Vol. 124: 289–299, 1995 | MARINE ECOLOGY PROGRESS SERIES Mar Ecol Prog Ser | Published August 10

Simple procedure for simultaneous recovery of dissolved inorganic and organic nitrogen in ^{15}N-tracer experiments and improving the isotopic mass balance

Gerd Slawyk*, Patrick Raimbault

Centre d'Océanologie de Marseille, Faculté des Sciences de Luminy, case 901, F-13288 Marseille Cedex 9, France

ABSTRACT: We developed a simple and reliable method which allows simultaneous isotope-ratio analysis of inorganic (DIN) and organic (DON) forms of nitrogen extracted from seawater. All forms of nitrogen under analysis are converted to ammonium, by diffusion with magnesium oxide, prior to collection on glass-fiber filters appropriate for mass spectrometric assay of ^{15}N. Oxidized DIN forms (nitrate, nitrite) are reduced to ammonium in the presence of Devarda alloy. Conversion of DON to ammonium is performed by wet oxidation using potassium persulfate and subsequent reduction of the nitrate formed. Recovery tests, both for total nitrogen and ^{15}N content, showed that this procedure is suitable for application in DI^{15}N-isotope dilution experiments and DON-release studies. Recovery of total nitrogen from DIN and DON was nearly complete (94 to 97%). The variability in the experimental determination of ^{15}N abundance was <2% and <4% for DIN and DON, respectively. We used the method to balance the ^{15}N budget in nitrate and ammonium uptake experiments conducted in an oligotrophic area (tropical North Atlantic) by including, in addition to the substrate (DIN) and biomass (PON) pool, the DON pool. However, the use of glass-fiber filters (GF/F) for the collection of particulate matter produced a significant artifact, i.e. a large amount of small particles (<0.7 µm, $PON_{<GF/F}$; prochlorophytes and/or bacteria) passed through these filters and were recovered together with the DON in a combined pool. While inclusion of this combined pool led virtually to a complete accounting for the ^{15}N label (99%) in all samples for nitrate uptake and in those for ammonium uptake incubated for <8 h, no mass balance was achieved during ammonium uptake lasting 10 to 24 h. We suggest that the ^{15}N that was still missing (13%) resulted mainly from bottle containment effects such as ammonium-ion adsorption and/or PON adherence onto incubation bottle walls. Transfer of ^{15}N label to the combined pool (nitrate experiment) and to the DON and $PON_{<GF/F}$ pools (ammonium experiment) represented up to 41, 38 and 20% of the total ^{15}N taken up as DIN, respectively, and depended strongly upon the length of incubation. Failure to take these pathways of the missing ^{15}N into account during traditional ^{15}N uptake experiments involves risk of substantially underestimating new and regenerated production, at least in oligotrophic areas. The latter fact has considerable significance in the design of future ^{15}N tracer methodologies.

KEY WORDS: Organic nitrogen · Inorganic nitrogen · ^{15}N methodology · Nitrogen uptake · ^{15}N partitioning · Oceanic waters

INTRODUCTION

The traditional ^{15}N-tracer method of measuring primary productivity in terms of nitrogen (Dugdale & Goering 1967) is based on a 2-compartment model which includes dissolved inorganic or organic nitrogen (DIN or urea, the source pool of ^{15}N-labelled N) and particulate organic nitrogen (PON, the target pool of ^{15}N-labelled N) and in which the net flux of nitrogen added as a tracer depends on the relative importance of assimilative and regenerative processes.

*E-mail: slawyk@com.univ-mrs.fr

Assuming that no further nitrogen pool is involved, one would expect to find a mass balance of ^{15}N between these 2 pools at any time during an experiment. However, many workers have failed to demonstrate such mass balance in ^{15}N inventory calculations of 2-compartment models (Glibert et al. 1982, 1985, Laws 1984, Kokkinakis & Wheeler 1987, La Roche & Harrison 1987, Hanson & Robertson 1988, Hansell & Goering 1989, Ward et al. 1989, Slawyk et al. 1990b). Generally, part of the ^{15}N label added to the dissolved phase (substrate) to initiate incubation was unaccounted for on the basis of the ^{15}N label remaining in the substrate and/or incorporated into the particulate phase at the end of the experiment, whether oxidized nitrogen forms (nitrate, nitrite) or reduced forms (ammonium, urea) were used for labelling. The same trend of missing ^{15}N has been documented in studies on nitrate utilization when changes in substrate concentration (disappearance uptake) have been compared with ^{15}N uptake (Price et al. 1985, Slawyk et al. 1990a, Eppley & Renger 1992).

Mechanisms responsible for this sink of ^{15}N suggest losses of substrate to another pathway, i.e. transformations involving only DIN, and/or losses of some form of nitrogen from the microplankton by processes subsequent to incorporation of ^{15}N-labelled substrate. Possible explanations for the nitrogen lost from the particulate phase given by the authors cited above are either methodological artifacts such as release of dissolved organic nitrogen (DON) due to cell lysis during filtration and DIN uptake of organisms not retained on GF/F filters, or genuine biological processes such as release (excretion) of DON by phytoplankton cells and grazer-induced losses of DON through 'sloppy' feeding.

Since the introduction of a suitable technique for the measurement of ^{15}N abundance in DON (Bronk & Glibert 1991) it has been shown that, among the latter possible avenues of missing ^{15}N, transfer of ^{15}N label both to the DON pool and to small-size PON (bacteria) passing through GF/F filters ($PON_{<GF/F}$) must be included in order to make up the ^{15}N deficit in final N budgets (Bronk & Glibert 1994).

In this study we made a further effort to locate the missing ^{15}N in DIN uptake experiments in which, in addition to the classical measurement of ^{15}N incorporation into PON retained on glass-fiber filters ($PON_{>GF/F}$), we inspected the organic nitrogen (DON + $PON_{<GF/F}$) in the filtrates for ^{15}N content. However, we did not apply Bronk & Glibert's (1991) method for the isolation of DON as it is rather labour intensive, especially for samples with low DON concentrations. Our field work was conducted in the very oligotrophic northern tropical Atlantic, which is characterized by a deep, nutrient-impoverished surface layer and by DON concentrations ranging from 4.4 to 6.5 µM. Under these conditions any method needing a large amount of sample processing is prone to contamination (DON, NH_4^+) problems. This prompted us to develop a method especially adapted for our own purposes. Our procedure is simple and allows us to extract inorganic and organic nitrogen pools from only 1 filtrate sample with little handling, and thus we foresee the possibility of simultaneously measuring DIN regeneration and DON release rates. A detailed description of the methodological protocol, including data on its performance, is presented.

