN):chlorophyll *a* (chl) ratio. A PN:chl ratio of ca 1 indicates the sample is composed of living phytoplankton. Ratios > 1 suggest a greater proportion of heterotrophic and/or detrital nitrogen in the sample (McCarthy & Nevins 1986).

The ^{15}N atom % enrichment of the unlabeled NH_4^+ or NO_3^- pool was assumed to be the ^{15}N atom % enrichment of the N_2 standard. In $^{15}\text{NH}_4^+$ uptake experiments the ^{15}N atom % enrichment of the $^{15}\text{NH}_4^+$ pool was measured on the GF/F filtrate (Table 1). In experiments with $^{15}\text{NO}_3^-$, the $^{15}\text{NO}_3^-$ atom % enrichment was calculated.

To calculate DON release rates, we used equations analogous to the equations used by Dugdale & Goering (1967) to calculate DIN uptake rates. The pool representing the source of ^{15}N label in our experiments was assumed to be the phytoplankton intracellular LMW DON pool (Mague et al. 1980), and the pool representing the fraction ultimately enriched with ^{15}N was the extracellular DON pool (either total or LMW). Thus:

DON release rate =

$$\frac{\text{atom \% excess extracellular DON pool}}{\text{atom \% excess intracellular LMW DON pool} \times \text{duration of incubation}} \times [\text{DON}] \quad (2)$$

where atom % excess of the extracellular and intracellular DO^{15}N pool was the ^{15}N atom % enrichment of the DON pool (calculated from Eq. 1) minus the ^{15}N atom % enrichment of the N_2 standard and $[\text{DON}]$ was the concentration of DON (total or LMW) within the incubation bottle at the end of the incubation. DON release was expressed in units of $\mu\text{g-at. N l}^{-1} \text{ h}^{-1}$.

Experimental protocol for testing selective retention and efficiency of ion retardation columns. For our method to result in an accurate measurement of DO^{15}N release, the ion retardation column must have a very high removal efficiency for inorganic ions, and must not selectively remove DON compounds at the same time. We present data below to demonstrate that these conditions were met.

Experiments were performed to test: the selective retention of DON components; the retention efficiency of the resin for inorganic ions; the possibility of carry-over of ^{15}N between samples due to inadequate resin regeneration; the loss of organic nitrogen during boiling; bacterial contamination of the resin; and the variability inherent in the ion retardation column method.

Table 2. Percent recovery of total DON, total DPA, and urea in 30 and 40 ml of eluate after the sample was passed through the ion retardation column. Data are from 3 experiments which used distilled water enriched with $4 \mu\text{g-at. N l}^{-1}$ urea, Choptank River water enriched with urea (3.8, 38, 11, 8, and $0.23 \mu\text{g-at. N l}^{-1}$ for NH_4^+ , NO_3^- , DON, urea, and DPA respectively), and Sargasso Sea water enriched with *Skeletonema* culture and AMP (5, 15, 10, 10, and $3.5 \mu\text{g-at. N l}^{-1}$ for NH_4^+ , NO_2^- , AMP, DON, and DPA respectively). Values are means \pm SD

	30 ml	n	40 ml	n
Total DON	51 \pm 31	6	97 \pm 14	10
Total DPA	64 \pm 21	8	89 \pm 18	9
Urea	57 \pm 20	8	95 \pm 15	8

A mixture of DON was needed to rigorously test for selective retention of DON within the columns. Senescent phytoplankton cultures contain a large number of DON compounds (Hellebust 1974). A *Skeletonema* culture obtained from the Center for the Culture of Marine Phytoplankton (Bigelow Laboratory, USA) was grown on f/2 + 2 Si media (Guillard 1975). Several days after NO_3^- depletion, the culture was filtered through a $0.2 \mu\text{m}$ Nuclepore filter and the filtrate was frozen.

To test DON retention efficiency, experiments were performed with DW enriched with urea, Choptank River water enriched with urea, and Sargasso Sea water enriched with senescent *Skeletonema* culture filtrate, adenosine monophosphate (AMP), and urea (enrichments shown in Table 2). Samples were filtered ($0.2 \mu\text{m}$ Nuclepore), passed through ion retardation column resin, and the concentrations of total DON, urea, and DPA were determined in the initial sample and in 30 and 40 ml samples of column eluate.

In addition, to test for retention of dissolved free amino acids (DFAA) or dissolved combined amino acids (DCAA) the above experiment was repeated and samples were analyzed using high pressure liquid chromatography (HPLC; Table 1). A senescent *Skeletonema* culture was used to examine DFAA retention and Choptank River water was used to examine DCAA retention.

To test the retention efficiency of the resin for DIN, the concentrations of inorganic nitrogen ions (NH_4^+ , NO_3^- , and NO_2^-) were measured before the sample was passed through the resin and in the column eluate. Autoclaved, GF/F filtered Sargasso Sea water enriched with DIN (concentrations shown in Fig. 2) was used in all experiments because its high salinity provided ions which would compete with the DIN species for active sites on the resin. Additional experiments with the same protocol were performed with DW to which 5 and $20 \mu\text{g-at. N l}^{-1} \text{ NO}_2^-$ were added.

To test for carry-over of label between samples, $^{15}\text{NH}_4^+$ and $^{15}\text{NO}_3^-$ solutions ($2.4 \mu\text{g-at. N l}^{-1}$, 9.7 atom % enrichment) were passed through the resin, the columns were regenerated, and column blanks were taken. The blank eluate was neutralized, boiled down, spotted onto a precombusted GF/F filter, and processed for mass spectrometric analysis (as outlined above). The atom % enrichment of the blank eluate was compared to the atom % enrichment of the initial sample.

