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Measurement of the stable isotope ratio of dissolved N_2 in ^{15}N tracer experiments

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Abstract

Stable isotope addition experiments seeking to trace the denitrification of combined forms of nitrogen (N) to gaseous N_2 in aquatic environments typically need to measure the stable isotope ratio of dissolved nitrogen gas. This measurement presents challenges because of the potential for contamination of samples by N in air, and because field experiments conducted in situ often do not produce a marked enrichment in the isotope ratio of the N_2 pool. Field experiments also require numerous samples, sometimes processed under arduous conditions, and thus methods have to be convenient and low in cost. This paper describes the methods for sampling and measurement of the N isotope ratio of dissolved nitrogen that were developed for the isotope addition experiments in the Lotic Intersite Nitrogen Experiment (LINX), a cross-site study examining N biogeochemistry in headwater streams. Headspace equilibration was performed in the field and gas samples were stored in re-evacuated glass vials (Exetainers). Samples were processed and stored underwater to minimize the potential for contamination of samples by air. Isotope ratios were measured using a gas chromatograph interfaced to the isotope ratio mass spectrometer and equipped with a custom sample entry system.

Studies applying stable isotopes in freshwater or marine research can be grouped into those that analyze patterns in natural abundance and those that produce experimental isotopic enrichments (i.e., tracer additions). Natural abundance studies are largely directed toward understanding the origins of materials or revealing the predominant biogeochemical processes within an ecosystem, and have been applied to determine rates of specific processes only in limited cases (e.g., Ostrom et al. 2002). Stable isotope addition experiments offer a potentially powerful approach to study rates of elemental cycling in cases such as nitrogen (N), where there are multiple chemical forms and processes present and natural abundance patterns can be difficult to interpret. Such experiments are often best conducted in situ in the field to avoid artifacts associated with containment of water and sediments in microcosms such as flasks or cores. In-situ experimental additions of ^{15}N have been employed in a wide range of aquatic ecosystems to study N cycling (Peterson et al. 2001, Holmes et al. 2000, Tobias et al. 2003, Mulholland et al. 2004, Smith et al. 2004, Böhlke et al. 2004, Gribsholt et al. 2005).

Experiments based on in situ ^{15}N additions to study nitrogen cycling in small streams have been conducted for the Lotic Intersite Nitrogen Experiment (LINX). The most recent set of 72 LINX experiments conducted across North America focused on quantifying removal of nitrate (NO_3^-) in headwater streams by benthic denitrification. Each experiment entailed a 24-h addition of $^{15}NO_3^-$. Sampling in downstream transects during the addition was designed to monitor the disappearance of $^{15}NO_3^-$ and the appearance of that ^{15}N in other N compartments, including N_2, ammonium, dissolved organic N, and benthic biomass. The appearance of tracer ^{15}N in the dissolved N_2 during the $^{15}NO_3^-$ addition provides an indication of denitrification (together with possible but likely small contributions from anaerobic ammonium oxidation). The LINX project has developed protocols to ensure that this measurement is adequate yet practical for field experiments and affordable for large numbers of samples.

Measurement of the stable isotope ratio in dissolved N_2 presents challenges because of the potential for contamination by N_2 in air. Dissolved gases need to be extracted into a headspace and the headspace gas sample needs to be stored

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until analysis in the laboratory by isotope ratio mass spectrometry. Even seemingly slight contamination by air, either as bubbles in the water sample or from leakage during sample processing or storage, can degrade the accuracy of the analysis because the partial pressure of N_2 in the headspace sample is far less than that in air. An additional challenge is imposed on ^{15}N tracer studies of denitrification because dissolved N_2 in surface waters continually exchanges with atmospheric N_2. Furthermore, the aquatic ecosystems of greatest interest for studies of denitrification are often shallow and characterized by a high surface area to volume ratio that enhances the impact of exchange with the atmosphere on the dissolved N_2. As a consequence of the high background levels and dynamic turnover of dissolved N_2, marked enrichments of ^{15}N in the dissolved N_2 are difficult to produce in in-situ tracer experiments. The amount of added tracer is constrained by the high cost of the isotope and the need to minimize the increase in NO_3 availability produced by the tracer addition, so as to not enhance denitrification rates. Thus isotope addition experiments commonly produce only modest increases in the $\delta^{15}N$ of the dissolved N_2, in which case measurement methods have to be optimized to quantify the tracer ^{15}N at enrichments that may be only a few ‰ above background (natural abundance). Spatial and temporal variability of the denitrification process often requires that many samples be collected in the field under conditions that can be arduous; consequently, an ideal protocol would be convenient in the field and as low in cost as possible to facilitate analysis of large numbers of samples.