MATERIALS AND METHODS

DIN and DON isolation. Fig. 1 outlines the experimental protocol for measuring the total N and ^{15}N content of the particulate (PON) and dissolved (DIN, DON) nitrogen fractions. We used the GF/F filtrate for the isolation of DIN and DON and performed no further filtration through a smaller-pore-size filter. Thus, we are operationally defining DON prime (DON') as the total organic nitrogen (DON + $PON_{<GF/F}$) recovered from this latter filtrate.

The procedure involves 3 analytical steps: (1) reduction of oxidized DIN (NO_3^-, NO_2^-) and simultaneous stripping off and concentration of evolved and initial ammonium as ammonium sulfate [$(NH_4)_2SO_4$] by diffusion with magnesium oxide (MgO) in the presence of Devarda alloy (DA; Devarda 1892); (2) digestion (wet oxidation) of the remaining DON' to NO_3^- with potassium persulfate ($K_2S_2O_8$); and (3) recovery of the formed NO_3^- as $(NH_4)_2SO_4$ by again adding DA and sodium hydroxide (NaOH) instead of MgO.

All chemicals were of reagent-grade quality and aqueous solutions were prepared using deionized water (Milli-Q). The chemicals used for the extractions were as follows: baked MgO (450°C, 4 h), baked (450°C, 4 h) and screened (230-mesh size = 63 µm) DA, 0.5 N H_2SO_4, 12.5 N NaOH and a digestion mixture according to Raimbault & Slawyk (1991). For the latter mixture, 15 g of $K_2S_2O_8$ was dissolved in 250 ml of 1.5 N NaOH.

A 300 ml filtrate was directly sampled in a 500 ml Pyrex bottle (Duran Schott) fitted with screwcaps and placed under the filtering device. No further decanting of the sample is required until the end of the experimental protocol, thus providing maximum protection against DON and/or DIN contamination problems. The sample was buffered with 300 mg of MgO to raise the pH above 9 and 300 mg of DA was added for reduction. For the initial NH_4^+ diffusion we used MgO instead of NaOH to prevent alkali-labile organic nitrogen compounds from decomposing with formation of ammonia

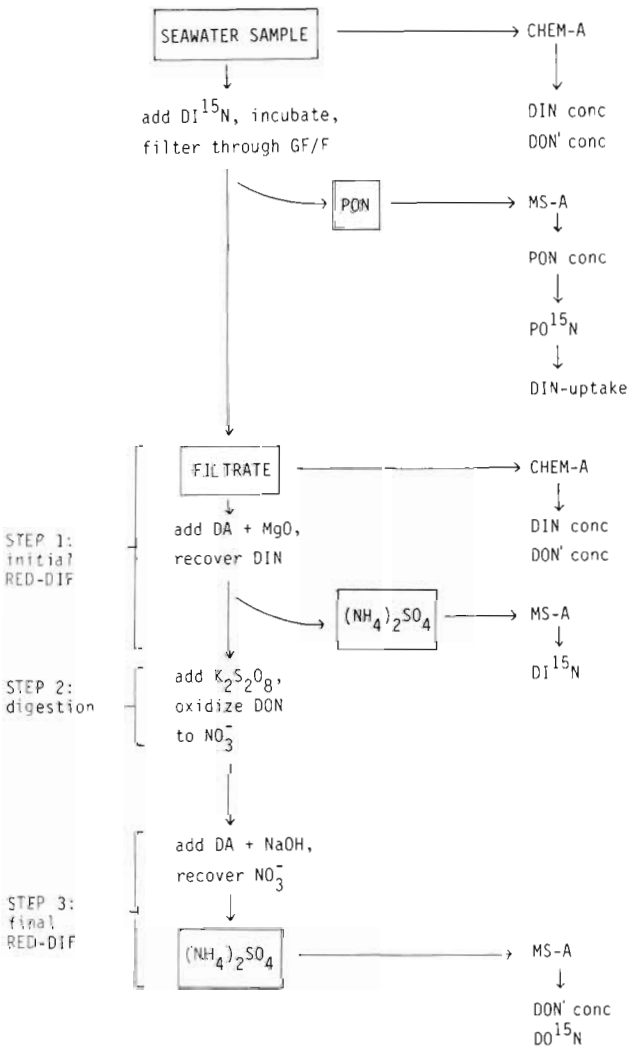

Fig. 1 Flow diagram of the experimental protocol used to recover dissolved and particulate nitrogen fractions from seawater for ^{15}N-isotope ratio analyses in nitrogen-flux studies. CHEM-A: chemical analysis; MS-A: mass spectrometric analysis; RED-DIF: reduction-diffusion; DA: Devarda alloy; conc: concentration

(NH_3) (Bremner & Keeney 1965). At this stage, unlabelled DIN was added as a carrier to raise the concentration to >2.0 µM and thus to provide sufficient nitrogen for mass spectrometric analysis. A strip of glass-fiber filter (GF/C) wetted with 50 µl of 0.5 N H_2SO_4 was suspended above the sample to trap the liberated ammonium (Paasche & Kristiansen 1982). The bottle was then capped tightly and left for a week at 60°C in an oven. DAPI (4,6-diamidino-2-phenylindole) counts on the filtrate sample at the beginning and the end of the week showed that the experimental conditions (elevated pH and temperature) prevented bacteria from flourishing during the diffusion period. The strip was then oven-dried at 60°C and stored in a desiccator until isotopic analysis of the DIN pool. The remaining DON' in the filtrate was then oxidized to NO_3^- by adding 30 ml of digestion mixture and autoclaving the sample in the capped bottle at 120°C (1 bar) for 30 min. After the digestion, a further 300 mg of DA and 5 ml of 12.5 N NaOH were added to the sample. No carrier addition was made for the DON' recovery. A new acidified filter strip was fitted and the capped bottle was again placed in the oven for 1 wk at 60°C. At the end of the reduction/diffusion period the GF/C strip was treated as described above before isotopic analysis.

Chemical analysis. Concentrations of NH_4^+ were measured manually according to Koroleff (1970) and those of NO_3^- were determined with a Technicon AutoAnalyzer according to Raimbault et al. (1990) for concentrations in the nanomolar range (0 to 100 nM) and Tréguer & Le Corre (1975) for concentrations >100 nM. Final PON concentrations (PON_f) were determined using mass spectrometer signals from the isotope-ratio analyses of the particles. Final DON' concentrations (DON'_f) were measured using a wet-oxidation method (Raimbault & Slawyk 1991).

