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A rapid chromatographic method for recovery of $^{15}\text{NO}_2^-$ and NO_3^- produced by nitrification in aqueous samples

Abstract—The sensitivity and comparative simplicity of ^{15}N stable isotopic tracer techniques has been used to quantify rates of nitrification in aquatic systems. However, the most commonly used method for recovery of inorganic oxidized nitrogen compounds from aqueous samples, which is based on liquid-liquid partitioning, is time consuming and contamination prone. We describe a solid-phase rapid chromatographic method for recovery of $^{15}\text{NO}_2^-$ and NO_3^- produced by nitrification in aqueous samples. Compared to liquid-liquid partitioning, the advantages are significantly reduced processing time and reduced potential for contamination. Typical results are presented for the tidal, freshwater reaches of the James River estuary.

Nitrification is an important nitrogen cycling process in marine and freshwater environments. The availability of ^{15}N stable isotopes as tracers and the comparatively inexpensive ^{15}N emission spectrometer has facilitated experimental approaches for the direct measurement of nitrification in various aqueous environments (Harrison 1983; Enoksson 1986). However, the most widely used method (Olson 1981) for conversion of NO_2^- and NO_3^- produced through oxidation of $^{15}\text{NH}_4^+$ to a chemical form recov-

erable for isotopic analysis remains time consuming, costly, and susceptible to contamination. We describe a modification of the existing method that uses solid-phase column chromatography to eliminate repetitive and time-consuming liquid-liquid extractions and to minimize the potential for isotopic dilution resulting from N contamination.

This method has been used to quantify nitrification rates in a tidal freshwater river and estuarine waters by measuring changes in abundance of the stable isotope ^{15}N in NO_3^- - NO_2^- pools over a time course begun after adding $^{15}\text{NH}_4^+$ at tracer concentrations. To determine enrichment in the NO_3^- - NO_2^- pool it was necessary to convert these oxidized compounds to a form suitable for analysis by emission spectrometry. Previously we had used the recovery method described by Olson (1981) that requires chemical complexation of sample NO_2^- with aniline sulfate under acidic conditions. This reaction yields a diazonium salt that is condensed with alkaline β -naphthol to form a base-soluble azo dye. The dye is acidified, reducing its solubility in water, and recovered through solute partitioning by repeated extractions from the aqueous phase into a nonpolar solvent (Olson 1981). Next, the dye is concentrated by solvent evaporation and transferred to an emission tube. Typically, a high vacuum train is used to remove atmospheric N_2 and other interfering gases, the tubes sealed, and the organic N converted to N_2 by a micro-Dumas combustion process (Fiedler and Proksch

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1975). The solvent-partitioning step of the dye recovery procedure is expensive, mechanically intensive, and contamination prone. Contamination of samples by N or C affects the emission spectra and yields inaccurate results (Fiedler and Proksch 1975). Potential sources of contamination include inorganic N in reagents and on glassware surfaces prior to dye formation, N contamination before and after dye formation owing to excess aniline sulfate or other reagents in the nonpolar solvent phase, and organic C from various sources.

Solid-phase column chromatography was adopted for recovery of the azo dye complex from aqueous samples to minimize contamination and decrease processing time. Other steps taken to reduce contamination included adjusting the concentrations of dye reagents, acid-washing of all glassware, use of N-free water, precombustion and storage of cleaned glassware in a drying oven, and rinsing of glassware with methylene chloride before use. In this note, the procedure is described and optimized for processing samples from the tidal, freshwater portion of the James River, Virginia. Sufficient detail is provided to minimize operational uncertainties that sometimes characterize reports describing N stable isotope recovery methods.

All chemicals were reagent grade or better. Solutions were prepared with nitrate-free water treated by reverse osmosis, ion exchange, organic C removal, and membrane filtration (Milli-Q, Millipore Corp.). Reagents were prepared as follows. A tracer solution ($^{15}\text{NH}_4$) $_2\text{SO}_4$ (99 atom % ^{15}N , Cambridge Isotope Labs.) was prepared immediately before starting a time-course experiment. The concentration added generally was very close to 10% of the estimated ambient NH_4^+ pool.