Previous studies have measured the $\delta^{15}N$ of dissolved N_2 by several methods. A very accurate and precise method employs large (e.g., 200-300 mL) evacuated glass flasks equipped with high-vacuum valves, which are partially filled with sample water to yield a headspace gas sample (Emerson et al. 1999). The headspace gas is cryogenically purified on a vacuum line and analyzed by mass spectrometry. That vacuum-flask method was deemed too expensive for the LINX project, but we did employ it to test alternative methods. Other studies have collected water samples by completely filling serum vials and sealing them with thick, plug-type septa, conducting headspace extractions of dissolved gases later in the laboratory (Smith et al. 2004, Böhlke et al. 2004). Another, more recently developed approach is the use of membrane-inlet mass spectrometry (An et al. 2001), but this requires special equipment and works best at greater ^{15}N enrichments than those attained in the LINX experiments.

This paper outlines methods developed for sampling and measurement of the $\delta^{15}N$ of dissolved N_2 as part of the LINX isotope addition experiments by static headspace equilibration performed in the field. The same sampling collection and storage methods also were used in the LINX project to measure concentrations of other dissolved gases (CO_2, CH_4, N_2O) by gas chromatography and $\delta^{15}N$ of nitrous oxide (N_2O) by isotope ratio mass spectrometry. However, the protocol has been developed with special attention to N_2 because of its greater potential for air contamination.

Materials and procedures

Selection and preparation of sample vessels—Isotopic analysis of large numbers of gas samples is facilitated by automated introduction systems that are now readily available (e.g., Barth et al. 2004). Use of such systems requires sample collection in serum type vials, and in the hope of using autosampler systems for the mass spectrometric analysis of N_2 samples, the LINX project chose Labco 12 mL Exetainers with chlorobutyl septa (Vial Type 3, Order code 839W/GL: Labco Ltd.; www.labco.uk.co). Although we later switched to the manual protocol described in this paper, the Exetainers proved to be a suitable choice for this purpose.

The Exetainer vials were purchased pre-evacuated, but they are evacuated mainly for the purpose of drawing sample from a syringe (D. Ashton, Labco Ltd., pers. comm.), and because even freshly prepared pre-evacuated Exetainers can contain N_2 at unacceptable levels for the present purposes. Furthermore, diffusion through the cap/septum assembly causes N_2 in the vials to increase with storage time.

Exetainers were re-evacuated within a few weeks prior to sample collection to eliminate N_2 contamination, and thereafter were stored underwater to minimize contamination by diffusion. The essential elements of an evacuation system are a mechanical roughing vacuum pump, a vacuum gauge, and a vacuum manifold with stopcocks and ports to interface to the sample vials. We used a custom-built glass vacuum line equipped with an Edwards E2M2 2-stage rotary vacuum pump (BOC Edwards) that generated vacuum of < 50 mTorr (= 6.7×10^{-5} atm or 6.7 Pa), as indicated by Hastings DV-5 vacuum gauges (Teledyne Brown Engineering). Assuming an initial condition of air at 1 atmosphere, evacuation reduces the N_2 partial pressure to < 51 ppmv, which is more than sufficient for this purpose. However, we have found that typical mechanical vacuum pumps used for general lab purposes may not be sufficient to render interference by N_2 insignificant. Furthermore, commonly used vacuum gauges often do not have sufficient sensitivity to indicate when appropriate vacuum levels have been obtained (i.e., $P < 0.005$ atm). The vacuum manifold we used consisted of ten ports with stopcocks, Cajon© tube fittings (Swagelock), and Luer slip male needle connections. The needle connections were cut-off syringe barrels from BD 1 mL polypropylene tuberculin syringes (Becton Dickinson), which fit within the 1/4-inch Cajon tube fittings with sufficiently good seals to obtain appropriate vacuum levels.