^{15}N analysis. All filters (GF/F for PON and GF/C strip for DIN and DON') were analyzed for ^{15}N content using a continuous-flow method (Europa Scientific) in which Dumas combustion (Roboprep-CN) is linked on-line to a triple-collector mass spectrometer (Tracermass) via a capillary interface based on the design of Preston & Owens (1983). A personal computer provides system control and data acquisition. Mass-spectrometric signals are used to calculate total N content and ^{15}N abundance. Calibrations were made against glycine standards. The mean natural atom % ^{15}N of a glycine standard containing 2 µmol of N was 0.36727 with a standard deviation (SD) of 0.00111 (n = 20). Mean total N was 2.08 µmol with a SD of 0.03. These values attest to the high stability in the analyzing system. The low background contribution of the system makes it possible to process samples safely with only 0.6 µmol of total N, which means that a 2 l sample from extremely oligotrophic waters produces enough N for ^{15}N work. Mass spectrometer signals were corrected for nitrogen contamination from reagents and glass-fiber filters.

Calculation of ^{15}N abundance in the DON' pool. The atom % excess ^{15}N in the DON' pool ($R_{DON'}$) is defined as

$$R_{DON'} = \frac{DO^{15}N' \times 100}{DON'_f} - R_n \quad (1)$$

where $DO^{15}N'$ is the concentration of ^{15}N (tracer + natural) in the DON' pool, DON'_f is the final concentration of DON' and R_n is the natural abundance of ^{15}N in DON'. A small amount of labelled DIN was unrecovered (DIN_{ur}) during the first extraction step and carried

over into the DON' pool, and it therefore had to be taken into account in the calculation of $R_{DON'}$.

DIN_{ur} was determined from 'time zero' samples (immediately after tracer addition) according to the equation

$$DIN_{ur} = N_T \frac{R_{mo} - R_n}{R_{mi} - R_n} \quad (2)$$

where R_{mi} and R_{mo} are the measured atom % ^{15}N of extracted N during analytical steps 1 and 3, respectively, and N_T is the concentration of total N (DON'_f + DIN_{ur}) extracted during analytical step 3 (measured by mass spectrometry).

The equation for computing $R_{DON'}$, which includes the correction for DIN_{ur} carry-over, was derived as follows: R_{mi} is given by

$$R_{mi} = \frac{DI^{15}N_{ur}}{DIN_{ur}} \times 100 \quad (3)$$

where $DI^{15}N_{ur}$ is the concentration of ^{15}N in DIN_{ur}, and R_{mo} by

$$R_{mo} = \frac{(DO^{15}N' + DI^{15}N_{ur})}{DON'_f + DIN_{ur}} \times 100 . \quad (4)$$

Substituting Eqs. (3) & (4) in Eq. (1) then yields

$$R_{DON'} = \frac{1}{DON'_f}[R_{mo}(DON'_f + DIN_{ur}) - R_{mi} \times DIN_{ur}] - R_n . \quad (5)$$

Since $N_T = DON'_f + DIN_{ur}$, Eq. (5) can be written as

$$R_{DON'} = \frac{1}{N_T - DIN_{ur}}(R_{mo} \times N_T - R_{mi} \times DIN_{ur}) - R_n . \quad (6)$$

Field work. Data from inorganic nitrogen uptake experiments (time-series) conducted during the EUMELI 3 cruise in September and October 1992 aboard the RV 'Atalante' in the northern tropical Atlantic were used in this paper for ^{15}N inventory and mass-balance calculations. Water from the 50% and 6% light-penetration depths was taken with a 30 l Niskin bottle. To mimic the irradiance levels at which the samples were obtained, the seawater samples were poured into 2 l polycarbonate (PC) bottles and incubated in a seawater-cooled deck incubator covered with perforated nickel screens. The ^{15}N enrichments applied are given with the results. Following incubation, the samples were filtered onto precombusted (450°C, 4 h) GF/F filters. Just before the sample was drained completely, a small amount of seawater was added to the filtration funnel to rinse the filter. After filtration, the filters were dried at 60°C and stored over desiccant for later analysis of ^{15}N abundance in the PON pool. One portion of the filtrate was used for immediate NH_4^+, NO_3^- and DON' analysis and another portion (300 ml) was recovered in 500 ml Pyrex bottles and frozen for later extraction of the DIN and DON' pool before ^{15}N analysis.

To investigate potential pathways of the missing ^{15}N during ^{15}N uptake, 2 further types of experiments were conducted. Firstly, in time-series experiments, aluminum-oxide membranes (Anopore, 0.2 µm pore size) were compared with the GF/F membranes used for filtering particulate matter, in order to check for possible passage of ^{15}N-labelled particles through the GF/F membranes. The Anopore membranes were cushioned with GF/F filters above and below and precombusted at 400°C for 4 h. To prevent breakage of the very brittle Anopore membranes the 3 membranes were fitted together in the filter holder as recommended by Altabet (1990). The membranes were separated after drying (60°C) and analyzed individually for their total N and ^{15}N content. GF/F filters alone were used in parallel as a control.

Secondly, we tested for the possibility of non-biological ^{15}N-NH_4^+ disappearance from prefiltered (0.2 µm) and ^{15}N-enriched (500 to 700 nM) seawater, enclosed for about 20 h in PC and Pyrex bottles, due to adsorption of NH_4^+ to the walls of the bottles. In one experiment seawater was poisoned with $HgCl_2$. Subsequent to 'incubation', 300 ml aliquots were processed for extraction of NH_4^+ as outlined above. Unlabelled NH_4^+ (carrier) was added to bring the final concentration to about 60 µM. Assuming that no isotope dilution occurred, we back-calculated the final NH_4^+ concentration from the ^{15}N abundance in the extracted NH_4^+ pool at the beginning and the end of the experiment.

RESULTS

The extraction procedure

We conducted several experiments to test our method of DIN and DON' recovery as well as the precision of isotopic determination in the 2 N pools. Nitrate reduction was faster than subsequent NH_4^+ recovery (Fig. 2). Nitrate was reduced almost completely after 2 d, whereas NH_3 diffusion lasted 2 further days before 90% NH_4^+ recovery was reached. Fig. 3 shows that the defined experimental conditions, i.e. MgO and DA additions and a 7 d period for diffusion, were adequate for the reduction of NO_3^- concentrations up to 60 µM. However, the slope of the linear regression line in Fig. 3 as well as data from replicate recovery tests (Table 1) show that a small amount of DIN (on average 3.4% of initial DIN) was not removed during the initial reduction/diffusion step (step 1). The time course pattern in Fig. 2 seems to indicate that NH_3 diffusion is the rate-limiting factor. DON' was recovered almost as efficiently as DIN at an average level of 94% (Table 1) so that no carrier addition was needed.