Experiments were also performed to determine the optimal eluate pH during boiling to maximize loss of NH_4^+ ions and minimize loss of DON components. Senescent *Skeletonema* culture was enriched with NH_4^+ , NO_2^- , AMP (concentrations shown in Table 5), divided into 120 ml subsamples, and then poured into 250 ml Erlenmeyer flasks. The pH of the flasks was ad-

justed to 7 to 10 and the flasks were weighed. Samples were boiled down to < 2 ml, DW was added to reconstitute it to its original weight, and the concentrations of DON, NH_4^+ , NO_3^- , urea, and DPA were determined and compared to the concentrations in unboiled samples. A second experiment was performed, using the same protocol, with unenriched Choptank River water to test the boiling effects on a natural water sample (concentrations shown in Table 5).

To test for bacterial contamination within the columns, acridine orange direct counts (Hobbie et al. 1977) were performed on column eluate from columns which remained unused for several weeks and on ion retardation resin which was vortexed with a surface reducing agent (0.05M sodium pyrophosphate) to dislodge any attached bacteria.

Samples were not routinely run in duplicate because of the large amount of time required in sample processing. To test the variability of the ion retardation column method, 3 experiments were performed in Chesapeake Bay and one in the Choptank River. In all experiments, water, collected from the surface using a plastic bucket, was incubated for 0.5 h with $5 \mu\text{g-at. N l}^{-1}$ $^{15}\text{NH}_4^+$, filtered, and passed through an ion retardation column. The eluates from 2 columns were kept separate and the ^{15}N atom % enrichments of each eluate were measured.

Field experiments. A 6 h time-course of NH_4^+ and NO_3^- uptake and DON release was performed at a site in the north Chesapeake Bay on May 22, 1990, aboard the RV 'Ridgely Warfield'. The experiment was performed during the decline of the spring bloom to maxi-

mize the chance of observing substantial DON release. Surface water was collected with a plastic bucket at 09:00 h, poured into clean polycarbonate bottles (2.7 l), and ca 10 % additions of $^{15}\text{NH}_4^+$ and $^{15}\text{NO}_3^-$ were made to separate bottles. The samples were incubated in on-deck incubators under 2 layers of neutral density screen. The concentrations of DON, NO_3^- , and NH_4^+ , as well as the rates of NH_4^+ and NO_3^- uptake and total and LMW DON release resulting from both NH_4^+ and NO_3^- uptake, were measured at ca 0.5, 1, 3, and 6 h after the ^{15}N additions (Table 1). In the second example, multiple short-term measurements of total and LMW DON release were performed. Water was collected at dawn from the Choptank River (34 km from the mouth) on August 14, 1990 with a plastic bucket. The water was prefiltered through a 202 μm Nitex mesh into a 20 l carboy. The carboy was incubated under in situ light and temperature conditions for 30 h. Samples were taken at ca 1, 3, 6, 12, 24, and 30 h. At each sampling point, NH_4^+ uptake and total and LMW DON release resulting from NH_4^+ uptake were measured from individual short-term experiments, each lasting 0.5 h (Table 1).

RESULTS

Efficiency and selective retention of ion retardation columns

The initial eluate from the ion retardation column contains the majority of the DON but as more sample is

Table 3. Mass of DFAA in a senescent *Skeletonema* culture and in ion retardation column eluate after the culture medium was passed through the resin as measured with HPLC. Percent recovery, (mass of DFAA in column eluate)/(mass of DFAA in initial culture), was calculated for both 30 ml (n = 2) and 40 ml (n = 2) of eluate collected. Values are means \pm SD

Amino acid	Mass in initial culture ^a (nmol)	Mass in 30 ml eluate (nmol)	% Recovery	Mass in 40 ml eluate (nmol)	% Recovery
Asp	0.092 \pm 0.020	0.053 \pm 0.030	58	0.108 \pm 0.015	118
Glu	0.643 \pm 0.069	0.221 \pm 0.267	34	0.427 \pm 0.134	66
Asn	0.038 \pm 0.010	0.019 \pm 0.011	50	0.021 \pm 0.006	56
Ser/Gln	0.748 \pm 0.172	0.376 \pm 0.161	50	0.503 \pm 0.080	67
His	0.203 \pm 0.120	0.133 \pm 0.075	66	0.208 \pm 0.037	102
Gly/Thr	1.211 \pm 0.223	0.830 \pm 0.143	69	1.161 \pm 0.072	96
Ala/Arg	0.712 \pm 0.094	0.521 \pm 0.108	73	0.655 \pm 0.054	93
Tyr	0.076 \pm 0.003	0.030 \pm 0.007	39	0.036 \pm 0.003	48
Met/Val	0.359 \pm 0.024	0.241 \pm 0.093	67	0.296 \pm 0.046	83
Try	0.120 \pm 0.006	0.021 \pm 0.002	18	0.081 \pm 0.001	68
Phe	0.170 \pm 0.014	0.068 \pm 0.082	40	0.113 \pm 0.041	66
Ile	0.372 \pm 0.044	0.289 \pm 0.164	78	0.341 \pm 0.082	92
Leu	0.518 \pm 0.031	0.360 \pm 0.092	69	0.451 \pm 0.046	87
Mean % recovery			55 \pm 18		80 \pm 20
^a Mass of amino acid nitrogen in the initial sample of 9 ml of culture					

Table 4. Mass of individual DCAA in Choptank River water and in 40 ml of ion retardation column eluate after the river water was passed through the resin, as measured using HPLC. Percent recovery was the mass of the DCAA in the column eluate divided by the mass of the DCAA in the initial river water sample. Each DCAA analysis was run in duplicate ($n = 2$). Values are means \pm SD