Exetainers were affixed to the vacuum line using fine needles (BD 27G 1/2, #305109), and a few drops of water were placed over the septum to ensure that air did not enter through the punctured septum. Once the target vacuum was achieved, each Exetainer was withdrawn individually while keeping it submersed in water. This was accomplished using 50 mL polypropylene centrifuge tubes (Corning© 430290,

plug seal cap) filled with deionized water freshly sparged with helium. We found that submergence in water avoided a brief influx of air as the needle was removed from the septum. A few drops of water would enter the Exetainer upon withdrawal, however, contamination from N_2 dissolved in that water was negligible. Upon withdrawal from the vacuum line the Exetainer in its water-filled centrifuge tube was immediately capped underwater in a large beaker of He-sparged water such that no air bubbles were visible inside the centrifuge tube. Exetainers were stored this way before sampling and between sample collection and analysis to reduce the potential for air contamination by taking advantage of much slower gas diffusion rates in water than in air. Sample codes were written in indelible ink on the Exetainer and also on the outer centrifuge tube because movement of the Exetainer within the centrifuge tube during subsequent transport sometimes obliterated the Exetainer labels.

Sample collection—Water samples were collected in polyethylene syringes of either 60 mL (Becton Dickinson) or preferably, 140 mL capacity (Monoject), fitted with polycarbonate 1-way male Luer stopcocks. Separate sets of syringes were used for background and ^{15}N-enriched samples, and syringes were replaced when they became noticeably harder to draw. Batches of samples were collected and processed together, but generally processing was complete within an hour of collection, and care was taken to avoid heating of the samples in that interval (significant warming could produce loss of dissolved N_2 into bubbles).

A water sample was collected by slowly drawing water from beneath the surface, eliminating air bubbles drawn in with the sample by tapping and expelling water while pointing the syringe upward. Removal of very small air bubbles may not be possible, and these would be a significant source of N_2 contamination only if their total volume exceeds a spherical diameter of ~2 mm (using the 60-mL syringe). Bubbles were removed only if they were drawn in with the sample, and not if they formed after sample collection. Once bubbles in the syringe and stopcock were removed, a sample of either 40 or 120 mL was collected, the stopcock was closed, and the syringe was stored temporarily in a bucket of water at ambient stream temperature. Water temperature in the stream was recorded to allow calculation of dissolved N_2 concentrations at atmospheric equilibrium from temperature-solubility relationships (Weiss 1970).

Extraction of dissolved gases—A small, portable tank of ultrapure He was used to provide gas for headspace equilibrations at the field sites. A flexible line of 1/8-inch outer diameter (O.D.) polytetrafluoroethylene tubing introduced He gas from the tank regulator to a plastic box filled with water (approximately 40 × 40 cm by 30-cm deep). A 30 mL polyethylene syringe fitted with a polycarbonate 3-way male Luer stopcock served as the "gas delivery syringe;" the polytetrafluoroethylene tubing was attached to the male Luer stopcock port with a sleeve of tightly fitting Tygon tubing. While maintained underwater, the gas delivery syringe was filled with He and emptied twice through the side port of the stopcock before filling it with ~25 mL He, followed by expulsion of He to yield 20.0 mL He at atmospheric pressure. This He was immediately added to the sample syringe by connecting the side port of the gas-delivery syringe stopcock to the sample syringe stopcock, then simultaneously pushing gently on the plunger of the gas-delivery syringe while pulling the plunger of the sample syringe. Sample syringes were stored briefly underwater before conducting the headspace extraction.

Dissolved gases were equilibrated with the headspace in the sample syringes by vigorous agitation for 5 min. Batches of syringes could easily be agitated together by placing them horizontally on a tray affixed to a cart with wheels. The time that the syringes are not submersed should be minimized in case of leakage, although we did not observe leakage over such short periods. The temperature of the gas-water system needs to be known and can be measured in the residual water of a representative sample immediately after transfer of the headspace gas to the Exetainer; normally it is quite close to that of the water bath in which samples were stored up to that point. The volumes of water and headspace enter into calculations of the original dissolved gas concentrations, as detailed later.

After equilibration, the headspace gas was immediately transferred into an Exetainer (Fig. 1), taking the following precautions to minimize contamination with air. A needle extender was made that consisted of 15 cm of 1/16-inch O.D./1/32-inch inner diameter (I.D.) Teflon-FEP tubing (Cole-Parmer 6406-60), connected to the sample syringe stopcock via a 20G 1 1/2 needle (Becton Dickinson 305176) with its point filed off and inserted tightly into the tubing, which leads to a Chemfluor miniature fluid flow fitting (Mini M Luer adaptor, Part no. 06391-90) fitted with a 27G 1/2 needle (Becton Dickinson 305109). This flexible needle extender allowed the sample headspace to be expelled while holding the sample syringe pointed upward, and the headspace gas to be injected downward into the Exetainer, which sat within its water-filled centrifuge tube (Fig. 1). The point where the needle punctured the Exetainer septum was maintained underwater, thereby avoiding introduction of air.