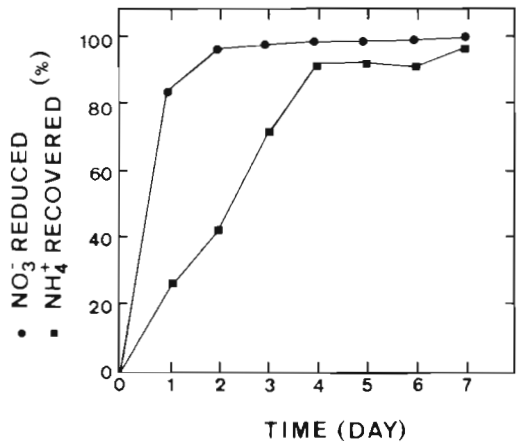

Fig. 2. Time course of nitrate (plus nitrite) reduction and of subsequent ammonium recovery during analytical step 1 of the extraction procedure. DIN concentrations at 'time zero': $N\text{-}NO_3^- + N\text{-}NO_2^- = 8.48$ µM, $N\text{-}NH_4^+ = 0.17$ µM

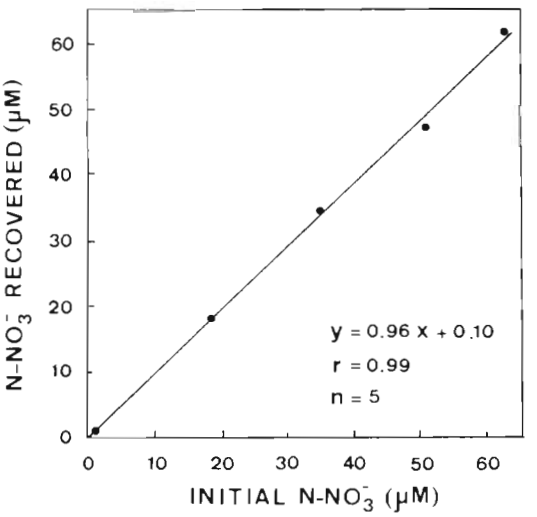

Fig. 3. Nitrate recovery from seawater as a function of initial nitrate concentration for a NO_3^- reduction/NH_4^+ diffusion period of 7 d. Line describes to the equation given in the figure

Since the unremoved DIN (DIN_{ur}) is ^{15}N-labelled and is carried over into the remaining DON' pool during analytical steps 2 and 3, it will contribute to the ^{15}N content of this pool. The extent of this contribution is evident from the measured atom % ^{15}N values in Table 2. In Expt 2 (with $^{15}N\text{-}NO_3^-$ addition) the nitrogen recovered (essentially DON') during step 3 was slightly enriched ($R_{mo} = 0.39670$ atom % ^{15}N) compared to the natural level found in Expt 1 with no ^{15}N-label addition ($R_{mo} = 0.37082$ %). The amount of DIN_{ur}

Table 1. Average amounts of total nitrogen removed from the DIN and DON' pools of filtered (GF/F) seawater sampled off Marseille, France, during analytical steps 1, 2 and 3 of the extraction procedure. Values in parentheses are standard deviations. For details of experimental conditions see Table 2

Expt	^{15}N tracer	DIN			DON'			n
		Initial (µM)	Extracted (µM)	Recovered of initial (%)	Initial (µM)	Extracted (µM)	Recovered of initial (%)	
1	Natural	17.05	16.62 (0.53)	97.5	6.35	6.28 (0.60)	98.9	3
2	$^{15}N\text{-}NO_3^-$	17.93	17.29 (0.93)	96.4	6.35	5.78 (0.20)	90.9	2
3	^{15}N-urea	17.88	17.15 (1.06)	95.9	6.40	5.84 (0.45)	91.2	3
				$\bar{x} = 96.6$			$\bar{x} = 93.7$	

Table 2. Experimental conditions and mean values of measured atom % ^{15}N of nitrogen extracted in Expts 1, 2 and 3 (Table 1) during analytical steps 1 (R_{mi}) and 3 (R_{mo}). Values in parentheses are standard deviations

Expt	Experimental conditions							Measured atom % ^{15}N		n
	^{15}N tracer	DIN[a] (µM)	DI^{15}N[b] (µM)	DON'[a] (µM)	DO^{15}N[b] (µM)	DIN[c] (µM)	DIN_{ur} (µM)	R_{mi}	R_{mo}	
1	Natural	0.38	–	6.35	–	16.67	–	0.36741 (0.00205)	0.37082 (0.00205)	3
2	$^{15}N\text{-}NO_3^-$	0.38	0.05[d]	6.35	–	17.50	0.62 (0.14)	0.63604 (0.00307)	0.39670 (0.00569)	2
3	^{15}N-urea	0.38	–	6.35	0.05[e]	17.50	0.62 (0.14)	0.37201 (0.00573)	1.05667 (0.02303)	3

[a]Initial; [b]^{15}N tracer added; [c]Carrier; [d]97.4 atom % ^{15}N; [e]99.0 atom % ^{15}N

Table 3. Comparison between theoretical and experimental atom % excess ^{15}N values for the DIN and DON' pools extracted in Expts 2 and 3 (Table 1)

Expt	^{15}N tracer	N pool	Atom % excess ^{15}N Theoretical	Experimental
2	^{15}N-NO_3^-	DIN	11.28341	$\bar{x}$ 11.20125
				SD 0.12794
				CV 1.14%
				n 2
3	^{15}N-urea	DON'	0.77059	$\bar{x}$ 0.75853
				SD 0.02547
				CV 3.36%
				n 3

(0.62 µM), which was obtained as outlined in the method section, represented 3.5% of the total DIN pool [DIN + DIN_c (carrier) = 17.88 µM] and corresponded very closely to the average amount found from DIN-recovery tests (Table 1).

The precision of the isotopic analysis was tested by running replicate sets of seawater spiked with known amounts of ^{15}N-labelled NO_3^- (Expt 2) and ^{15}N-labelled urea (Expt 3) (Table 3). The coefficient of variation (CV) calculated from the experimental determination of ^{15}N abundance was 1.14 and 3.36% for the extracted NO_3^- and DON' pool, respectively. The mean experimental ^{15}N abundance was lower (0.73 and 1.62% for DIN and DON', respectively) than the theoretical abundance. This indicated that, in spite of the care we took in sample processing, the ^{15}N content of the DIN and DON' was diluted by contaminating nitrogen introduced in the filtrate during extraction. The concentration of the additional nitrogen in the filtrate causing this dilution was calculated from the equation:

$$\mu M\ N\ contamination = DIN\ (or\ DON') \times (R_{theoretical}/R_{experimental} - 1) \quad (7)$$

(where R represents atom % excess ^{15}N) and yielded values of 3 and 106 nM for the DIN and DON' extraction, respectively.