Fresh dye reagents were prepared for each batch of samples to be processed. Standard curves were obtained weekly for each new set of dye reagents and before processing a batch of samples. Dye reagents are light-sensitive and were stored in the dark at 4°C. Under these conditions they were useful for at least 1 month. Aniline sulfate solution was prepared by dissolving 2.0 g of reagent-

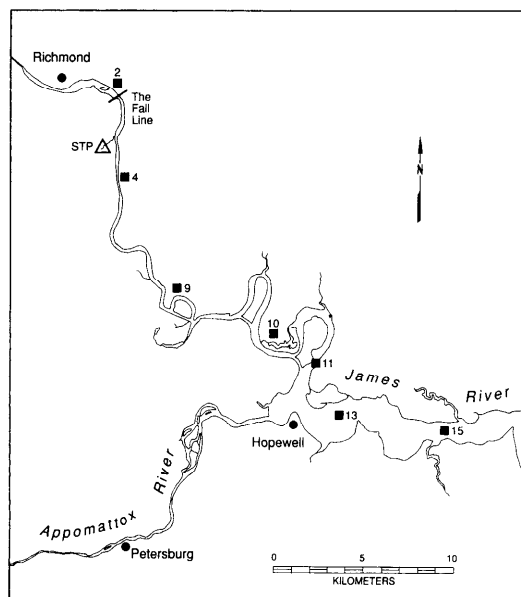


Fig. 1. Upper James River showing locations of sampling stations. Richmond municipal sewage treatment plant—STP. Distance between Sta. 2 and Sta. 15 is 66.3 km.

grade aniline sulfate in 500 ml of 2 N HCl. β -naphthol reagent was prepared by dissolving 1.0405 g of reagent grade β -naphthol (2-naphthol) in 500 ml of 3 N NaOH.

Reagents for the modified Dumas procedure were prepared by combustion at high temperature before sealing emission tubes containing dye samples under high vacuum (Fiedler and Proksch 1975). CaO was combusted by bringing it to 1,000°C and then adjusting the temperature to 700°C. Cuprox (Perkin-Elmer) and CuO reagents were placed in the furnace when the temperature reached 700°C. Reagents were held at 550°C when not being used to process samples.

All glassware was acid washed with 10% HCl and final rinsed with Milli-Q water. After cleaning, emission tubes were wrapped in commercial aluminum foil and combusted at 500°C overnight.

Water samples were collected at 1-m depths along a transect in the James River (Fig. 1). Samples were collected as close to slack-before-flood tidal conditions as possible. Station 4 is just downstream of the Richmond STP outfall (54 MGD). Ambient

NH_4^+ pools were determined at each sampling station the day before collection of field samples to estimate the tracer additions required to approximate 10% of the ambient pools.

All samples, which included controls and three replicates from each site, were collected in opaque containers to avoid light inhibition of nitrification (Lipschultz et al. 1985). Each container had sufficient headspace volume to prevent oxygen limitation. Procedural controls, sampled initially to enumerate nitrifying bacteria, were autoclaved and allowed to cool. All samples and controls were treated with an appropriate amount of $(^{15}\text{NH}_4)_2\text{SO}_4$ (99 atom% ^{15}N) solution and incubated without agitation in the dark at average in situ temperatures. At selected time intervals 250-ml volumes were removed and filtered through precombusted (4 h at 500°C) glass-fiber filters (GFA, Whatman Inc.). This volume requirement was based on prior determinations of ambient NO_3^- pools. Filtrates were subsampled for immediate processing to determine NH_4^+ concentrations and the remainder quick frozen by immersion in liquid N for subsequent determination of ^{15}N enrichment. Incubation intervals of 12, 24, and 48 h were generally used for time-course nitrification assays at temperatures above 10°C.

Sample filtrates were thawed, equilibrated at room temperature, and passed through a copperized Cd reduction column (Strickland and Parsons 1972) to determine ^{15}N enrichment. NO_2^- present in the elutriate (indigenous $\text{NO}_3^- + \text{NO}_2^-$ derived from reduction of NO_3^-) was incorporated into an azo dye (Olson 1981) as previously described. Optimal dye development occurred if 5 min was allowed to elapse after addition of each dye reagent. Dye development times, amounts of dye reagents used, and elution protocols were optimized in a separate series of experiments. The following reagent additions were used per 50-ml sample volume and assume a NO_3^- - NO_2^- pool equivalent to at least 1.5 μM : add 1.5 ml of 2 N HCl and mix; add 2.5 ml of aniline sulfate solution and mix; wait 5 min; add 2.5 ml of β -naphthol solution and mix; wait 5 min; add 2.5 ml of 2 N HCl and mix (final pH of 5–6).