Before inserting the needle into the Exetainer septum, water in the needle extender and a few milliliters of headspace gas from the syringe were expelled into the water beside the Exetainer. Next, the needle was quickly moved over and into the septum, while avoiding contact with overlying air. The remainder of the headspace (~17 mL) was then injected into the Exetainer, and the needle quickly withdrawn. If samples were collected at high elevations (e.g., > 2000 meters above sea level) and analyzed at low elevations, the headspace gas volume in the syringe was increased by 5 mL to produce enough sample to maintain positive pressure in the Exetainers with respect to barometric pressure. The Exetainer was stored at room temperature in its water-filled centrifuge tube. Samples were analyzed as soon as possible, normally within 2 weeks of collection.

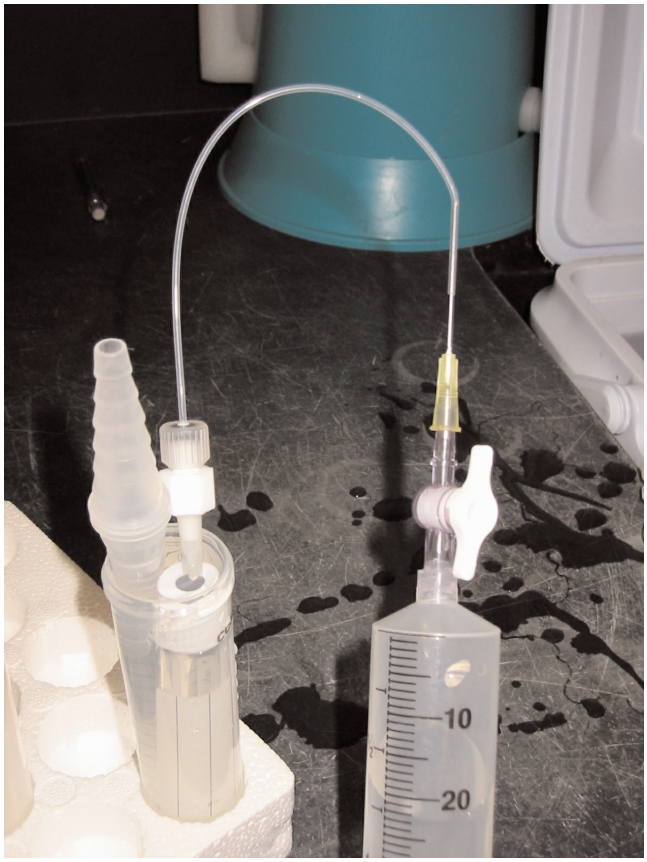

Fig. 1. Injection of the headspace gas into the Exetainer vial. The needle extender allows the sample syringe to be maintained with the headspace at the top while injecting the gas into the Exetainer, the septum of which remains submersed under a few millimeters of water to ensure against air contamination. The plastic tube fitting jams the Exetainer in place so it does not float out of its water bath.

Measurement of N_2 partial pressures and $\delta^{15}N$ in N_2—The partial pressures of N_2 and other gases in the Exetainers were measured using a Shimadzu GC14A gas chromatograph (GC) equipped with a thermal conductivity detector (operated at 100 mA). The sample was injected through a 1 mL sample loop by displacement of the gas in the Exetainer with 10 mL He-sparged deionized water, using a long, thin hypodermic needle to add water to the bottom of the Exetainer quickly but with minimal turbulence. The contents of the sample loop were transferred to the carrier gas stream with a switching valve, and a 2-m column of molecular sieve (5A) was used to separate O_2 + Ar and N_2 with time. Water vapor was removed from the column by backflushing. Certified standards of partial pressures comparable with the samples were used for calibration of each measured gas (Scott Specialty Gases, Inc.). Air diluted in syringes with known quantities of ultrapure He also served as calibration standards in the case of N_2. The precision of this measurement of dissolved N_2 concentration, as indicated by the mean coefficient of variation of seven sets of triplicate stream samples, was 1.1%.