^{15}N inventories and mass-balance studies

The time course of nitrate and ammonium uptake was studied in water from the nitrate-depleted mixed layer (nitracline between 100 and 120 m) of the euphotic zone. In these experiments we measured changes in concentration and atom % ^{15}N enrichment of NO_3^- and NH_4^+ and followed the incorporation of ^{15}N-labelled nitrogen into the PON and DON' pool (Table 4). During NO_3^- uptake, DIN concentrations decreased steadily, whereas the atom % ^{15}N enrichment remained constant, thus indicating that no isotope dilution due to nitrification occurred. The ^{15}N isotope accumulated essentially in the PON pool (up to 3 atom % excess) and only a small percentage (0.07 atom % excess) appeared in the DON' pool. During NH_4^+ uptake, isotope dilution in the dissolved fraction was significant, giving a clear indication of NH_4^+ regeneration. Large amounts of ^{15}N (up to 21 atom % excess) were incorporated into the PON pool, but to a much lesser extent into the DON' pool (max. of 0.4 atom % excess). Results from ^{15}N inventory and ^{15}N mass-balance studies using the latter

Table 4. Results from time-series experiments of nitrate and ammonium utilization in oligotrophic waters of the North Atlantic. Local time of start of experiment was 10:00 h and 09:00 h for Stns RH1 and 02-P, respectively

Stn (location)	Depth (m) (% surface light)	Incubation duration (h)	DIN source	DIN_f (µM)	R_{DIN}[a] (%)	PON_f (µM)	R_{PON} (%)	DON'_f (µM)	$R_{DON'}$ (%)
RH1	5	0	NO_3^-	0.045	92.3	0.44	0.000	5.65	0.000
(21° 02' N,	(50)	3.5		0.040	94.3	0.42	0.529	5.13	0.031
31° 08' W)		6.0		0.037	92.5	0.43	1.288	4.77	0.050
		11.0		0.031	89.2	0.42	3.116	5.25	0.049
		19.0		0.028	92.2	0.45	2.321	5.59	0.069
02-P	90	0	NH_4^+	0.50	95.2	0.30	0.000	5.64	0.000
(21° 02' N,	(6)	2.0		0.52	88.9	0.39	1.448	5.40	0.103
31° 08' W)		6.0		0.51	80.5	0.37	6.218	6.15	0.148
		8.0		0.59	70.5	0.39	6.408	5.77	0.197
		9.5		0.51	72.0	0.42	9.310	6.45	0.166
		15.0		0.49	66.9	0.42	14.494	5.68	0.276
		24.0		0.63	50.5	0.38	21.424	6.20	0.397

[a] R designates atom % excess ^{15}N

Table 5. Mass balance of ^{15}N label calculated from data in Table 4. DON' = $PON_{<GF/F}$ + DON

Stn	DIN source	Incubation duration (h)	^{15}N added (nM)	^{15}N in: DIN	$PON_{<GF/F}$ (% of ^{15}N added)	DON'	Total ^{15}N recovered (%)
RH1	NO_3^-	3.5	41.5	90.9	5.4	3.8	100
		6.0		82.4	13.4	5.8	102
		11.0		66.6	31.5	6.2	104
		19.0		62.2	25.2	9.3	97
02-P	NH_4^+	2.0	476	97.1	1.2	1.2	100
		6.0		86.3	4.8	1.9	93
		8.0		87.4	5.3	2.3	95
		9.5		77.1	8.2	2.2	88
		15.0		68.8	12.8	3.4	85
		24.0		66.8	17.1	5.2	89

data are shown in Table 5. Strikingly, at any time during incubation the sum of ^{15}N label remaining in the substrate (DIN) and of ^{15}N incorporated into PON retained on GF/F filters ($PON_{>GF/F}$) was less (by 2 to 16%) than the amount of label initially added to start the experiment. Closer examination of the data reveals that in the NO_3^- uptake experiment, 5.4 to 31.5% of the added ^{15}N label was recovered in $PON_{>GF/F}$ and 3.8 to 9.3% in the DON' pool. The ^{15}N label was completely accounted for by including the ^{15}N found in the latter pool. In the case of NH_4^+ uptake, 1.2 to 17.1 and 1.2 to 5.2% of the added ^{15}N entered the $PON_{>GF/F}$ and DON' pool, respectively. In contrast to the NO_3^- uptake experiment, even with the inclusion of $DO^{15}N'$, a complete mass balance could only be achieved or approached in the first samples of the NH_4^+ time-series (between 2 and 8 h). Significant discrepancies between added and recovered ^{15}N label then appeared in the subsequent samples (>8 to 24 h) and increased in rough correspondence with the length of the incubation period. For example, <90% of the tracer added to the seawater at the beginning of the time-series was recovered in the dissolved and particulate fractions of samples which had been incubated for >8 h.

Pathways of missing ^{15}N due to methodological artifacts

One potential pathway was detected in time-series experiments of NH_4^+ uptake carried out on samples from the bottom of the euphotic layer (Table 6). In these experiments Anopore membranes underlay GF/F membranes during filtration of PON. The GF/F membranes contained on average only 42.1% of the total particulate matter recovered on both filters but 79.6% of the total ^{15}N incorporated, indicating that significant amounts of the missing ^{15}N entered PON from small cells (<0.7 µm, $PON_{<GF/F}$) removed (together with DON) from our GF/F filtrates. Evidence for another potential pathway was obtained from experiments testing for the possibility of non-biological NH_4^+ disappearance, i.e. adsorption of NH_4^+ ions to the walls of incubation bottles. Indeed, a small amount of NH_4^+ (6.1 to 9.4% of the initial amount) disappeared from prefiltered (0.2 µm) and poisoned ($HgCl_2$) seawater enclosed for a period of about 1 d in different types of bottles (Table 7).

DISCUSSION

Recoveries of nitrogen from the DIN (NO_3^-, NO_2^-, NH_4^+) pool were nearly complete (>96%) and comparable to those obtained by other workers for ammonium isolation in seawater (Glibert et al. 1982) and lake water (Fisher & Morrissey 1985, Axler & Reuter 1986). Therefore, fractionation between ^{14}N and ^{15}N during diffusion (Kristiansen & Paasche 1989, Glibert & Capone 1993) may be safely excluded. The small amount of DIN which is not recovered during the initial analytical step seems to result from incomplete NH_3 diffusion, since NO_3^- reduction is complete. The DIN extraction efficiency is constant over a wide range of concentrations (0 to 60 µM) and no detectable amount of DON' is removed (lost) during this extraction step, thus assuring that the nitrogen recovered is specific to DIN.

DON removal efficiency with this method should be high enough to enable collection of nitrogen representative of the DON pool as a whole, including biologically 'inert' fractions as well as fractions capable of fuelling nutrient demands in oligotrophic waters (Jackson & Williams 1985). This statement is, of course, based on the assumption that the wet oxidation totally recovers all organic compounds (Raimbault & Slawyk 1991, Williams et al. 1993).

