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Sub-Parts-Per-Billion Nitrate Method: Use of an N_2O-Producing Denitrifier to Convert NO_3^- or $^{15}NO_3^-$ to N_2O†

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A more sensitive analytical method for NO_3^- was developed based on the conversion of NO_3^- to N_2O by a denitrifier that could not reduce N_2O further. The improved detectability resulted from the high sensitivity of the ^{63}Ni electron capture gas chromatographic detector for N_2O and the purification of the nitrogen afforded by the transformation of the N to a gaseous product with a low atmospheric background. The selected denitrifier quantitatively converted NO_3^- to N_2O within 10 min. The optimum measurement range was from 0.5 to 50 ppb (50 μg/liter) of NO_3^- N, and the detection limit was 0.2 ppb of N. The values measured by the denitrifier method compared well with those measured by the high-pressure liquid chromatographic UV method above 2 ppb of N, which is the detection limit of the latter method. It should be possible to analyze all types of samples for nitrate, except those with inhibiting substances, by this method. To illustrate the use of the denitrifier method, NO_3^- concentrations of <2 ppb of NO_3^- N were measured in distilled and deionized purified water samples and in anaerobic lake water samples, but were not detected at the surface of the sediment. The denitrifier method was also used to measure the atom% of ^{15}N in NO_3^-. This method avoids the incomplete reduction and contamination of the NO_3^--N by the NH_4^+ and N_2 pools which can occur by the conventional method of $^{15}NO_3^-$ analysis. N_2O-producing denitrifier strains were also used to measure the apparent K_m values for NO_3^- use by these organisms. Analysis of N_2O production by use of a progress curve yielded K_m values of 1.7 and 1.8 μM NO_3^- for the two denitrifier strains studied.

Over the past century, numerous methods have been developed to measure NO_3^-. Among the currently available methods, the choice of which method to use is based on whether the objective is high sample capacity (autoanalyzer), sensitivity (ion chromatography), or simplicity and low cost (batch colorimetric methods). While these methods serve many needs, there are two situations in which they are deficient. First, in certain samples from terrestrial, aquatic, and animal habitats, the NO_3^- concentration is below the detection limits of existing NO_3^- methods, yet biological transformations of nitrate still occur. Knowledge of the NO_3^- pool size is usually important in understanding the dynamics of the NO_3^- transformation process. The second limitation of current methods is that in some samples there are compounds that interfere with the NO_3^- analysis (6).

The most sensitive methods currently available are based on prior purification of NO_3^- by ion chromatography, followed by NO_3^- quantitation with UV spectrophotometric or conductometric detectors; the detection limits range from 2 ppb (2 μg/liter) of N (R. M. Edwards, A. J. Sexstone, and J. M. Tiedje, Agron. Abstr. p. 152, 1980) to 35 ppb of N (9, 13), respectively, for the two types of detectors.

The new method described here is based on the analysis of N_2O after its production from NO_3^- by an N_2O-producing denitrifier. The method takes advantage of the following principles: (i) that N_2O N detection by electron capture gas chromatography is three orders of magnitude more sensitive than the autoanalyzer measurements of NO_3^- N, for example; (ii) that specificity for NO_3^- can be increased by using an enzymatic reaction; (iii) that substantial purification from interfering substances is achieved by conversion of the measured atom to a gas; and (iv) the low background of N_2O. The concept of NO_3^- measurement by conversion to N_2O has been used before by Lensi et al. (8) and M. Müller (Department of Microbiology, University of Helsinki, personal communication, 1982), but they used anaerobic soil slurries to catalyze the NO_3^- reduction and acetylene to cause N_2O to accumulate. This procedure probably does not work at low NO_3^- concentrations, unless acid soil samples or soil samples with very low microbial activity are used, as the acetylene inhibition of N_2O reduction often fails for more standard soil samples under these conditions (12, 17). Furthermore, it is more difficult to standardize procedures and achieve very low detection limits with a variable and less well characterized catalyst like soils. We overcame these limitations by using a denitrifying pure culture that lacks N_2O-reducing capacity.