Amino acid	Mass in initial river water ^a (nmol)	Mass in 40 ml eluate (nmol)	% Recovery
Asp	0.015 \pm 0.003	0.015 \pm 0.003	100
Glu	0.057 \pm 0.001	0.047 \pm 0.010	82
Ser	0.266 \pm 0.136	0.216 \pm 0.098	81
His	0.093 \pm 0.049	0.086 \pm 0.037	93
Gly/Thr	0.567 \pm 0.044	0.405 \pm 0.198	71
Ala/Arg	0.481 \pm 0.014	0.305 \pm 0.130	63
Tyr	0.095 \pm 0.016	0.059 \pm 0.009	62
Met/Val	0.094 \pm 0.054	0.062 \pm 0.029	66
Phe	0.028 \pm 0.007	0.021 \pm 0.008	75
Ile	0.046 \pm 0.007	0.034 \pm 0.009	74
Leu	0.093 \pm 0.022	0.056 \pm 0.007	60
Mean % recovery			75 \pm 13

^a Mass of amino acid nitrogen in the initial sample of 9 ml of Choptank River water

added, the DIN ions dissociate and begin to appear in the eluate. The dissociation occurs because no bond is formed between the ion and the resin; the ions are merely attracted to the resin and thus slowed down. The volume retained as sample was chosen to maximize the amount of DON collected while minimizing the amount of DIN ions in the sample. A quantitative collection of all the organic fraction is not necessary for mass spectrometric analysis. The eluate which is collected, however, must be representative of the DON pool as a whole.

We found DON recovery increased with increasing eluate volume collected up to 40 ml. Of the DON components measured, 51 to 64 % were recovered when 30 ml of eluate were collected, and 89 to 97 % when 40 ml of eluate were collected (Table 2). Recovery of DFCAA averaged 55 ± 18 % when 30 ml of eluate were collected and 80 ± 20 % when 40 ml of eluate were collected (Table 3). No apparent differences in recovery were seen between groups of amino acids when they were separated according to their chemical characteristics (uncharged, charged, acidic, basic). We believe the reduced recovery and variability associated with some amino acids were due to the very low concentrations involved and not to selective retention by the resin. We found no significant retention of DCAA within the columns (Table 4). The mean % recovery was 75 ± 13 % when 40 ml of eluate was collected; which was slightly lower but still within the range observed for other DON components (Tables 2 & 3).

The percent retention of DIN in Sargasso Sea water enriched with inorganic nitrogen was described by a rectangular hyperbola when plotted against nutrient concentration (Fig. 2). At concentrations lower than $5 \mu\text{g-at. N l}^{-1}$, retention efficiency decreased and variability in retention efficiency increased, in part due to greater variability associated with substrate measurements at low concentrations. In addition, we found retention of inorganic ions decreased as the amount of eluate collected increased (Fig. 2A, B). Retention of NO_2^- in DW was 100 % when a solution of 5 or $20 \mu\text{g-at. N l}^{-1} \text{NO}_2^-$ was passed through the resin.

A pH range of 7 to 8 was chosen for evaporating subsequent column eluates as loss of urea, DPA, and total DON was lowest and more consistent within this range (Table 5). In the case of NH_4^+ , when the eluate pH was adjusted to 7 or higher prior to boiling, > 97 % of the

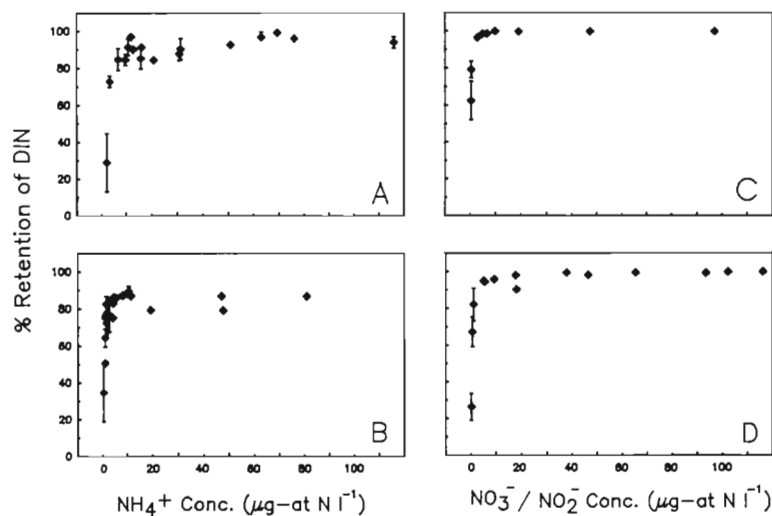


Fig. 2. Percent retention of the ion retardation resin for dissolved inorganic nitrogen ions as a function of ion concentration. (A) NH_4^+ retention in 30 ml of eluate, (B) NH_4^+ retention in 40 ml of eluate, (C) NO_3^- retention in 30 ml eluate, (D) NO_2^- retention in 30 ml eluate