The amount of NO_2^- -N processed (i.e. the amount that can be added to a combustion tube) must be determined for each sample. Sample NO_2^- concentration was determined colorimetrically with a standard curve relating absorbance of the azo dye N-complex (at 500 nm) to known NO_2^- concentrations. The volume of sample required for stable emission was equivalent to that needed to yield 200–300 nmol N. NO_2^- concentrations used to construct the azo dye standard curve ranged from 1 to 10 μM NO_2^- . A blank should verify that laboratory water is NO_2^- -free.

The absolute amount of sample nitrogen that can be assayed is influenced by the emission tube volume because the partial pressure of N_2 required for stable emission is limited to a relatively narrow window equal to 5 ± 3 Torr (Fielder and Proksch 1975). The theoretical amount of N corresponding to this range can be calculated from the ideal gas law. A partial pressure of 5.0 Torr equates to ~ 538 nmol N_2 or 1,076 nmol N per tube with 2.0-ml (final volume) emission tubes. In practice, amounts of sample in the range 200–300 nmol N were used, as these values provided stable emission and reduced sample size. This amount is equal to half of the total N added because the aniline reagent contributes one N atom for each molecule of azo dye formed. The amount chosen was also evaluated for mass effects using samples of equivalent enrichment but varied amounts of total N.

Once a target N value was chosen, machine enrichment values were corrected with a standard curve relating theoretical atom% N enrichment to enrichment measured for an appropriate range of values. Our working range for nitrification assays in riverine samples was 0.0–5.0 atom%. It is recommended that standard curves be constructed for each new batch of dye reagents and to corroborate mass effect curves (over longer intervals) to check for possible errors or procedural problems.

Construction of standard enrichment curves using ambient water samples should be performed to assess the possibility of isotopic dilution owing to contaminant N. Spiking selected volumes of ambient samples with appropriate concentrations of azo dye of known atom% ^{15}N enrichment may

be useful. Sample volumes ≥ 200 ml may be necessary if samples contain small NO_3^- pools (i.e. $\leq 1.2 \mu\text{M}$) to obtain an amount of N necessary for stable emission. Although in our application pools this small were very infrequent, there was an indication that when sample volumes exceeding 200 ml (300–500 ml) were processed with the columns described (300-mg packing bed), the enrichment of recovered standards was reduced. We have not established if this dilution effect was related to overloading of the sorbent phase, perhaps by decreasing its ability to bind the azo dye and affecting elution of unwanted compounds. If significant autochthonous sources of N contamination are suspected, overall recovery efficiencies should be assessed and routinely monitored by spiking ambient or time-course samples with azo dye containing a known atom% N. If feasible, standard curves should be developed with ambient water.

Prepacked columns containing octadecyl (C_{18}) nonpolar bonded silica sorbent (Fisher PrepSep, 12-ml reservoir, 300-mg bed) were placed in a commercial column chromatographic vacuum manifold (e.g. Visiprep solid phase extraction manifold). Vacuum did not exceed 100 mm of Hg.

The chromatographic procedure is as follows. Activate columns with 10 ml of 95% methylene chloride: 5% methanol solution (columns must never be permitted to dry once activated); condition columns with 10 ml of distilled water (DHOH); pass entire sample volume containing azo dye through column; wash column with 10 ml of 1% HCl, discard wash; wash column with 10 ml of DHOH, discard wash; wash column with 10 ml of 0.1 N NaOH, discard wash; wash column with 10 ml of DHOH, discard wash; turn vacuum off; open manifold and insert sample collection rack containing precombusted emission tubes; replace manifold top and apply vacuum; elute azo dye with 2 ml of 95% methylene chloride 5% methanol solution; remove emission tubes from manifold and blow down to dryness with nitrogen gas using a water bath ($\sim 40^\circ\text{C}$).