This method can also be used to avoid a major problem in the isotopic analysis of $^{15}NO_3^-$. The standard $^{15}NO_3^-$ method relies on prior removal of NH_4^+ from the sample and then reduction of NO_3^- to NH_4^+, which is subsequently oxidized to N_2 for introduction into the mass spectrometer (7). The NO_3^- reduction is often incomplete and is contaminated by residual reduced N forms when it passes through the NH_4^+ pool. Other methods of avoiding the NH_4^+ pool have been suggested, but they are complex, e.g., NO_3^- conversion to NO (16) or diazotization and coupling to form an extractable dye (10). Our method also avoids the problems associated with reduction to NH_4^+, is simple, and produces N_2O rather than N_2 and thus avoids errors due to atmospheric leaks.

The purpose of this study was to describe a denitrifier method for measuring NO_3^- at very low concentrations, to evaluate its performance relative to an established method,

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and to illustrate its use with samples of several types. Furthermore, we also used it to provide apparent K_m values for NO_3^- use by denitrifiers, which have been difficult to obtain because of the lack of sensitive methods for NO_3^- analysis.

MATERIALS AND METHODS

Organism and its kinetic characterization. The denitrifying bacterium used in this study was obtained from a Danish agricultural field (4) and was isolated on streak plates from a tube containing nitrate broth (Difco Laboratories, Detroit, Mich.) inoculated with a serial dilution of soil. When grown with NO_3^- and under He, N_2O and not N_2 was detected as the product (4). All subsequent cultivations of the organism were done on tryptic soy broth (Difco) with 0.1% KNO_3. The organism was identified as *Pseudomonas chlororaphis* (formerly *P. fluorescens* biotype D) by Microbial ID, Inc., Newark, Del. A fatty acid microbial identification system (5898A; Hewlett-Packard Co., Palo Alto, Calif.) was used for identification. The culture has been deposited with the American Type Culture Collection, Rockville, Md., and has accession no. 43928.

To evaluate the kinetic parameters and the stoichiometry of the NO_3^- conversion to N_2O, we inoculated resting cells of the denitrifier into a chemostat vessel bubbled with an Ar gas stream (4a). The vigorous mixing and gas stripping provided a more instantaneous recovery of N_2O in the gas effluent, which could be more quickly and accurately measured by gas chromatography than by the more conventional shaking and sampling of the headspace gas. The measured flow rate × N_2O concentration gave an N_2O flux which could be integrated and corrected for dissolved N_2O (as described below) to obtain a pattern of N_2O accumulation. The integrated Michaelis-Menten equation in product appearance form was fit by nonlinear regression (by the method of Marquardt [12]) to the cumulative N_2O concentrations to obtain estimates of V_{max} and K_m for NO_3^- use. This model was obtained by substituting $S_0 - k_eP$ for S in the integrated Michaelis-Menten equation in substrate disappearance form (11), where S and P are substrate and product concentrations, respectively; S_0 is the initial substrate concentration; and k_e is the efficiency with which substrate is converted to product. Algebraically, the model is expressed as follows:

$$k_eP - K_m[(S_0 - k_eP)/S_0] - V_{max}B_0t = 0, \quad (1)$$

where B_0 is the initial cell density. Within the nonlinear regression procedure, roots of equation 1 were found for given values of t (time) by Newton-Raphson iteration. This study was done with 900 ml of culture (0.30 g [dry weight] of cells per liter) in tryptic soy broth, to which 277 μg of NO_3^- N and 0.22 g of chloramphenicol (to arrest enzyme synthesis) were added. The vessel was maintained at 16°C, flushed with 1 liter of Ar per min, and stirred at 1,100 rpm. The K_m values were estimated from three separate NO_3^- additions.

