Direct Determination of Nitrification in Marine Waters by Using the Short-Lived Radioisotope of Nitrogen, ^{13}N

DOUGLAS G. CAPONE,^{1*} SARAH G. HERRIGAN,² SUSAN E. DUNHAM,² AND JOANNA FOWLER³

*Center for Environmental and Estuarine Studies, Chesapeake Biological Laboratory, Solomons, Maryland 20688-0038¹;
Marine Sciences Research Center, State University of New York, Stony Brook, New York 11794²; and
Division of Chemistry, Brookhaven National Laboratory, Upton, New York 11973³*

Received 18 October 1989/Accepted 9 January 1990

Biological oxidation of radiolabeled $^{13}\text{NH}_4^+$ (half-life = 10 min) was observed within minutes in assays of an estuarine ammonium oxidizer and in natural populations of nitrifiers in coastal waters. Our estimates of turnover of the ammonium pool and rates of nitrification based on experiments using ^{13}N are consistent with previous values in the literature based on longer-term ^{15}N tracer experiments or on indirect methods and thus provide corroboration for the estimates by other researchers.

In the sea, autotrophic nitrification is considered an important component of the marine nitrogen cycle since the largest pool of inorganic nitrogen is nitrate, the end product of nitrification (9). Furthermore, nitrification appears to be a major natural source of N_2O , a trace greenhouse gas of current concern (3). However, little is known about the in situ rates of this process since direct estimates of the rate of nitrification are rare and hard won (9), a result of the apparently low activity of marine nitrifiers and the inherent insensitivity of available means of direct determination of nitrification rate. Several sensitive, indirect procedures have been developed (11) but do not provide a straightforward means of quantitating nitrification rate (9, 11).

In contrast to biogeochemical studies of carbon, phosphorus, and sulfur, investigations of nitrogen have been hampered by the lack of a convenient radioisotope. However, with access to appropriate facilities, a short-lived radioactive isotope of nitrogen (^{13}N , half-life of about 10 min) is available. The disadvantages of the short half-life of the isotope and the need to perform experiments at or near the site of isotope production are offset by the high sensitivity inherent with ^{13}N (13), which allows assays over brief periods and quick results compared with experiments with stable isotopes. In fact, with the radioactive yields obtainable (>10 mCi), the effective use time of the isotope can be in excess of 1 h, which is sufficient for direct determination of rate constants for many transformations of interest. Furthermore, it is possible to perform true tracer experiments and directly assess the rate of particular transformations of nitrogen since a natural pool can be labeled without any significant change in the pool size. While the ^{13}N radioisotope approach cannot practically supplant routine approaches for determining in situ rates of biologically catalyzed reactions of nitrogen, it provides complementation and independent confirmation of more widely used approaches. Furthermore, it is an important adjunct tool for addressing specific ecological or physiological questions.

^{13}N has been used in biomedical, biochemical, physiological, and ecological studies (2). Previous ecological studies have examined inorganic nitrogen uptake by lake phytoplankton (8) and denitrification of soil (14) and lake sediment (7). Except for our recent efforts (5, 16, 17), however, to our knowledge ^{13}N has never been used in studies of nitrogen

cycling in marine ecosystems or in any investigations of nitrification. This is somewhat surprising, given the difficulty in measuring the nitrification process by other means and the perceived importance of nitrification in nitrogen cycling in terrestrial and aquatic systems. It is noteworthy that Schell (13) specifically considered the potential advantages of a ^{13}N approach to nitrification studies. We report here the first direct determination with ^{13}N of rates of nitrification in natural samples.

^{13}N was produced by the $^{16}\text{O}(\text{p},\alpha)^{13}\text{N}$ reaction using the Brookhaven National Laboratory 40- or 60-in. (ca. 100- or 150-cm) research cyclotron to bombard small volumes (0.5 ml) of H_2^{16}O with 16 to 18 MeV protons (15). The irradiated H_2O contained predominantly $^{13}\text{NO}_3^-$. Checks on radiochemical purity of $^{13}\text{NO}_3^-$ were routinely run by anion-exchange high-pressure liquid chromatography (HPLC) radiochromatography (1). Yields of up to 100 mCi were delivered from the target. $^{13}\text{NH}_4^+$ was produced by reacting the target water containing $^{13}\text{NO}_3^-$ with 0.5 g of deVarda alloy and 0.5 g of NaOH (6, 15) and purging and trapping the $^{13}\text{NH}_3$ in 1 ml of dilute acid, deionized water, or seawater (with similar recoveries). This volume was passed through 1 ml of an anion-exchange resin (Bio-Rad AG 1 $\times$ 8, Cl^-) to remove traces of $^{13}\text{NO}_3^-$ and $^{13}\text{NO}_2^-$. The resin was rinsed with 1 ml of water.