Modified Dumas reagents were added to emission tubes at the following estimated weights with a spatula: 200 mg of CaO, 500 mg of CuO, and 500 mg of Cuprox. Hot reagents should not come in direct contact

with the dye, so time was allowed for cooling before adding each reagent. Tubes were then attached to a vacuum manifold, evacuated, sealed at $\sim 1-2 \times 10^{-4}$ Torr, and combusted at 550°C for 12 h. Tubes were allowed to cool for at least 4 h before measuring enrichment by emission spectrometry (Jasco N-15 analyzer).

Enrichment in incubated samples was calculated by converting observed sample enrichment to corrected enrichment with a standard curve obtained by fitting the data to a linear regression model. Corrected enrichment values were used to calculate ^{15}N specific activity of each sample. The rate of nitrification R was determined as follows:

$$R = \frac{k[\text{NO}_x]_{\text{pool}}}{t} \quad (1)$$

where

$$k = \frac{\text{atom}\% \ ^{15}\text{NO}_t - \text{atom}\% \ ^{15}\text{NO}_{t=0}}{[^{15}\text{NH}_4^+]/[\text{NH}_4]_{\text{pool}} \text{ at } t = 0} \quad (2)$$

Combining equations yields

$$R = \left\{ \frac{\text{atom}\% \ ^{15}\text{NO}_t - \text{atom}\% \ ^{15}\text{NO}_{t=0}}{([^{15}\text{NH}_4^+]/[\text{NH}_4^+]_{\text{pool}}) \times t} \right\} \times [\text{NO}_x]_{\text{pool}} \text{ at } t \quad (3)$$

where R is rate of nitrification in $\mu\text{mole liter}^{-1} \text{ h}^{-1}$, $[\text{NO}_x]$ the concentration of oxidized N as $\text{NO}_2^-/\text{NO}_3^-$, $^{15}\text{NO}_t$ and $^{15}\text{NO}_{t=0}$ the corrected atom% enrichment in NO_x at time t , $[^{15}\text{NH}_4^+]/[\text{NH}_4^+]_{\text{pool}}$ the specific activity of $^{15}\text{NH}_4^+$ added to the ambient NH_4^+ pool at $t = 0$, and t the time between successive incubation intervals. Average rates for the entire incubation time-course were calculated with simple linear regression when possible.

Olson (1981) developed a method for recovery of $^{15}\text{NO}_2^-$ from seawater as a modification of the method of Wada and Hattori (1972) because of label dilution (reduced sensitivity) attributed to carryover of excess dye components. In our hands, attempts to use Olson's method, which is similar in terms of dye components used to the method of Schell (1978), were generally unsuccessful. Standard curves obtained with this liquid-liquid extraction method (Olson