Table 6. Results from 2 ammonium-uptake time-series experiments conducted in oligotrophic waters of the North Atlantic (21° 02' N, 31° 08' W). Water was sampled at the 6% light penetration depth (~100 m) and filtered on glass-fiber filters (GF/F) with inorganic membranes (Anopore, pore size 0.2 µm) underlying them. Both filter types were analysed for their PON and ^{15}N content to calculate ^{15}N incorporation ($\Delta PO^{15}N$). Local starting time of the experiments was 11:00 h and 15:00 h for Stns 02-8 and 02-55, respectively. At these times the ambient NH_4^+ concentration (initial + ^{15}N-tracer) was 0.94 and 0.25 µM, respectively

Stn	Incubation duration (h)	GF/F PON_f (µM)	GF/F $\Delta PO^{15}N$[a] (nM)	Anopore PON_f (µM)	Anopore $\Delta PO^{15}N$[a] (nM)		$PON_{GF/F}$ (% of total[b])	$\Delta PO^{15}N_{GF/F}$ (% of total[b])
02-8	5.5	0.44	21.1	0.88	7.0		33.3	75.1
	21.8	0.47	48.2	0.90	20.8		34.3	69.9
	26.8	0.50	59.8	0.77	14.3		39.4	80.7
	34.0	0.50	69.2	0.74	16.3		40.3	80.9
02-55	6.8	0.39	13.5	0.79	3.0		33.1	81.8
	19.5	0.49	43.2	0.71	9.9		40.8	81.4
	24.5	0.52	65.9	0.40	8.9		56.5	88.1
	30.5	0.57	69.8	0.40	18.6		58.8	79.0
						$\bar{x}$	42.1	79.6
						SD	10.1	5.3
						n	8	8

[a] $\Delta PO^{15}N = \frac{(PON_f \times R_{PON})}{100}$, where R_{PON} is the atom % excess ^{15}N in PON_f

[b] Total = GF/F + Anopore

The overall accuracy achieved in the determination of ^{15}N abundance, taking account of N contamination and limitations in the mass-spectrometric analysis, is satisfactory and adequate for the measurement of DIN regeneration and DON release. Variabilities in atom % excess enrichment of the DIN pool (CV < 2.0%) and DON' pool (CV < 4.0%) are lower than those found for PON (Dugdale & Wilkerson 1986) and close to those reported for urea (Slawyk et al. 1990b) and DON (Bronk & Glibert 1991).

Our method is simple and suitable for use under field conditions owing to the fact that: (1) no special glassware or apparatus is required, (2) continued intervention of the operator is not needed, (3) DIN and DON are directly recovered in the sample bottle containing the filtrate without any decanting, thus limiting the risk of contamination, (4) ^{15}N cross-contamination between samples is impossible, and (5) DIN and DON extraction from very different types of seawater (with DIN and DON concentrations ranging from 0 to 60 µM) is feasible without any further change in the experimental protocol.

One possible improvement in the extraction method would be to reduce the diffusion period by accelerating NH_3 diffusion with a magnetic stirring bar in the filtrate or by shaking the sample bottles (Kristiansen & Paasche 1989). Furthermore, instead of freezing samples, we now poison our filtrates with $HgCl_2$ (20 mg l^{-1}) and store them at room temperature. This eliminates possible interference of DIN extraction (loss of ammonium?) with freezing and subsequent thawing of filtrates (Macdonald & McLaughlin 1982) and prevents bacteria from growing in the filtrate after the filtration step. The poison does not affect the extraction efficiency for DIN and DON. Finally, given the passage of small particles through GF/F membranes, the filtrate should be subjected to a further filtration step using a small-pore-size filter (0.2 µm), especially when DON release rates have to be estimated.

Our fieldwork data show that, in all samples, a $DI^{15}N$ spike produced a significant increase in ^{15}N abundance (> natural ^{15}N) of DON'. Thus, our findings support the hypothesis that during DIN uptake part of the ^{15}N label is not entering the traditional target pool for ^{15}N ($PON_{>GF/F}$) and instead is lost to this additional pool. The use of Anopore membranes together with GF/F filters clearly demonstrated that a large proportion of particles (>50% of the total) was not retained on GF/F filters and was added to the DON pool in the filtrate. As mentioned in the 'Materials and methods', our DON' pool in fact corresponds to a combined pool of organic nitrogen including DON and $PON_{<GF/F}$. Therefore, we cannot claim that all the missing ^{15}N has been transferred to the DON pool due to phytoplankton excretion (DON release), since the atom % excess ^{15}N in the combined N pool may have resulted to a large extent from the passage of ^{15}N-containing cells through the GF/F filters. Our observation of PON passage through GF/F filters is not new and has already been reported by Altabet (1990), who found that in open-ocean (Sargasso Sea, North Atlantic) samples, 16 to 54% of the total PON and about the same percent-

Table 7. Results from experiments measuring non-biological ammonium disappearance (adsorption) in prefiltered (0.2 µm) nutrient-impoverished seawater filled into glass (Pyrex) or polycarbonate (PC) bottles, spiked with ^{15}N-labelled ammonium and 'incubated' for about 1 d. In the experiment with the 5 l Pyrex bottle, $HgCl_2$ was added to prevent bacterial development during the incubation period. nd: no data

Bottle	$DIN_f(0)$[a] (nM)	$DIN_f(t)$[b] (nM)	DIN disappearance: $DIN_f(0) - DIN_f(t)$ (nM)	% of $DIN_f(0)$
Pyrex (2 l)	690	658.8	42.0	6.1
		651.8		
		633.5		
		$\bar{x}$ 648.0		
		SD 13.1		
PC (2 l)	690	665.5	37.8	5.5
		638.8		
		nd		
		$\bar{x}$ 652.2		
		SD 18.9		
Pyrex (5 l)	480	430.8	45.3	9.4
		461.3		
		428.9		
		417.7		
		$\bar{x}$ 434.7		
		SD 18.7		

[a, b] DIN_f concentration at times 0 and t, respectively

age of total N uptake collected on Anopore membranes passed through GF/F filters.