HPLC analysis of the trapping solution after it passed through the anion-exchange column found only $^{13}\text{NH}_4^+$ with no traces of $^{13}\text{NO}_3^-$ or $^{13}\text{NO}_2^-$. Yields of up to 30 mCi (actual activity after 5 to 10 min of reaction) of $^{13}\text{NH}_4^+$ have been achieved. $^{13}\text{NH}_4^+$, $^{13}\text{NO}_3^-$, and $^{13}\text{NO}_2^-$ were separated on a Partisil SAX column (10 μm) with a mobile phase of 0.03 M NaH_2PO_4 (pH 3) flowing at 1.5 ml/min (1); fractions were collected and radioactivity was quantified by gamma spectrometry. Retention times were confirmed by UV absorption at 210 nm with standards of NO_3^- and NO_2^- . Approximate retention times for NH_4^+ , NO_2^- , and NO_3^- were 2, 5, and 7 min, respectively.

In general, experiments were initiated by combining 5 or 10 ml of either an enrichment culture of an estuarine ammonium oxidizer or a natural water sample with about 0.5 ml of the $^{13}\text{NH}_4^+$ solution. Two methods were used to monitor $^{13}\text{NO}_2^-$ production: (i) discrete separation of NO_3^- and NO_2^- pools from NH_4^+ by anion-exchange HPLC (7-min run time; see above) and counting of fractions and (ii) a more rapid procedure in which samples taken over a period of time

* Corresponding author.

FIG. 1. $[^{13}\text{N}]$ ammonium oxidation to NO_2^- by an estuarine ammonium oxidizer as analyzed (i) by direct HPLC radiochromatography and (ii) by determination of radioactivity passing through a cation resin bed of Dowex 50W-X, Na form (resin rinse method).

were passed through a cation resin to remove NH_4^+ and its associated radioactivity and then the radioactivity in the eluate (resin rinse) was counted. Over the course of about 60 min, small subsamples (100 μl to 1 ml) of the assay mixture were periodically removed and applied to a 1- to 2-ml wetted bed of Dowex 50W-X contained in a 3-ml syringe barrel or a small, fritted, plastic chromatography column. The resin was immediately rinsed with three 1-ml washes of distilled water, and the combined rinses were counted for total radioactivity. For HPLC, a 1-ml subsample of the rinse was used after it was counted.

For the resin rinse method, experiments were also performed with samples of 0.2 μM filtered seawater to assess the extent of any breakthrough of $^{13}\text{NH}_4^+$ past the cation resin. In later experiments on natural seawater, a nitropryrin (N-Serve, a nitrification inhibitor) control was also used. Attempts to use the HPLC approach on the seawater samples were unsuccessful, possibly because of much lower nitrifier densities and aggregate activities in the natural samples than in the enrichment cultures.

We initially developed our methods using an enrichment

TABLE 1. Nitrification in enrichment cultures of an estuarine ammonium oxidizer, clone B19, as estimated by tracers and by mass balance

Date (day/mo/yr)	^{13}N analysis method	$[\text{NH}_4^+]$ (μM)	^{13}N (%/h)	Nitrification rate ($\mu\text{mol/liter per h}$)		
				^{13}N	$^{15}\text{NH}_4^+$	ΔNO_2^-
15 Jan 1985	RR ^a	46	3.12	1.43	ND ^b	2.36
	HPLC	46	3.59	1.65	ND	2.36
26 Feb 1985	RR	100	1.37	1.36	ND	5.6
	HPLC	100	1.43	1.42	ND	5.6
24 Apr 1985		ND	ND	ND	1.55	8.0
22 May 1985		ND	ND	ND	1.38	3.5

^a RR, Cation resin rinse.