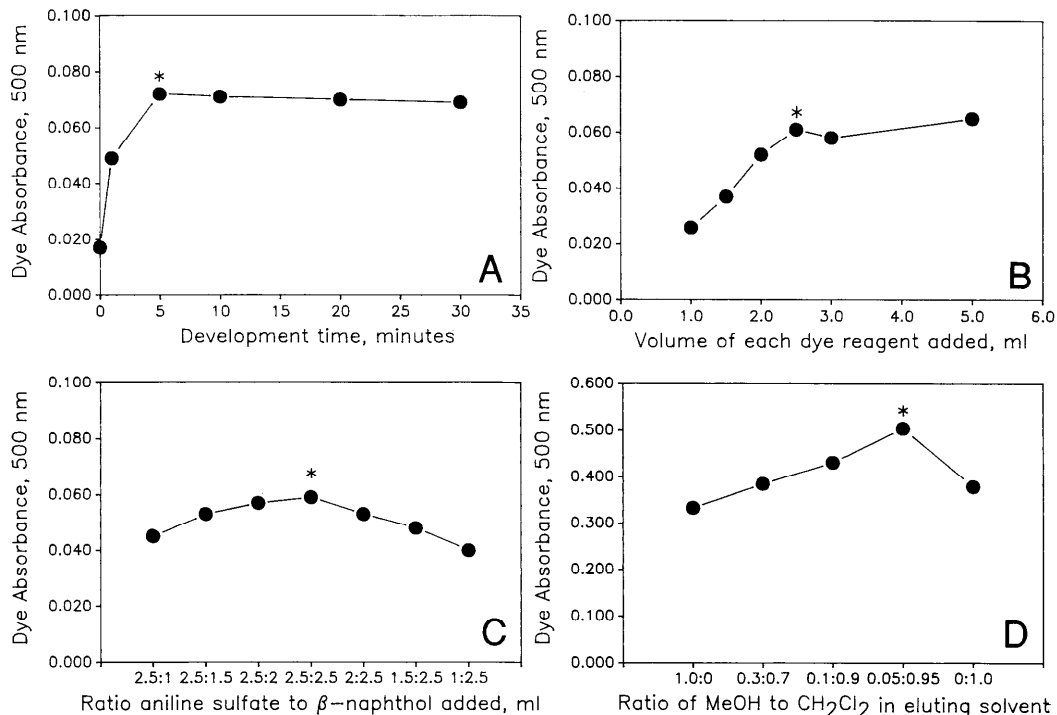


Fig. 2. Optimization of dye reagent volumes, development time, and solvent elution parameters. Azo dye formation was measured spectrophotometrically at 500 nm. Tests were performed with 50- or 100-ml samples of reagent-grade water to which was added 400 nmol N as NO_3^- . Asterisks identify the values adopted for our procedure. A. Time required for maximum dye development following addition of the β -naphthol reagent. B. Effect of increasing the volumes of dye reagents (concentrations given in text) on dye formation. A volume of 2.5 ml was chosen to minimize unreacted dye reagents and to maximize dye formation. C. Effect of varying the volumes of added dye reagents below the 2.5-ml target volume on dye formation. D. Recovery of azo dye from a solid-phase column using various mixtures of methanol to methylene chloride.

1981) were characterized by low r^2 values, unacceptable variation between replicates, and poor analytical sensitivity. We attributed these undesirable characteristics also to carryover of unreacted dye components during liquid-liquid partitioning and to variation of the extraction procedure efficiency.

Reversed phase chromatography has been used for recovery of $^{15}\text{NH}_4^+$ (Selmer and Sorensson 1986; Dudek et al. 1986) and was viewed as an alternative recovery method to improve recovery sensitivity and overall precision by retention of the azo dye. Evaluation of solid phase chromatography as a recovery method required testing to optimize (minimize) the concentrations of dye components, determine the effects of pH and reaction time on dye formation and elution of dye components, and maximize dye re-

covery by selection of appropriate eluants and eluant mixtures. We have incorporated the results of these experiments in the procedure presented here. Representative examples of these results are shown in Fig. 2. These experiments showed that dye reagents added at equimolar concentrations were more effective in dye formation than the nonequimolar concentrations used by Olson (1981). In addition to reducing the amounts used and exposure to toxic organic solvents required for the liquid-liquid method, the processing time for recovery of the dye combined $^{15}\text{NO}_2^-$ from water samples was significantly reduced. Processing 10 samples that previously required 4 h by the Olson liquid-liquid partitioning was now accomplished in <20 min.

Figure 3 compares the relationship between atom% ^{15}N enrichment calculated

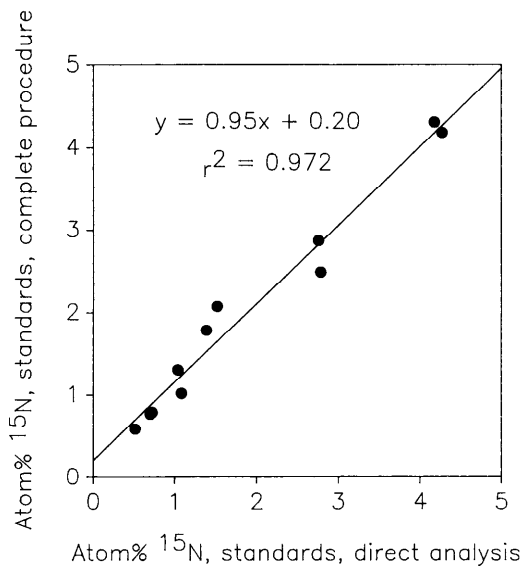


Fig. 3. Linear regression plot of $^{15}\text{NO}_2^-$ standards processed by direct addition of standards to combustion tubes or by addition of standards to water samples followed by azo dye formation and recovery by solid-phase chromatography. Both sets of standards were converted to N_2 with modified Dumas treatment as described.