Direct evidence for a combined pool (DON + bacteria) has recently been obtained by Bronk & Glibert (1994). These authors reported that under oligotrophic conditions (Caribbean Sea, Chesapeake Bay), comparable to our situation in the North Atlantic, 6 to 10% of the ^{15}N label initially added entered the DON + bacteria pool after 0.5 to 1 h incubations. We found similar amounts of ^{15}N in our DON' pool (1.2 to 9.3%), although incubation durations were much longer (2 to 24 h). The inclusion of the DON' pool in the ^{15}N inventory led generally to a better accounting for ^{15}N-label, but mass balance was more difficult to achieve in NH_4^+- than in NO_3^--uptake experiments, especially in samples incubated for longer periods (>8 h). In those samples >10% of the ^{15}N added remained undiscovered at the end of the experiment. These latter discrepancies may be attributable to bottle containment effects. For example, ^{15}N label may have been lost through NH_4^+ uptake by bacteria growing on the surface of incubation bottle walls. Note that final PON concentrations from PC bottles have been shown to be lower (20%) than those from Pyrex bottles, suggesting increased PON adherence to PC bottles (Wilkerson & Dugdale 1992). This effect is likely to be more important during long incubation periods. Another possible effect is adsorption of NH_4^+ ions onto bottle walls. Adsorption of NH_4^+ onto particles is well known from studies on sediments (Mackin & Aller 1984). Bottle walls also seem to have the ability to adsorb NH_4^+ since glass and plastic can act as cation exchangers (Riley 1965). This may explain why mass balance is more difficult to achieve in NH_4^+- than in NO_3^--uptake experiments. To our knowledge, NO_3^- does not adsorb onto particles and probably also not onto bottle walls. We observed that in PC bottles almost 6% of the NH_4^+ stuck to the walls. This would make up about one-half of the ^{15}N that was still missing in samples taken at the end of the incubation.

Losses of ^{15}N-NH_4^+ to the nitrification pathway are unlikely since initial NO_3^- and NO_2^- in filtrates, resulting from NH_4^+ oxidation, were undetectable using a very sensitive method (Raimbault et al. 1990).

Regarding the fate of ^{15}N during DIN uptake, our mass-balance studies revealed an interesting feature. In Fig. 4, the amount of ^{15}N found in the different organic pools is compared with the amount of ^{15}N taken up as DIN. In the NO_3^- experiment the ^{15}N label taken up was nearly equally distributed between

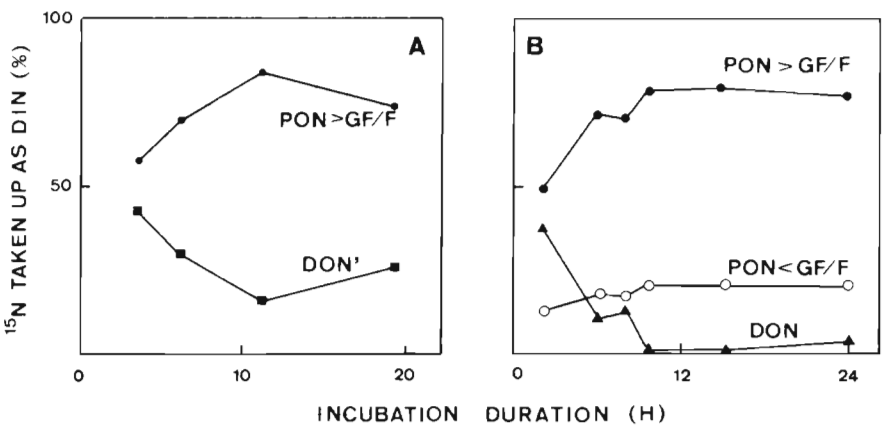

Fig. 4. Time course of % ^{15}N taken up as (A) NO_3^- and (B) NH_4^+, and incorporated into particulate ($PON_{>GF/F}$, $PON_{<GF/F}$) and dissolved (DON) organic nitrogen pools. ^{15}N-NH_4^+ incorporation into $PON_{<GF/F}$ was estimated using findings in Table 6 (i.e. 20.4% of total ^{15}N passed through GF/F filters). ^{15}N-NH_4^+ incorporation into DON was calculated from the amount of ^{15}N in the extracted DON' pool minus the amount of ^{15}N found in $PON_{<GF/F}$

$PON_{>GF/F}$ and DON' at the beginning of the incubation (Fig. 4A). The proportion of the total ^{15}N-NO_3^- uptake represented by ^{15}N incorporation into DON' then decreased with further incubation, down to about 20%. In the NH_4^+ experiment, we attempted to partition the ^{15}N in the combined DON' pool using the data on cell passage through GF/F filters in Table 6. After 2 h of incubation, 50% of the ^{15}N taken up as NH_4^+ was incorporated into $PON_{>GF/F}$, 10% into $PON_{<GF/F}$ and almost 40% were released as DON. The amount of released DON then dropped to about 1% during the following 6 h of incubation, probably as a result of stoppage of DON release and/or bacterial consumption of released DON. This further illustrates that, even when only a small amount of the initially added ^{15}N label is recovered in the DON' pool (1 to 2%), DON release may be highly significant during DIN uptake (Bronk & Glibert 1994). Unless the high DON release early in the incubation was due to stress experienced by the planktonic population when the experiment was set up, it may have reflected the nutritional state of the organisms (Glibert 1993), i.e. nitrogen deficiency in our oligotrophic area. Our findings are also consistent with those reported by Collos et al. (1992), Bronk et al. (1994) and Glibert & Bronk (1994) suggesting that primary production rates measured with the classical ^{15}N method are seriously underestimated (by 50 to 74%) due to DON losses from PON subsequent to DIN uptake or N_2 fixation.

This work has shown that several problems plague the traditional ^{15}N method. We stress that in future ^{15}N uptake studies small-pore-size filters (Anopore or silver filters) should be used in parallel with GF/F filters for collection of PON, at least for samples that are likely to contain small organisms. In addition to DON release, PON losses through GF/F filters may lead to significant underestimation of primary production in oligotrophic waters (up to 20% in the case of NH_4^+ uptake). Our results from the NH_4^+ uptake expriment also point out the need for further evaluation of the magnitude of methodological artifacts (PON adherence and NH_4^+ ion adsorption onto incubation bottle walls) in resolving the problem of the missing ^{15}N.

Given the clear time dependence of DON release during NH_4^+ uptake (Bronk & Glibert 1993, present study) and N_2 fixation (Glibert & Bronk 1994), the most effective approach for studying N cycling in an ecosystem in detail would be to run time-course experiments. Finally, we urge ^{15}N workers to measure $DO^{15}N$ release in order to remove further uncertainties associated with traditional estimates of new and regenerated production. The inclusion of our DON extraction procedure into the experimental protocol of the classical ^{15}N method requires only a limited effort.

Acknowledgements. We thank N. Garcia for her technical assistance throughout the development of the method, F. van Wambeke for bacterial counting and M. C. Bonin for drawing figures and for editing. Three anonymous reviewers provided critical comments on earlier versions of this manuscript. This work is a contribution to the EUMELI (JGOFS-France) Program. Funding was provided from the Centre National de la Recherche Scientifique (INSU).