Table 5. Effect of pH on the % recovery of nitrogen when the column eluate was boiled. Data are from 2 experiments which used Sargasso Sea water enriched with senescent culture and AMP (5, 15, 10, 10, and 3.5 $\mu\text{g-at. N l}^{-1}$ for NH_4^+ , NO_2^- , AMP, DON, and DPA respectively) and Choptank River water (3.8, 38, 11, 4, and 0.23 $\mu\text{g-at. N l}^{-1}$ for NH_4^+ , NO_3^- , DON, urea, and DPA respectively). Values are means \pm SD

pH	% Recovered				
	NH_4^+	$\text{NO}_3^-/\text{NO}_2^-$	Urea	DPA	Total DON
7.0	2.0 ± 1.9	101.0 ± 2.0	85.8 ± 4.0	100.0 ± 6.9	97.6 ± 4.0
7.5	2.8 ± 2.3	99.0 ± 1.5	93.4 ± 6.2	96.7 ± 3.1	96.2 ± 10.9
8.0	1.3 ± 1.4	98.4 ± 2.6	91.4 ± 6.3	88.8 ± 1.6	99.8 ± 8.7
8.5	3.3 ± 2.3	100.7 ± 3.1	— ^a	86.4 ± 10.5	76.7 ± 16.4
9.0	3.7 ± 1.5	103.2 ± 2.4	— ^a	74.6 ± 9.6	76.2 ± 17.2

^aNot measured

NH_4^+ which was not removed by the resin was lost during the subsequent boiling of the eluate (Table 5). Thus, when the ion retardation column method was applied to a sample there was minimal contribution of $^{15}\text{NH}_4^+$ to the DO^{15}N analysis.

Boiling does not affect the concentrations of NO_3^- and NO_2^- (Table 5), and no additional means of removing the ions which appear in the eluate were pursued. Instead, smaller volumes (30 ml) of eluate were collected when samples from $^{15}\text{NO}_3^-$ or $^{15}\text{NO}_2^-$ incubations were processed, to ensure the minimal amount of DIN in the final sample (Fig. 2). Also, in the case of NO_3^- and NO_2^- the retention efficiency of the resin was higher so the amount of NO_3^- and NO_2^- which appeared in the final sample was smaller. No significant carry-over of ^{15}N label from one sample to the next was found. The regeneration protocol outlined above was adequate to achieve NH_4^+ and NO_2^- blanks of $0.06 \pm 0.05 \mu\text{g-at. N l}^{-1}$ (mean \pm SD; $n = 9$) and $0.03 \pm 0.01 \mu\text{g-at. N l}^{-1}$ ($n = 6$), respectively, after $50 \mu\text{g-at. N l}^{-1}$ NH_4^+ and $50 \mu\text{g-at. N l}^{-1}$ NO_2^- were passed through the resin. The behavior of NO_3^- within the column was the same as that of NO_2^- . The ratio of the ^{15}N atom % enrichment in the blank eluate to the ^{15}N atom % enrichment of the initial sample had a mean of $0.09 \pm 0.04 \%$ ($n = 6$) for $^{15}\text{NO}_3^-$ experiments and $0.06 \pm 0.05 \%$ ($n = 5$) for $^{15}\text{NH}_4^+$ experiments.

Bacteria, free or attached, were not observed in eluates from columns which had been unused for several weeks.

The variability in DO^{15}N atom % enrichment found when duplicate samples were processed with the ion retardation column method is shown in Table 6. The variability between replicate samples was between 0.8 and 3.6 % of the original atom % enrichment.

6 h time-course of DON release

The DIN concentrations in the northern Chesapeake Bay are extremely high during spring: in our May 1990

experiment, NO_3^- concentrations exceeded $70 \mu\text{g-at. N l}^{-1}$ and NH_4^+ concentrations exceeded $6 \mu\text{g-at. N l}^{-1}$ in surface waters. No significant decrease in the concentration of NO_3^- was observed during the 6 h incubation, but there was a $1.36 \mu\text{g-at. N l}^{-1}$ decrease in NH_4^+ concentration over the same interval.

The rate of NH_4^+ uptake did not vary significantly during the experiment (Fig. 3A). The rate of NO_3^- uptake, however, was almost 5 times higher than NH_4^+ uptake rate during the first 0.5 h of incubation, but then decreased in near exponential fashion throughout the incubation (Fig. 3A). Of the $^{15}\text{NO}_3^-$ taken up during the time course, only $4 \pm 2 \%$ was assimilated into LMW DON. In sharp contrast, up to 84 % of the $^{15}\text{NH}_4^+$ taken up was assimilated into LMW DON in 6 h. Despite the initial disparity in uptake rates in the experiment, no preference for NO_3^- was observed as evidenced by relative preference indexes (RPI) of < 1 (McCarthy et al. 1977). The RPIs for NH_4^+ were > 1 at all time points and increased throughout the experiment, reaching 7 at 6 h.

Rates of total DO^{15}N release resulting from $^{15}\text{NH}_4^+$ uptake were significantly higher than release rates resulting from $^{15}\text{NO}_3^-$ uptake (Fig. 3). In fact, rates of total DO^{15}N release resulting from $^{15}\text{NH}_4^+$ uptake were found to exceed the uptake rates in incubations lasting longer than 1 h.

The rate of release of LMW DO^{15}N resulting from $^{15}\text{NH}_4^+$ uptake displayed little variability during the

Table 6. Variation in DO^{15}N atom % enrichment in duplicate samples processed using the ion retardation column method

Sample description	DO^{15}N atom %	
	1	2
Chesapeake Bay:		
Mesohaline-April	0.432	0.448
Mesohaline-June	0.560	0.549
Oligohaline-August	0.726	0.720
Choptank River-June	0.475	0.470