from replicate ($n = 3$) 50-ml aqueous samples spiked with standard solutions of known atom% ^{15}N enrichment, recovered by reversed-phase chromatography and modified Dumas recovery procedure (Y axis), and enrichment calculated for these same standard solutions directly added to emission tubes, evaporated to dryness by freeze drying, and combusted by modified Dumas treatment (X axis). The linear regression of atom% ^{15}N enrichment values obtained with these procedures yielded a good fit, $r^2 = 0.97$, and a slope of 0.95 ($y = 0.95x + 0.20$). A slope of 0.95 indicated the reversed-phase procedure produced a 5% reduction in atom% ^{15}N enrichment compared to direct addition of standard solutions. The small positive intercept can be interpreted as background signal owing to the carryover of reagents or contaminants whose combustion products may affect the intensity of the nitrogen emission spectra.

The usefulness of the recovery method presented is a function of its precision relative to changes in isotopic enrichment measured in treated samples. Figure 4 shows

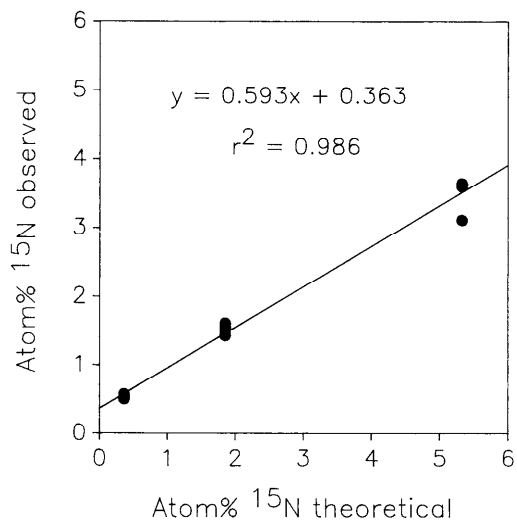


Fig. 4. Linear regression plot of typical calibration curve relating calculated atom% ^{15}N enrichment in standards to observed machine response. Four replicate determinations were performed for each enrichment value.

a typical calibration curve relating calculated atom% enrichment to observed machine response. Four replicate determinations were performed at each of three enrichment values. Although the standard errors between replicates were low, i.e. 2.6–3.7% (0.04–0.12 atom% ^{15}N enrichment), error associated with construction of the calibration line must also be considered to calculate overall precision (Larsen and Wagner 1975). Table 1 shows the effect of replicate sample determinations on the 95% confidence levels for values of X' calculated for two observed enrichment values of Y' .

These results clearly support replication as essential to maximize analytical precision and verify the need for careful technique to minimize scatter in data used to

Table 1. Comparison of 95% confidence intervals for values of X' corresponding to replicate samples of Y' equal to 1.0 and 2.0 atom% enrichment. Calculations based on the regression equation shown in Fig. 2.

Replicates	Y'	
	1.0 atom%	2.0 atom%
$n = 2$	1.07 ± 0.47	2.76 ± 0.45
$n = 3$	1.07 ± 0.27	2.76 ± 0.25
$n = 4$	1.07 ± 0.22	2.76 ± 0.20

Table 2. Field data and rates of nitrification in the upper freshwater, tidal portion of the James River, Virginia. (Not available—na; not detected—ND.)

Sta.	Temp. (°C)	DO (mg liter ⁻¹)	[NH ₄ ⁺] _{pool} (μM)	[NO ₂ ⁻ + NO ₃ ⁻] (μM)	Nitrification (μmol liter ⁻¹ h ⁻¹)
Jul 86					
2	29.5	7.1	1.8	2.4	ND
4	30.0	6.7	75.4	8.0	ND
9	34.6	5.1	40.1	34.9	0.058
10	32.0	5.4	15.0	36.0	0.045
11	30.0	5.8	8.2	44.4	0.131
13	30.0	5.9	11.5	54.2	0.020
15	29.0	6.5	6.5	56.6	0.141
Jan 87					
2	5.0	na	5.3	35.8	ND
4	4.6	na	34.7	23.0	ND
9	7.0	na	40.2	29.5	ND
10	6.0	na	37.7	28.6	ND
11	5.8	10.6	37.1	18.1	0.003
13	5.8	10.3	48.1	29.5	0.004
15	5.0	10.8	108.6	20.7	ND
Jul 87					
2	31.5	7.5	2.4	1.5	0.002
4	31.5	6.5	46.8	5.8	0.352
9	34.1	6.6	19.4	8.3	0.296
10	31.9	7.4	11.1	11.2	0.031
11	31.0	7.1	6.9	26.7	0.014
13	31.2	7.2	32.6	29.5	0.122
15	29.5	7.3	0.9	24.4	0.003
Aug 87					
2	31.2	7.4	2.4	1.9	ND
4	31.4	7.8	29.2	9.8	0.018
9	36.0	7.3	19.7	19.3	0.015
10	32.9	6.4	14.3	27.9	0.150
11	30.1	6.0	4.2	18.0	0.082
13	30.6	6.3	8.3	15.5	0.151
15	30.5	7.3	0.6	10.8	0.016