LITERATURE CITED

Altabet MA (1990) Organic C, N and stable isotopic composition of particulate matter collected on glass-fiber and aluminium oxide filters. Limnol Oceanogr 35:902–909

Axler RP, Reuter JE (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

Bremner JM, Keeney DR (1965) Steam distillation methods for determination of ammonium, nitrate and nitrite. Analyt Chim Acta 32:485–495

Bronk DA, Glibert PM (1991) A ^{15}N tracer method for the measurement of dissolved organic nitrogen release by phytoplankton. Mar Ecol Prog Ser 77:171–182

Bronk DA, Glibert PM (1993) Contrasting patterns of dissolved organic nitrogen release by two size fractions of estuarine plankton during a period of rapid NH_4^+ consumption and NO_2^- production. Mar Ecol Prog Ser 96: 291–299

Bronk DA, Glibert PM (1994) The fate of the missing ^{15}N differs among marine systems. Limnol Oceanogr 39:189–195

Bronk DA, Glibert PM, Ward BB (1994) Nitrogen uptake, dissolved organic nitrogen release, and new production. Science 265:1843–1846

Collos Y, Döhler G, Biermann I (1992) Production of dissolved organic nitrogen during uptake of nitrate by *Synedra planctonica*: implications for estimates of new production in the oceans. J Plankton Res 14:1025–1029

Devarda A (1892) Ueber die direkte Bestimmung des Stickstoffs im Salpeter. Chemiker Ztg 16:1952

Dugdale RC, Goering JJ (1967) Uptake of new and regenerated forms of nitrogen in primary production. Limnol Oceanogr 12:196–206

Eppley RW, Renger EH (1992) Nitrate utilization by plankton in the Equatorial Pacific March 1988 along 150° W. J geophys Res 97:663–668

Fisher TR, Morrissey KM (1985) A new method for the recovery of ammonium from natural waters for measurement of ^{15}N composition in isotope dilution experiments. Mar Chem 16:11–21

Glibert PM (1993) The interdependence of uptake and release of NH_4^+ and organic nitrogen. Mar microb Food Webs 7:53–67

Glibert PM, Bronk DA (1994) Release of dissolved organic nitrogen by marine diazotrophic cyanobacteria, *Trichodesmium* spp. Appl environ Microbiol 60:3996–4000

Glibert PM, Capone DG (1993) Mineralization and assimilation in aquatic, sediment, and wetland systems. In: Knowles R, Blackburn TH (eds) Nitrogen isotope techniques. Academic Press, San Diego, p 243–272

Glibert PM, Lipschultz F, McCarthy JJ, Altabet MA (1982) Isotope dilution models of uptake and remineralization of ammonium by marine plankton. Limnol Oceanogr 27: 639–650

Glibert PM, Lipschultz F, McCarthy JJ, Altabet MA (1985) Has the mystery of the vanishing ^{15}N in isotope dilution experiments been resolved? Limnol Oceanogr 30:444–447

Hansell PA, Goering JJ (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

Hanson BB, Robertson CY (1988) Spring recycling rates of ammonium in turbid continental shelf waters off the southeastern United States. Cont Shelf Res 8:49–68

Jackson GA, Williams PM (1985) Importance of dissolved organic nitrogen and phosphorus to biological nutrient cycling. Deep Sea Res 32:223–235

Kokkinakis SA, Wheeler PA (1987) Nitrogen uptake and phytoplankton growth in coastal upwelling regions. Limnol Oceanogr 32:1112–1123

Koroleff F (1970) Direct determination of ammonium in natural waters as indophenol blue. ICES. Interlab Rep 3: 19–22

Kristiansen S, Paasche E (1989) An improved method for determining relative ^{15}N abundance in ammonium regeneration studies by direct diffusion. Mar Ecol Prog Ser 54: 203–207

La Roche J, Harrison WG (1987) Compartmental models of nitrogen cycling in tropical and temperate marine environments. Mar Ecol Prog Ser 38:137–149

Laws E (1984) Isotope dilution models and the mystery of the vanishing ^{15}N. Limnol Oceanogr 29:379–386

Macdonald RW, McLaughlin FA (1982) The effect of storage by freezing on dissolved inorganic phosphate, nitrate and reactive silicate for samples from coastal and estuarine waters. Water Res 16:95–104

Mackin JE, Aller RC (1984) Ammonium adsorption in marine sediments. Limnol Oceanogr 29:250–257

Paasche E, Kristiansen S (1982) Ammonium regeneration by microzooplankton in the Oslo fjord. Mar Biol 69:55–63

Preston T, Owens NJP (1983) Interfacing an automatic elemental analyser with an isotope ratio mass spectrometer: the potential for fully automated total nitrogen and nitrogen-15 analysis. Analyst 108:971–977

Price NM, Cochlan WP, Harrison PJ (1985) Time course of uptake of inorganic and organic nitrogen by phytoplankton in the Strait of Georgia: comparison of frontal and stratified communities. Mar Ecol Prog Ser 27:39–53

Raimbault P, Slawyk G (1991) A semiautomatic, wet-oxidation method for the determination of particulate organic nitrogen collected on filters. Limnol Oceanogr 36:405–408

Raimbault P, Slawyk G, Coste B, Fry J (1990) Feasibility of using an automated colorimetric procedure for the determination of seawater nitrate in the 0 to 100 nM range: examples from field and culture work. Mar Biol 104: 347–351

Riley JP (1965) Analytical chemistry of sea water. In: Riley JP, Skirrow G (eds) Chemical oceanography, Vol 2. Academic Press, London, p 295–424

Slawyk G, Raimbault P, Gentilhomme V (1990a) On the discrepancies between a colorimetric and isotopic method for measuring nitrate utilization in nutrient-depleted waters: implications for the design of experimental protocols in new production studies. Hydrobiologia 207:333–339

Slawyk G, Raimbault P, L'Helguen S (1990b) Recovery of urea nitrogen from seawater for measurement of ^{15}N abundance in urea regeneration studies using the isotope-dilution approach. Mar Chem 30:343–362

Tréguer P, Le Corre P (1975) Manuel d'analyse des sels nutritifs dans l'eau de mer. Utilisation de l'AutoAnalyzer 2 Technicon, 2nd edn. Univ Bretagne Occidentale, Lab Océanogr Chim, Brest, p 1–110

Ward BB, Kilpatrick KA, Renger EH, Eppley RW (1989) Biological nitrogen cycling in the nitracline. Limnol Oceanogr 34:493–513

Wilkerson FP, Dugdale RC (1992) Measurements of nitrogen productivity in the equatorial Pacific. J geophys Res 97: 669–679

Williams PM, Bauer JE, Robertson KJ, Wolgast DM, Occelli ML (1993) Report on DOC and DON measurements made at Scripps Institution of Oceanography, 1988–1991. Mar Chem 41:271–281

This article was submitted to the editor

Manuscript first received: August 1, 1994
Revised version accepted: March 8, 1995