Stable isotope ratios of N_2 gas in headspace samples were analyzed using a GC system interfaced to an isotope ratio mass spectrometer, modified from the design of Roberts et al. (2000) to enable direct transfer of gas from the Exetainers to a 3 mL sample loop with minimal dead volume. A glass barrel from a 100 μL gas-tight syringe (VICI Pressure-Lok©) was affixed to a 1/4-inch Cajon fitting leading to an on-off valve and the sample loop. Switching valves (6- and 4-port) controlled the sample loop system. The sample loop, a small water trap containing Ascarite, and associated steel tubing lines were evacuated with the aid of an in-line liquid N_2 trap, and then isolated from vacuum. The syringe barrel was not evacuated, but its dead volume was very small relative to the Exetainer. A fine needle (27G 1/2) on the syringe was pierced into the Exetainer septum, and the on-off valve between the barrel, and the sample loop opened to allow the sample gas to expand into the sample loop. The valve between the loop and the Exetainer was then closed, and the contents of the sample loop were flushed by a He carrier gas stream through an 8 m packed column of molecular sieve (5A) to separate N_2 from O_2 and Ar before introduction of the N_2 into the inlet of the mass spectrometer. All samples were analyzed on a GV Instruments Prism (series II) mass spectrometer versus a calibrated pure tank N_2 standard. Values are reported with respect to N_2 in air. Aliquots of atmospheric air were analyzed prior to samples as a daily test of accuracy and precision. Precision of replicate samples and of atmospheric air was 0.3‰ or better.

Calculation of dissolved N_2 concentrations—Calculation of approximate dissolved N_2 concentration in the sample water was useful as an indicator of air contamination, which can readily produce large excesses over the amount of N_2 expected for air-equilibrated water. The original dissolved N_2 concentration was calculated from the N_2 quantity measured in the headspace after equilibration, the solubility coefficient at the temperature of equilibration, and the mass balance for the gas-liquid system. Volumes of liquid and gas for the equilibration were adjusted for the 1 mL dead space in the stopcock by adding 1 mL to the liquid volume and subtracting 1 mL from the gas volume. Headspace N_2 partial pressures measured by GC were converted to micromoles gas L^{-1} for the calculations using the Ideal Gas Law. Alternatively, an estimate of the molar quantity of N_2 in the Exetainer may be available from the mass 28 beam intensity within the mass spectrometer, which after correction for subsampling of the headspace (e.g., if 17 of 19 mL were injected into the Exetainer) yields an estimate of the total moles of N_2 in the headspace.

Determination of the abundance of N_2 is based on the following mass-balance equation for the headspace equilibration in the sample syringe, in which concentration units can be μM and volume units can be liters:

$$(C^0_{liq})(V_{liq}) = (C_{liq})(V_{liq}) + (C_{gas})(V_{gas})$$

where C^0_{liq} is the original gas concentration, V_{liq} and V_{gas} are the volumes of liquid and gas in the syringe, and C_{liq} and C_{gas}

are the concentrations in the liquid and gas phases after equilibration. The equilibrium concentration (liters gas per liter of solution) of a particular gas dissolved in the liquid phase (C_{liq}) is related to its partial pressure in the gas phase (P_{gas} in atm) by the Bunsen solubility coefficient:

$$C_{liq} = P_{gas} \cdot \beta_T \cdot P_{BAROMETRIC}$$

where β_T is the solubility coefficient in units of L L^{-1} atm^{-1}, calculated for the temperature at which the air-water equilibration took place. Atmospheric partial pressure was corrected for the vapor pressure of water-saturated air (0.012 atm at 10°C and 0.031 atm at 25°C) above a water surface. The barometric pressure (in atm) corrects for the reduced total pressure at higher elevations, and/or short-term variations in barometric pressure that can be measured during sampling.

A small equilibrium isotopic fractionation occurs during dissolution of N_2 in water (α = 0.99915; Klots and Benson 1963), and thus dissolved N_2 is enriched in ^{15}N by 0.85‰ relative to the atmospheric N_2 that serves as the standard for isotopic measurements (0‰). However in the headspace equilibrations described above, almost all of the originally dissolved N_2 enters the headspace. Below we show that correction for equilibrium fractionation is insignificant.