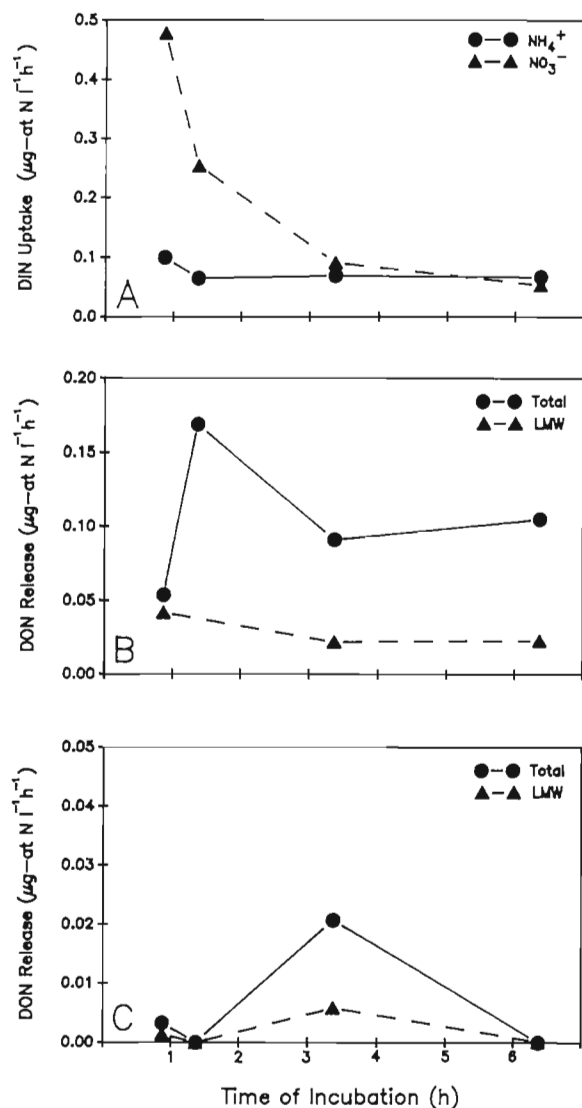


Fig. 3. Uptake rates of $^{15}\text{NO}_3^-$ and $^{15}\text{NH}_4^+$ (panel A), and total and LMW DO^{15}N release resulting from $^{15}\text{NH}_4^+$ uptake (panel B), and $^{15}\text{NO}_3^-$ uptake (panel C) during a 6 h time-course conducted in north Chesapeake Bay on May 22, 1990

6 h. However, LMW DO^{15}N release as a percentage of total DO^{15}N release decreased from 78 to 21 % during the 6 h incubation (Fig. 3B).

Multiple short-term measurements of DON release

In the large carboy experiment conducted with Choptank River water in August 1990 the $6.86 \mu\text{g-at. N l}^{-1}$ of NH_4^+ present at 1 h was completely consumed by 30 h. There was only a slight increase ($0.53 \mu\text{g-at. N l}^{-1}$) in the DON concentration during the same time period. The C:N ratios were approximately constant during the experiment (5.0 ± 0.1).

Table 7. $^{15}\text{NH}_4^+$ uptake and total and LMW DO^{15}N release rates measured in a large carboy of Choptank River contained for 30 h. At each time point, samples were removed and individual 0.5 h incubations were performed. All rates are expressed as $\mu\text{g-at. N l}^{-1}\text{h}^{-1}$

Time of sampling (h)	$^{15}\text{NH}_4^+$ uptake	Total DO^{15}N release	LMW DO^{15}N release
1	0.521	0.052	0.004
3	0.367	0.074	0.000
6	0.437	0.144	0.002
12	0.416	0.124	0.020
24	— ^a	0.138	0.005
30	0.605	0.199	0.029

^aNot measured

Throughout the 30 h period, no direct relationship was observed between the rates of release of LMW and total DO^{15}N (Table 7). LMW DO^{15}N release rates ranged from 0 to 16 % of total DO^{15}N release with no obvious pattern. The ratio of LMW DO^{15}N release to $^{15}\text{NH}_4^+$ uptake was only 2 ± 2 %, and the ratio of total DO^{15}N release to NH_4^+ uptake was 25 ± 10 %

DISCUSSION

Considerations in measuring DON release

Release rates of DON are inherently difficult to measure for at least 4 reasons: phytoplankton cells are easily stressed during experimental manipulation; simultaneous uptake of DON occurs; DON release can result from a large number of different processes; and the methods for determining DON concentrations are controversial. In our experiments we cannot discount the possibility that cell stress and/or breakage occurred during handling and filtration. We attempted to minimize cell stress by using Nuclepore filters, shown by Paerl & MacKenzie (1977) to minimize cell breakage, with low vacuum pressures ($< 50 \text{ mm Hg}$), and incubating all samples under as close to in situ conditions as possible. However, since even gravity filtration can cause the release of dissolved free amines (Kirchman et al. 1989) some artifactual release may have occurred.

Simultaneous uptake of released DO^{15}N occurs on the time scales of our experiments and affects the estimates of DO^{15}N release. Uptake of released DO^{15}N by organisms $> 0.2 \mu\text{m}$ would lead to an underestimate of the calculated rate of DON release, as the ^{15}N incorporated by these organisms would be removed by the $0.2 \mu\text{m}$ filtration. Uptake of released DO^{15}N by organisms

< 0.2 μm , however, would not affect the calculated DO^{15}N release rate. Since we are operationally defining DON as the 0.2 μm filtrate, the ^{15}N contained in these very small organisms would be assayed along with the DO^{15}N .

Uptake of DI^{15}N by organisms < 0.2 μm may also impact our estimates of DON release. Our method is based on the premise that DI^{15}N is incorporated into phytoplankton and released as DO^{15}N via direct or indirect pathways. Uptake of NH_4^+ , however, can also occur by bacteria and other small cells passing the 0.2 μm filter (Li 1990, Stockner et al. 1990). Uptake of NO_3^- by bacteria is generally insignificant (Chan & Campbell 1978, Wheeler & Kirchman 1986). The uptake of $^{15}\text{NH}_4^+$ by these small organisms would result in an ^{15}N enrichment of particles in the < 0.2 μm filtrate. The result in this case would be an overestimate of DON release.