Protocol for denitrifier nitrate method. The organism was cultured for 5 days at room temperature in 100 ml of medium in 160-ml stoppered serum bottles. Although all NO_3^- was consumed early, use of the longer incubation period was the easiest way to reduce residual N_2O production by the culture; this was essential for the analysis of low levels of NO_3^-. The cells were concentrated by centrifugation at 7,400 × g for 10 min and suspended in old medium of sufficient volume to achieve a 10× concentration of cells. Use of old medium was important since, when new medium without added NO_3^- was used, N_2O was always produced for a certain period of time. Suspended cells (2.5 ml) were added to 25-ml serum bottles and stoppered. The atmosphere and residual N_2O were removed by flushing the bottle with Ar for 10 min by using a vent needle and a longer needle to bubble the cell suspension. The assay contained approximately 7.5 mg of cells per bottle.

Ten milliliters of sample to be assayed for NO_3^- concentration was added to the flushed culture with a syringe. An extra needle was inserted through the stopper during sample addition to maintain atmospheric pressure. Oxygenated water samples did not need to be preflushed to remove dissolved O_2. Immediately after the sample was added, 0.2 ml of headspace was sampled with a syringe and analyzed for N_2O on a gas chromatograph. This initial sampling was important for establishing the background N_2O from all sources. The bottle was incubated at room temperature (usually 25°C) and, 10 to 15 min later, vigorously shaken for a few seconds to achieve gas equilibrium; a 0.2-ml headspace sample was then taken for analysis.

N_2O analysis can be done on any gas chromatograph equipped with a ^{63}Ni electron capture detector of sufficient sensitivity to detect 0.01 ppm (0.01 nl/ml) of N_2O. We used a Porapak Q column (1.8 m by 0.32 cm [outer diameter]) operated at 55°C and a carrier gas of 5% CH_4 in argon. The N_2O retention time was approximately 1.45 min. The detector was obtained from the Perkin-Elmer Corp., Norwalk, Conn., and was operated at 325°C. Electron capture detectors from some manufacturers have higher CO_2/N_2O sensitivity ratios, which can require an increased chromatographic separation of the two gases.

NO_3^- N was calculated by measuring the increase in N_2O between the two sampling times and converting this value to N_2O concentration in the headspace by using a standard curve constructed by diluting a previously analyzed N_2O standard. The total N_2O content (M) of the assay bottle was then calculated from the headspace N_2O concentration by the following equation:

$$M = C_g(V_g + V_l\alpha), \quad (2)$$

where M is the total amount of N_2O in the water plus gas phases, C_g is the concentration of N_2O in the gas phase, V_g is the volume of the gas phase, V_l is the volume of the liquid phase, and α is the Bunsen absorption coefficient. Bunsen coefficient values for N_2O in water at 1 atm are 0.632 at 20°C, 0.544 at 25°C, and 0.472 at 30°C (15). The NO_3^- N concentration is then calculated by the following equation, which contains a constant, K, that converts the molar volume of N_2O to molecular weight and corrects for the molar N ratio in N_2O (K = 1.15 at 20°C and 1.17 at 25°C):

$$\text{ppb of } NO_3^- \text{ N} = MK/(\text{ml of sample analyzed}) \quad (3)$$

Other methods. For comparative purposes, NO_3^- was also analyzed by high-pressure liquid chromatography (HPLC) by using a 25-cm column (Partisil SAX; Whatman, Inc., Clifton, N. J.) operated at a flow rate of 1.8 ml of 50 mM phosphate buffer (pH 3.0) per min (14). Detection was done by determining the UV adsorption at 210 nm. A twofold dilution series from 13.86 to 0.22 ppb of NO_3^- N was analyzed in triplicate by both HPLC and the denitrifier method. A 500-μl sample was analyzed by HPLC.

The ^{15}N content of the N_2O produced by the denitrifier method was analyzed in a gas chromatograph-mass spectrometer (HP5985; Hewlett-Packard) equipped with a Porapak Q column (1). The assay bottles were amended with 1.1 mg of NO_3^- N of 1.00 and 0.366 atom% of ^{15}N and incubated for 15 min. Prior to analysis, 1 ml of 10 N NaOH was added