^b ND, Not determined.

culture of an estuarine ammonium oxidizer raised in batch culture for which we could estimate ammonium oxidation rates over scales of hours to days by monitoring NH_4^+ and NO_2^- pools. $^{13}\text{NO}_2^-$ production was clearly shown by radiochromatography over approximately 0.5-h time courses, and there was very good agreement between the resin rinse and HPLC radiochromatography methods in several experiments (Fig. 1, Table 1). Use of the resin rinse method allowed more frequent and elaborate sampling design than HPLC radiochromatography. On two occasions, we measured rates of nitrification in this culture by using the heavy stable isotope ^{15}N following the incorporation of label into the NO_2^- pool over a period of several hours. Rates measured with either ^{13}N and ^{15}N (on separate days) during the exponential phase of growth were in close agreement (Table 1). However, rates calculated from changes in concentration of NO_2^- or NH_4^+ were often two- to fourfold higher. We suspect that the lower, comparable rates measured with either ^{13}N or ^{15}N may have been an artifact of the lower pH (about 6.7) of the cultures sampled near stationary phase compared with that at the initiation of the batch cultures (typically at seawater pH of 7.8) and the resultant lower availability of the presumed substrate, NH_3 , at the lower pH. The equilibration time of the isotope and ambient pools, if slow relative to the assay period, may also contribute to our finding. We plan further experiments to examine this result more closely.

TABLE 2. Direct estimates of rate of nitrification in water column sample from Long Island Sound using the short-lived radioisotope $^{13}\text{N}^a$

Date (day/mo/yr)	Site ^b	N-Serve added	$[\text{NH}_4^+]$ (μM)	Nitrification rate ^c		NSA ^d		Ratio (NSA/TOT) ^e
				%/h	nmol of NH_4^+ / liter per h	%/h	nmol of NH_4^+ / liter per h	
23 July 1985	CN	—	1.1	8.01	88			
18 Sept 1985	CN	—	1.0	7.66	77			
6 May 1986	CB	—	2.1	3.98	84			
		+		0.84		3.14	66	0.79
13 May 1986	CB	—	4.0	4.03	161			
		+		0.66		3.37	135	0.84
20 May 1986	CB	—	6.0	4.90	294			
		+		0.50		4.40	264	0.90
17 June 1986	CB	—	4.2	1.78	75			
		+		0.67		1.11	47	0.62

^a Nitropryrin (N-Serve, or 2-chloro-6-trichloromethyl pyridine) was added to a final concentration of 100 ppm.

^b CN, Crane Neck Beach; CB, Cedar Beach.

^c Corrected for any NH_4^+ breakthrough past the cation resin.

^d NSA, N-Serve-sensitive activity, or (total nitrification - N-Serve-insensitive activity).

^e N-Serve-sensitive nitrification/total nitrification (TOT).

[^{13}N]ammonium added to samples freshly collected from Long Island Sound and assayed within 3 h was rapidly converted to oxidized end products (Table 2). In 1985 studies, about 8% of the NH_4^+ pool per h was converted in samples collected in the summer, while a somewhat lower pool turnover (1.8 to 5%/h) was noted in the samples collected in May and June 1986. When the daily observed concentrations of ammonium were used, nitrification rates varied from 75 to about 300 nmol/liter per h. The addition of nitrapyrin resulted in 60 to 90% inhibition of the counts passing the resin, confirming that the activity was predominantly attributable to nitrifiers. The absence of complete inhibition may be a result either of nitrapyrin-insensitive nitrifiers or of products of ammonium oxidation which do not arise through the classical autotrophic nitrification pathway.

The overall impact of nitrification on water column nitrogen cycling appears to change dramatically throughout the season. Parallel estimates of ammonium uptake at the Crane Neck site in May indicated utilization of the NH_4^+ pool at a rate of about 1.5%/h (C. A. Suttle, J. Fuhrman, and D. G. Capone, *Limnol. Oceanogr.*, in press) (cf. 3.1 to 4.4%/h for nitrification; Table 1). As temperatures increased and NH_4^+ concentrations decreased, ammonium uptake accounted for 460%/h by the end of June, far surpassing nitrification rates.