The measurement of DON release is also complicated by the fact that there many different processes which can result in DON release, and that variations in cellular physiological state can impact the measured DON release rates. In the determination of intracellular DON atom % enrichment, we extract the intracellular DON from the phytoplankton and any bacteria present on the filter. In effect, we measure the atom % enrichment of the combined intracellular pool. As long as the released DON originates from the labeled intracellular pools we isolate, an accurate DON release rate is measured. Note, however, that any process which involves the release of unlabeled DON (i.e. release from dying cells which no longer take up nitrogen, unlabeled bacterial release, or zooplankton excretion) into the extracellular DON pool will decrease the atom % enrichment of the extracellular DON pool and result in an underestimate of the DON release rate. Additional experimentation is needed to decipher more precisely how the rate of uptake and physiological state of the phytoplankton community will impact the measurement of DON release rates.

Finally, the measurement of DON release rates is complicated by the recent controversy surrounding the measurement of DON concentrations which began with the introduction of high temperature combustion techniques (Suzuki et al. 1985, Walsh 1989). The question of the appropriate method to use to measure DON concentrations is still open. Regardless of which method is used, the ion retardation column method is applicable for isolation of DON.

Time-course patterns in DON release

The 6 h time-course of DI^{15}N uptake and DO^{15}N release was conducted in waters containing high am-

bient concentrations of NH_4^+ and NO_3^- . We have a number of lines of evidence to suggest that this experiment was conducted during the end of the spring bloom when there was a rapid decline in the population and productivity of the phytoplankton. Primary productivity decreased from $15.2 \mu\text{g C l}^{-1} \text{ h}^{-1}$ on May 16, 1990, to $6.6 \mu\text{g C l}^{-1} \text{ h}^{-1}$ on May 22, 1990, and chlorophyll concentrations decreased from 10.5 to 3.1 $\mu\text{g chl l}^{-1}$ over the same interval (Malone unpubl.). Uptake rates of NH_4^+ measured on May 16, 1990, were over 5 times higher than those measured on May 22, 1990 (Glibert unpubl.).

The uptake rates of $^{15}\text{NO}_3^-$ declined sharply during the 6 h time-course, while the uptake rates of $^{15}\text{NH}_4^+$ remained relatively constant. The peak in NO_3^- uptake observed after 0.5 h of incubation (ca 10:00 h) is consistent with previous reports of maximum observed NO_3^- uptake occurring during the morning hours (Berman et al. 1984, Glibert & Garside 1991). The approximate exponential decline in the uptake rates of NO_3^- and the constant atom % excess of the PN (data not shown) during the time-course also strongly suggest a state of isotopic equilibration between internal and external NO_3^- pools. Once isotopic equilibration has been reached, the calculated rate of uptake during a time-course will be reduced proportionately by the length of the incubation. Larger and more persistent internal pools of NH_4^+ under nutrient saturated conditions (DeManche et al. 1979, Dortch 1982) would reach isotopic equilibration much more slowly (Wheeler et al. 1982).

Though NO_3^- uptake was higher than NH_4^+ uptake for the first 3 h of incubation and the intracellular NO_3^- pool appeared to reach isotopic equilibrium much faster, the rate of assimilation of NO_3^- into the intracellular LMW DON pool was extremely low: only 6 % of the NO_3^- taken up over 6 h was incorporated into intracellular organic compounds. The very low rates of DO^{15}N release resulting from $^{15}\text{NO}_3^-$ uptake are consistent with these low assimilation rates. In contrast, > 80 % of the NH_4^+ taken up during the 6 h incubation was incorporated into cellular organic material.

As a percentage of DI^{15}N uptake, release from the incorporation of NH_4^+ was greater than that of NO_3^- . This is partially a function of the more rapid assimilation of NH_4^+ into organic molecules than that of NO_3^- (as discussed above). The ratio of LMW DO^{15}N release to DIN uptake varied from 32 to 42 % in the NH_4^+ incubation and from 0 to 7 % of $^{15}\text{NO}_3^-$ uptake in the NO_3^- incubation. The ratio of total DO^{15}N release to DIN uptake was 54 to 260 % in the NH_4^+ incubation and < 4 % in the NO_3^- incubation. However, direct comparisons between rates of uptake of DI^{15}N and rates of release of DO^{15}N are problematic. Release processes occur independently of uptake, yet, analytically, we

use components of cellular atom % to calculate both rates. During a time-course, the very processes which would result in cell breakage (i.e. senescence, viral infection, sloppy feeding, filtration stress) are also processes which would result in an underestimate of the total uptake that occurred during that time interval. For example, if filtration results in cell rupture, cellular DI^{15}N and DO^{15}N would be released into the medium, resulting in a high calculated rate of DO^{15}N release but a very low rate of DI^{15}N uptake. If the phytoplankton assemblage in May was indeed in a period of rapid decline, it may have been more susceptible to such breakage, resulting in the very high release:uptake ratios we observed. Sloppy feeding by zooplankton during the incubation would have a similar effect.

The difference in LMW and total DO^{15}N release rates and their proportion to one another is a useful index in deciphering what release processes may be important under various conditions. When the rate of LMW DO^{15}N release represents a relatively high percentage of the total DO^{15}N release rate, we can speculate that direct release by phytoplankton is the dominant release process. Direct release by phytoplankton would involve such compounds as small amino acids, amino sugars, small polypeptides, etc. which would readily diffuse across the cell membrane (reviewed by Antia et al. 1991). If total DO^{15}N release rates were significantly higher than rates of LMW DO^{15}N release, it is likely that processes such as cell lysis, viral infection, or sloppy feeding were occurring, as these processes would result in a suite of larger DON compounds being released.