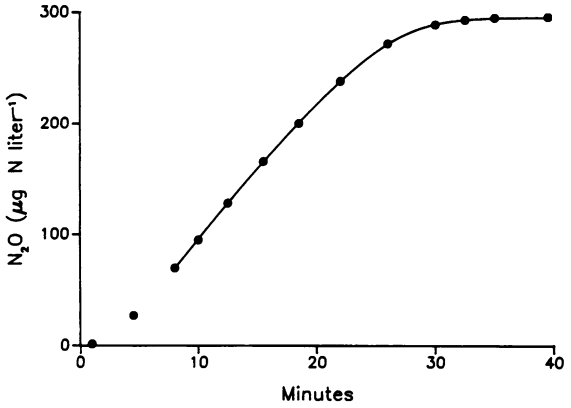

FIG. 1. Time course of N_2O production (●) after NO_3^- was added at time zero. The line was computed by using the Michaelis-Menten parameters derived from a progress curve analysis of the data and solved for substrate concentration fitted to the last 11 observations.

to reduce any interference by CO_2 at *m/e* 44. One-milliliter headspace samples were analyzed.

All analyses were carried out in triplicate, unless indicated otherwise, and the means and standard deviations are reported.

RESULTS AND DISCUSSION

Kinetics of NO_3^- reduction to N_2O. In order for this method to work, the conversion of very low NO_3^- concentrations must be complete (or nearly so) and not too lengthy in time. The culture transformed 277 μg of NO_3^- N to N_2O (Fig. 1) with an efficiency of conversion that did not differ from 100%, given experimental accuracy. This was expected since tryptic soy broth is rich in organic NH_4^+ sources, which should repress synthesis of the NO_3^- assimilation pathway. Accordingly, for the nonlinear regression analyses, k_e, the efficiency of substrate to product conversion, was set equal to 1.

Fitting of the Michaelis-Menten equation to the N_2O production data to obtain apparent K_m and V_{max} values for NO_3^- use was justified for the following two reasons. First, no phase transfer limitation existed in this incubation system, as shown by a plateau in gas transfer at these stirring speeds (Tiedje and Christensen, in press) and because spot checks on liquid-phase aqueous N_2O concentrations corresponded to the concentration calculated from the gas phase at the same time. Second, the monophasic production of N_2O and the stoichiometry between NO_3^- and N_2O suggested that N_2O production kinetics reflected the effect of NO_3^- concentration on the rate of NO_3^- consumption by the denitrifier.

The kinetic parameters were estimated for the *P. chlororaphis* strain used in the NO_3^- method plus one other N_2O-producing strain, *Pseudomonas aureofaciens* (5), which was used for comparison (Table 1). These parameters also show that the conversion of part-per-billion concentrations of NO_3^- should be first order. This prediction is supported by the proportional rate increase noted for the two NO_3^- concentrations studied (Fig. 2). By using the derived first-order rate constant (Table 1), we calculated that the reaction for NO_3^- N concentrations below the K_m (1.7 μM or 24 ppb of N) should be 99.9% complete after 5.9 min. The results in Fig. 2 confirm that very low concentrations of

TABLE 1. Kinetic constants for the two denitrifying bacteria determined from N_2O product formation curves

Organism	K_m (μM NO_3^-)	V_{max} (μg of N g^{-1} cells min^{-1})	k (min^{-1})[a]	$t_{1/2}$ (min)
P. chlororaphis	1.71	47.6 ± 0.8[b]	1.19	0.59
P. aureofaciens	1.79	51.3 ± 2.9	1.23	0.56

[a] First-order rate constant derived from $V_{max}B_0/K_m$ reflecting twofold greater concentrations of cells in assay bottles than those in chemostat vessels.
[b] Standard deviation.

NO_3^- N were converted in stoichiometric amounts to N_2O N within 10 min.

Nitrate K_m values for denitrifier cultures are scarce, because the rate-sensitive NO_3^- concentrations are below the detection limits of the nitrate methods. In other kinetic analyses (3) it has been suggested that the K_m values must be below 15 mM; and in one study in which we attempted to measure them directly by the then most sensitive method (HPLC), values of 5 to 10 μM were suggested (R. M. Edwards and J. M. Tiedje, Abstr. Annu. Meet. Am. Soc. Microbiol. 1981, N-48, p. 131). Since both strains had similar apparent K_m values (Table 1), it might be that values in this range are typical for denitrifiers.