Our ^{13}N -based values for Long Island Sound water column samples fall within the range of recent estimates of nitrification in estuaries; these estimates were based on longer-term assays with $^{15}\text{NH}_4^+$ (9, 10) and shorter-term, dark $^{14}\text{CO}_2$ -nitrification inhibitor assays (4, 12) and are considerably higher than estimates for open ocean waters (9). Thus, we can provide independent confirmation of the validity of these prior estimates and are able to confirm that nitrification is an active and at times a quantitatively important process in the water column of estuaries.

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LITERATURE CITED

1. Chasko, J. H., and J. R. Thayer. 1981. Rapid concentration and purification of ^{13}N -labelled anions on a high performance anion exchanger. *Int. J. Appl. Radiat. Isot.* 32:645-649.
2. Cooper, A. J., A. S. Gebard, and B. R. Freed. 1985. Nitrogen-13 as a biochemical tracer. *Adv. Enzymol.* 57:251-356.
3. Elkins, J. W., S. C. Wofsy, M. B. McElroy, C. E. Kolb, and W. A. Kaplan. 1978. Aquatic sources and sinks for nitrous oxide. *Nature (London)* 27:602-606.
4. Enoksson, V. 1986. Nitrification rates in the Baltic Sea: comparison of three isotope techniques. *Appl. Environ. Microbiol.* 51:244-250.
5. Fuhrman, J., S. G. Horrigan, and D. G. Capone. 1988. Use of ^{13}N as a tracer for bacterial and algal uptake of ammonium from seawater. *Mar. Ecol. Prog. Ser.* 45:271-278.
6. Gelbard, A. S., L. P. Clarke, J. M. McDonald, W. G. Monahan, R. S. Tilbury, K. Y. T. Kuo, and J. S. Laughlin. 1975. Enzymatic synthesis and organ distribution studies with ^{13}N -labelled L-glutamine and L-glutamic acid. *Radiology* 116:127-132.
7. Gersberg, R., K. Krohn, N. Peek, and C. R. Goldman. 1976. Denitrification studies with ^{13}N -labeled nitrate. *Science* 192:1229-1231.
8. Gersberg, R. M., R. P. Axler, K. Krohn, N. Peek, and C. R. Goldman. 1978. Nitrate uptake by phytoplankton: measurements utilizing the radiotracer ^{13}N . *Verh. Internat. Verein. Limnol.* 20:388-392.
9. Kaplan, W. A. 1983. Nitrification, p. 139-190. *In* E. J. Carpenter and D. G. Capone (ed.), *Nitrogen in the marine environment*. Academic Press, Inc., New York.
10. Lipschultz, F., S. C. Wofsey, and L. E. Fox. 1986. Nitrogen metabolism of the eutrophic Delaware River ecosystem. *Limnol. Oceanogr.* 31:701-716.
11. Oremland, R. S., and D. G. Capone. 1988. Use of specific inhibitors in microbial ecological and biogeochemical studies. *Adv. Microb. Ecol.* 10:285-383.
12. Owens, N. J. P. 1986. Estuarine nitrification: a naturally occurring fluidized bed reaction? *Estuar. Coastal Shelf Sci.* 22:31-44.
13. Schell, D. M. 1978. Chemical and isotopic methods in nitrification studies, p. 292-295. *In* D. Schlessinger (ed.), *Microbiology—1978*. American Society for Microbiology, Washington, D.C.
14. Tiedje, J. M., R. B. Firestone, M. R. Firestone, M. R. Betlach, M. S. Smith, and W. H. Caskey. 1979. Methods for the production and use of nitrogen-13 in studies of denitrification. *Soil Sci. Soc. Am. J.* 43:709-719.
15. Vaalberg, W., J. A. A. Kaurphais, H. D. Beerling-van der Molen, S. Reiffers, A. Rijkskamp, and M. G. Woldring. 1975. An improved method for the cyclotron production of ^{13}N -labeled ammonia. *Int. J. Appl. Radiat. Isot.* 26:316-318.
16. Zehr, J., P. Falkowski, and D. G. Capone. 1988. Coupling between ^{13}N -ammonium uptake and incorporation in a marine diatom. *Limnol. Oceanogr.* 33:518-527.
17. Zehr, J. P., D. G. Capone, and P. G. Falkowski. 1989. Rapid incorporation of $^{13}\text{NO}_3$ by NH_4 -limited phytoplankton. *Mar. Ecol. Prog. Ser.* 51:237-241.