As expected, rates of total DO^{15}N release were higher and more variable than LMW DO^{15}N release rates in both 6 h incubations. In the incubation with $^{15}\text{NH}_4^+$, LMW DO^{15}N release was a relatively high percentage of total DO^{15}N release during the 0.5 h sampling point but then decreased with time. Thus, as the incubation progressed, additional release mechanisms, other than direct release, apparently increased in importance. In the incubation with $^{15}\text{NO}_3^-$, the rates of both LMW and total DO^{15}N release were very low, and LMW DO^{15}N release represented a higher proportion of total release at the start of the experiment than at 3 h of incubation.

The experiment conducted on August 14, 1990, in which short-term DO^{15}N release rates were measured on a sample of water contained in a large carboy, revealed different patterns of DON release compared to the 6 h time-course. Rates of phytoplankton growth at this time were rapid, as evidenced by the rapid decline in ambient NH_4^+ concentrations, high nitrogen uptake rates, and low C:N ratios measured during the experiment. The release of LMW DO^{15}N was a small percentage (0 to 16 %) of total DO^{15}N release, sug-

gesting that direct release by phytoplankton was not an important release mechanism. Furthermore, the low percentages of LMW DO^{15}N release to $^{15}\text{NH}_4^+$ uptake (2 ± 2 %) are consistent with reports from both laboratory and field studies (reviewed by Sharp 1984) which indicate that phytoplankton do not lose much of their recently assimilated organic material under steady state conditions, or under conditions of rapid growth.

CONCLUSIONS

The method we have described allows us to quantify previously unmeasurable rates of DO^{15}N release resulting from incorporation of DI^{15}N -labelled substrates by phytoplankton. Combined with ultrafiltration, this method can be a valuable tool in defining assimilation efficiencies of DIN and the sources and composition of recently released DON.

Acknowledgements. This research was supported by NSF grant OCE-8817585 to P. Glibert. This is contribution no. 2256 from the Center for Environmental and Estuarine Studies, University of Maryland. We gratefully acknowledge Susan Banahan for assistance throughout the development of the method. We also thank Elisabeth Sahlsten and John Ludlam who assisted with field sample collection; David Kirchman for use of the HPLC; and Rick Keil for assistance with HPLC. Susan Banahan, Hugh Ducklow, Matt Hoch, Dave Kirchman, Carolyn Miller and 2 anonymous referees reviewed earlier versions of the manuscript. Finally, we thank the captain and crew of the RV 'Ridgely Warfield'.

LITERATURE CITED

- Antia, N. J., Harrison, P. J., Oliveira, L. (1991). The role of dissolved organic nitrogen in phytoplankton nutrition, cell biology and ecology. *Phycologia* 30: 1-89
- Axler, R. P., Reuter, J. E. (1986). A simple method for estimating the ^{15}N content of dissolved organic matter (DO^{15}N) in N-cycling studies. *Can. J. Fish. Aquat. Sci.* 43: 130-133
- Berman, T., Sherr, B. F., Sherr, E., Wynne, D., McCarthy, J. J. (1984). The characteristics of ammonium and nitrate uptake by phytoplankton in Lake Kinneret. *Limnol. Oceanogr.* 29: 287-297.
- Chan, Y. K., Campbell, N. E. R. (1978). Phytoplankton uptake and excretion of assimilated nitrate in a small Canadian shield lake. *Appl. environ. Microbiol.* 35: 1052-1060
- Clayton, Jr., J. R., Dortch, Q., Thoresen, S. S., Ahmed, S. I. (1988). Evaluation of methods for the separation and analysis of proteins and free amino acids in phytoplankton samples. *J. Plankton Res.* 10: 341-358
- DeManche, J. M., Curl, Jr., H. C., Lundy, D. W., Donaghay, P. L. (1979). The rapid response of the marine diatom *Skeletonema costatum* to changes in external and internal nutrient concentration. *Mar. Biol.* 53: 323-333
- Dortch, Q. (1982). Effect of growth conditions on accumulation of internal pools of nitrate, ammonium, amino acids,