Method performance. Results obtained by the denitrifier method were compared with those obtained by HPLC UV analysis, as this was previously the most sensitive method (Fig. 3). The precision of the chromatographic method as performed here was in the same range or slightly above that reported previously (13). The accuracy of the denitrifier method was in the same range as the HPLC method for NO_3^- concentrations above 2 ppb of N (Fig. 3); the standard deviation in relation to the mean (coefficient of variation) was 1 to 6% for both methods. Lower NO_3^- concentrations could not be measured by HPLC, as the coefficient of variation rapidly increased to 60%. However, the denitrifier method had coefficients of variation below 10% until the NO_3^- concentration reached 0.2 to 0.4 ppb, for which the coefficients of variation were 36 and 28%, respectively. Therefore, the denitrifier method showed a detection limit of about 0.2 ppb of N (for which the signal-to-noise ratio dropped below 3:1). The detection limit could easily be decreased by (i) injecting >0.2-ml gas samples, (ii) reducing the gas-to-liquid ratio in the bottle, and (iii) using more than 10 ml of sample in the assay.

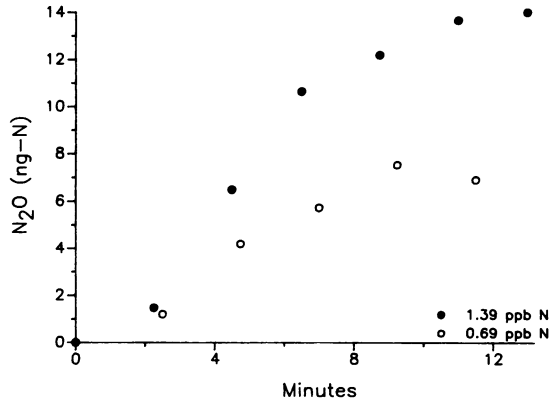

FIG. 2. Conversion of 10 ml of 0.69- and 1.39-ppb NO_3^- N solutions to N_2O.

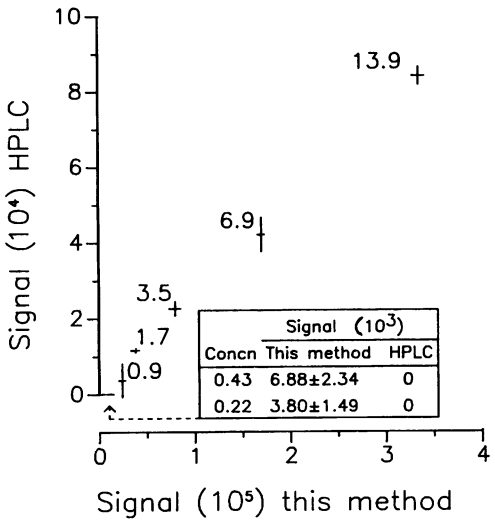

FIG. 3. Relationship between the integrator signal of the NO_3^- peak determined by HPLC and of the N_2O peak determined by the denitrifier gas chromatographic method (this method) for seven different nitrate concentrations (13.9 to 0.22 ppb of N). The five highest concentrations gave a signal by both methods and are depicted. The two lowest concentrations (detectable by the denitrifier method) are given in the inset. Standard deviations are given as bars in the graph and as values in the inset.

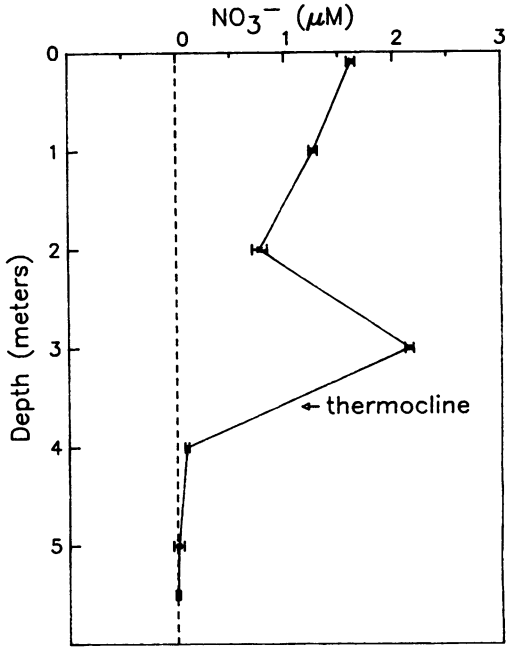

FIG. 4. NO_3^- profile of a hypereutrophic lake water column sample measured by the denitrifier method. Measurements were made in duplicate, and bars represent the standard deviation.