fit the standard curve regression line. Larsen and Wagner (1975) noted that the confidence interval will be decreased by choosing values of X which bracket the anticipated values of X and if these values are close to the mean values of X' and Y' on the calibration line. Experiments should be designed so that final sample enrichment will exceed the calculated precision.

This analytical procedure has been applied to various riverine and estuarine samples over a range of seasonal conditions and NH₄⁺ pool concentrations to calculate average nitrification rates. Representative data from four surveys of the tidal, freshwater portion of the upper James River (Fig. 1)

are shown in Table 2. Station 2 is above the fall line and station 4 is situated immediately below the Richmond sewage treatment plant discharge. The distance from station 2 to 15 is ~66 km. Rates observed are in the upper range of those reported for oxidation of NH₄⁺ to NO₂⁻ in the York River, Virginia, and in Chesapeake Bay (Kaplan 1983).

We have developed and applied a solid-phase extraction technique to measure nitrification that provides distinct advantages in terms of analytical speed and freedom from reagent contamination. Nitrification rate data obtained with this method are consistent with previous estimates and provide temporal and spatial information about this process in the tidal, freshwater portion of the James River. Future applications will focus on the relationships between nitrification and factors such as N pool concentration, temperature, nitrifier biomass, total suspended solids, distance from discharges, water depth, and light in the tidal, estuarine reaches.

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Nitrate and organic N analyses with second-derivative spectroscopy

Abstract—Simple and reliable procedures have been developed for analyses of NO_3^- , total N, and organic N in freshwaters. NO_3^- is determined by second-derivative UV spectroscopy. Total N and organic N are determined based on second-derivative analyses of NO_3^- following persulfate digestion. Resolution of organic N determinations was increased by using ion-exchange resins to remove NO_3^- from samples with high concentrations of NO_3^- prior to persulfate oxidation of the organic N.

Although numerous methods have been developed for the determination of N in aqueous samples, there is continued need for simple and reliable procedures for both inorganic and organic N analyses. The most widely accepted methods for NO_3^- analysis include Cd reduction and ion chromatography (Am. Public Health Assoc. 1989). Both methods have been adapted for automated analysis and, with proper consideration of potential interferences, both are accurate and precise. Ion chromatography has the advantage of also measuring several additional anions in a single aliquot of sam-

ple. Ion chromatography and Cd reduction, however, are relatively complicated and expensive. Samples must normally be filtered to remove suspended matter before analysis, and in the case of Cd reduction, sample color, metal ions, and phosphate are potential interferences (Olson 1980).

The principal methods for organic N determination are based on conversion of organic N to NH_4^+ or NO_3^- and subsequent analysis of these inorganic forms. Separate analysis of ambient inorganic N allows organic N levels to be calculated by difference. For freshwater samples, conversion of organic N to inorganic forms is most commonly based on either Kjeldahl digestion (U.S. EPA 1979; Am. Public Health Assoc. 1989), photo-oxidation (Armstrong et al. 1966), or persulfate digestion (Koroleff 1976; D'Elia et al. 1977; Solórzano and Sharp 1980). Photo-oxidation requires more specialized equipment and may be ineffective for some compounds (Henriksen 1970). Kjeldahl digestion is a complicated and expensive procedure and may be unsuitable for systems receiving high inorganic N loads. High concentrations of NO_3^- severely interfere with standard Kjeldahl N determinations (Schlueter 1977; U.S. EPA 1979; Am. Public Health Assoc. 1989). NO_3^- -N concentrations 10–20 times greater than those of organic N result in >90% inhibition of Kjeldahl N determinations (Schlue-

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