- and protein in three marine diatoms. *J. exp. mar. Biol. Ecol.* 61: 243–264
- Dugdale, R. C., Goering, J. J. (1967). Uptake of new and regenerated forms of nitrogen in primary production. *Limnol. Oceanogr.* 12: 196–206
- Fiedler, R., Proksch, G. (1975). The determination of nitrogen-15 by emission and mass spectrometry in biochemical analysis: a review. *Anal. Chim. Acta.* 78: 1–62
- Fuhrman, J. (1987). Close coupling between release and uptake of dissolved free amino acids in seawater studied by an isotope dilution approach. *Mar. Ecol. Prog. Ser.* 37: 45–52
- Fuhrman, J. (1990). Dissolved free amino acid cycling in an estuarine outflow plume. *Mar. Ecol. Prog. Ser.* 66: 197–203
- Glibert, P. M., Garside, C., Fuhrman, J. A., Roman, M. R. (1991). Time-dependent coupling of inorganic and organic nitrogen and NH_4^+ regeneration in the plume of the Chesapeake Bay estuary, USA, and its regulation by large heterotrophs. *Limnol. Oceanogr.* 36: 895–909
- Glibert, P. M., Garside, C. (1991). Diel variability in nitrogenous nutrient uptake by phytoplankton in the Chesapeake Bay plume. *J. Plankton Res.* 14: in press
- Glibert, P. M., Lipschultz, F., McCarthy, J. J., Altabet, M. A. (1982). Isotope dilution models of uptake and remineralization of ammonium by marine plankton. *Limnol. Oceanogr.* 27: 639–650
- Glibert, P. M., McCarthy, J. J. (1984). Uptake and assimilation of ammonium and nitrate by phytoplankton: indices of nutritional status for natural assemblages. *J. Plankton Res.* 6: 677–697
- Guillard, R. (1975). Culture of phytoplankton for feeding marine invertebrates. In: Smith, W., Chanley, M. (eds.) *Culture of marine invertebrate animals*. Plenum Press, New York, p. 29–60
- Hansell, P. A., Goering, J. J. (1989). A method for estimating uptake and production rates for urea in seawater using ^{14}C urea and ^{15}N urea. *Can. J. Fish. Aquat. Sci.* 46: 198–202
- Hellebust, J. A. (1974). Extracellular products. In: Stewart, W. D. P. (ed.) *Algal physiology and biochemistry*. Blackwell, Oxford, p. 838–863
- Hobbie, J. E., Daley, R. J., Jasper, S. (1977). Use of Nuclepore filters for counting bacteria by fluorescence microscopy. *Appl. environ. Microbiol.* 33: 1225–1228
- Keil, R. G., Kirchman, D. L. (1991). Dissolved combined amino acids in marine waters as determined by a vapor-phase hydrolysis method. *Mar. Chem.* 33: 243–259
- Kendall, C., Grim, E. (1990). Combustion tube method for measurement of nitrogen isotope ratios using calcium oxide for total removal of carbon dioxide and water. *Anal. Chem.* 62: 526–529
- Kirchman, D. L., Keil, R. G., Wheeler, P. A. (1989). The effect of amino acids on ammonium utilization and regeneration by heterotrophic bacteria in the subarctic Pacific. *Deep Sea Res.* 36: 1763–1776
- Li, W. K. W. (1990). Particles in 'particle-free' seawater growth of ultraphytoplankton and implications for dilution experiments. *Can. J. Fish. Aquat. Sci.* 47: 1258–1268
- Lindroth, P., Mopper, K. (1979). High performance liquid chromatographic determination of subpicomole amounts of amino acids by precolumn fluorescence derivatization with *o*-phthalaldehyde. *Anal. Chem.* 51: 1667–1674
- Mague, T. H., Friberg, E., Hughes, D. J., Morris, I. (1980). Extracellular release of carbon by marine phytoplankton: a physiological approach. *Limnol. Oceanogr.* 25: 262–279
- McCarthy, J. J., Nevins, J. L. (1986). Utilization of nitrogen and phosphorus by primary producers in warm-core ring 82-B following deep convective mixing. *Deep Sea Res.* 33: 1773–1788
- McCarthy, J. J., Taylor, W. R., Taft, J. L. (1977). Nitrogenous nutrition of phytoplankton in the Chesapeake Bay. I. Nutrient availability and phytoplankton preferences. *Limnol. Oceanogr.* 22: 996–1011
- Paerl, H. W., Mackenzie, L. A. (1977). A comparative study of the diurnal carbon fixation patterns of nanoplankton and net plankton. *Limnol. Oceanogr.* 22: 732–738
- Parsons, T. R., Maita, Y., Lalli, C. M. (1984). *A manual of chemical and biological methods for seawater analysis*. Pergamon Press, Oxford, p. 3–22
- Paul, J. H., Jeffrey, W. H., DeFlaun, M. F. (1987). Dynamics of extracellular DNA in the marine environment. *Appl. environ. Microbiol.* 53: 170–179
- Procter, L. M., Fuhrman, J. A. (1990). Viral mortality of marine bacteria and cyanobacteria. *Nature, Lond.* 343: 60–62
- Sharp, J. H. (1983). The distribution of inorganic nitrogen and dissolved and particulate organic nitrogen in the sea. In: Carpenter, E. J., Capone, D. G. (eds.) *Nitrogen in the marine environment*. Academic Press, New York, p. 1–35
- Sharp, J. H. (1984). Inputs into microbial food chains. In: Hobbie, J., Williams, P. J. LeB. (eds.) *Heterotrophic activity in the sea*. Plenum Press, New York, p. 101–120
- Solorzano, L. (1969). Determination of ammonia in natural waters by the phenohypochlorite method. *Limnol. Oceanogr.* 14: 799–801
- Stockner, J. G., Klut, M. E., Cochlan, W. P. (1990). Leaky filters: a warning to aquatic ecologists. *Can. J. Fish. Aquat. Sci.* 47: 16–23
- Suttle, C. A., Chan, A. M., Cottrell, M. T. (1990). Infection of phytoplankton by viruses and reduction of primary productivity. *Nature, Lond.* 347: 467–469
- Suzuki, Y., Sugimura, Y., Itoh, T. (1985). A catalytic oxidation method for the determination of total nitrogen dissolved in seawater. *Mar. Chem.* 16: 83–97
- Valderrama, J. C. (1981). The simultaneous analysis of total nitrogen and total phosphorus in natural waters. *Mar. Chem.* 10: 109–122
- Walsh, T. W. (1989). Total dissolved nitrogen in seawater: a new high-temperature combustion method and a comparison with photo-oxidation. *Mar. Chem.* 26: 295–311
- Wheeler, P. A., Glibert, P. M., McCarthy, J. J. (1982). Ammonium uptake and incorporation by Chesapeake Bay phytoplankton: short-term uptake kinetics. *Limnol. Oceanogr.* 27: 1113–1128
- Wheeler, P. A., Kirchman, D. L. (1986). Utilization of inorganic and organic nitrogen by bacteria in marine systems. *Limnol. Oceanogr.* 31: 998–1009

This article was submitted to the editor

Manuscript first received: March 4, 1991

Revised version accepted: September 30, 1991