For measurement of NO_3^- concentrations in soil and sediment samples, it is important not to use an extractant with a high ionic strength; this could inhibit the denitrifying bacteria from reducing NO_3^-. This is not a major practical problem, however, since NO_3^- is easily extracted from these natural samples with a more dilute extractant, e.g., saturated $CaSO_4$ solution (B. G. Ellis, personal communication).

Assay of samples with low NO_3^- concentrations. Several laboratory water sources were analyzed for NO_3^- by the new method (Table 2). Previously nondetectable nitrate concentrations were found in distilled water condensed from the local steam plant and in water from a standard laboratory column purifier system (which included deionization).

The nitrate content of the water in a hypereutrophic lake (Wintergreen Lake, Hickory Corners, Mich.) was measured during summer stratification (Fig. 4). The profile showed a NO_3^- maximum just above the thermocline and measureable nitrate below the thermocline (4 m), where O_2 was not detected and the water had a distinct smell of sulfide. Samples at the 5 and 5.5 m water depth (near the sediment surface) had <0.2 ppb of NO_3^- N. Measurement of NO_3^- at the 4 m depth but not at 5 m below the surface showed that the method could distinguish NO_3^- in anaerobic waters where NO_3^- was previously undetectable and where organic constituents make analysis difficult by previous methods.

Conversion of $^{15}NO_3^-$ to ^{15}N gas. NO_3^- samples of two different ^{15}N enrichments were converted to N_2O by the procedure described above (Table 3). The differences between ^{15}N abundances in added NO_3^- and recovered N_2O of 2 to 3% were within the limits of accuracy of the stated enrichment of the NO_3^- source and the precision of the mass spectrometer.

Since this procedure is also quantitative, the total N (as total ion current) as well as the atom% enrichment could be measured in the same analysis. A denitrifier that produces N_2 could also be used if measurement of *m/e* 28 and 29 is preferred on the available mass spectrometer. However, potential contamination with atmospheric N_2 is more of a problem, especially if the NO_3^- concentration is low. In either case, contamination of the NO_3^- analysis by NH_4^+ is avoided.

Conclusions. The new method reduces the detection limit for NO_3^- by at least 1 order of magnitude, to 0.2 ppb of NO_3^- N. Moreover, the problems of interference from organic components of samples in an HPLC column or during NO_3^- reduction to NO_2^- on a cadmium column are avoided by the conversion of NO_3^- to N_2O. However, the microbial procedure is not the method of choice for NO_3^- concentrations above 20 to 50 ppb of N if a high-quality high-pressure liquid chromatograph is available; higher concentrations of N_2O are not as easily analyzed with the electron capture detector. The equipment for the denitrifier

TABLE 2. NO_3^- content of laboratory water sources

Water sample	NO_3^- N (ppb)
Tap	48.3 ± 0.3[a]
Distilled	1.37 ± 0.04
Distilled, deionized	0.67 ± 0.04

[a] Mean ± standard deviation of duplicate samples.

TABLE 3. Atom% ^{15}N in NO_3^- measured by the denitrifier method

^{15}N abundance (atom%) in:	
Added NO_3^-	Recovered N_2O
0.37	0.36 ± 0.01[a]
1.00	0.98 ± 0.02

[a] Mean ± standard deviation of duplicate samples.

method is relatively inexpensive, especially compared with the costs of ion chromatographs or autoanalyzers; a suitable ^{63}Ni-equipped gas chromatograph can be purchased for as little as $4,000 to $5,000, if one is not already available. The supplies are inexpensive, and the skill and time required for analysis are minimal.

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