The $\delta^{15}N$ measurement of the headspace gas can be corrected for any tracer ^{15}N that remains in solution after the equilibration with the following isotopic mass-balance equation:

$$[\delta^{15}N_{(ORIGINAL\ SAMPLE)} \times M_{(ORIGINAL\ SAMPLE)}] =$$
$$[\delta^{15}N_{(HEADSPACE)} \times M_{(HEADSPACE)}] + [\delta^{15}N_{(LIQUID)} \times M_{(LIQUID)}]$$

where M is mass (e.g., in μmol), $M_{(HEADSPACE)}$ is corrected for any subsampling if necessary, and

$$\delta^{15}N_{(LIQUID)} = \delta^{15}N_{(HEADSPACE)} - \varepsilon.$$

For example, streamwater equilibrated with the atmosphere at 25°C would carry a dissolved N_2 concentration of 486 μM. Suppose that a headspace equilibration is performed at 25°C with water and gas volumes for the equilibration of 0.121 and 0.019 L, respectively. The $M_{(ORIGINAL\ SAMPLE)}$ in 0.121 L sample was 58.84 μmol of N_2. If the final $\delta^{15}N_{(HEADSPACE)}$ had been measured at +1.0‰, then applying the above mass balance shows that the $\delta^{15}N_{(ORIGINAL\ SAMPLE)}$ was +1.07‰. Similarly, if the final $\delta^{15}N_{(HEADSPACE)}$ had been measured at +10.0‰, the $\delta^{15}N_{(ORIGINAL\ SAMPLE)}$ would have been +10.07‰. These corrections become significant only at low levels of enrichment, and only for relatively soluble gases, in contrast to N_2.

Assessment

Exetainers as sample vessels—The Labco Exetainers proved suitable as sample vessels once they were re-evacuated and stored in water. Analysis of 6 Exetainers that had been purchased pre-evacuated and stored for ~9 mo showed N_2 contents ranging from 21-76 μmol; re-evacuation reduced N_2 contents to < 0.6 μmol (based on GC measurements; data not shown). A set of re-evacuated Exetainers was monitored over time and analyzed by GC for N_2 content (Fig. 2). The increase in N_2 of ~0.01 μmol d^{-1} can be attributed to diffusive gas exchange with the water surrounding the Exetainer, but this slow rate is not problematic as long as sample storage time does not exceed a few months.

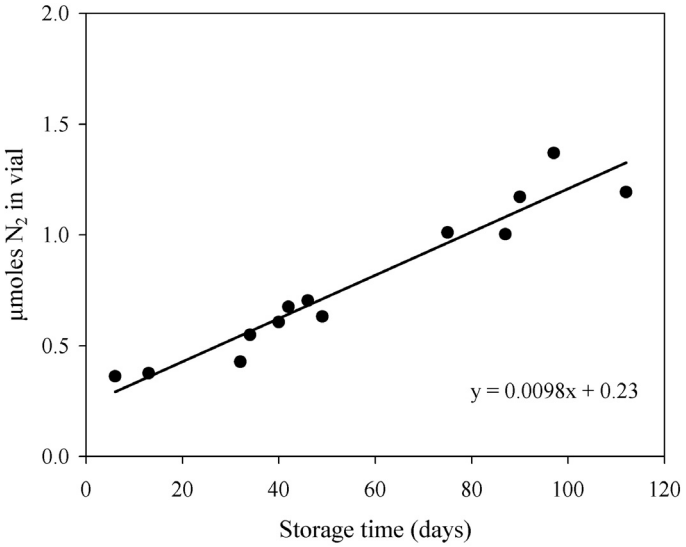

Fig. 2. Change in N_2 content in re-evacuated Exetainer vials during storage in water

A set of Exetainers filled with N_2 at partial pressures spanning the range of concentrations within typical samples was produced by dilution of pure N_2 with He and stored as described above. N_2 within these samples was monitored with time, and concentrations showed little change over 51 d, a period much longer than our normal sample storage time (Fig. 3).

Headspace equilibrations—The headspace equilibration procedure produced N_2 sample quantities that were adequate for GC and mass spectrometric analysis (Table 1). The use of 140 mL syringes produced 2.8 times more N_2 in the headspace than the 60 mL syringes, which facilitated analysis despite their higher cost and greater difficulty in handling. The larger sample size is not necessary with the measurement systems we employed, but could be important for other systems, and may be critical if the $\delta^{15}N$ of N_2O is also to be measured. Larger sample size also helps reduce the consequences of air contamination.

The agitation time was tested for N_2, O_2 + Ar, and CO_2, comparing 2, 5, and 10 min with 60 mL syringes (based on GC measurements; data not shown). Extraction was complete by 5 min for the less soluble gases (N_2 and O_2 + Ar). The more soluble CO_2, which is comparable in solubility to N_2O, showed a 7% increase in headspace partial pressure between 5 and 10 min of agitation. Smaller volumes of headspace relative to water than we used here might require longer agitation times.

Examples of data from four streams in southwestern Michigan demonstrate how the headspace equilibration methods

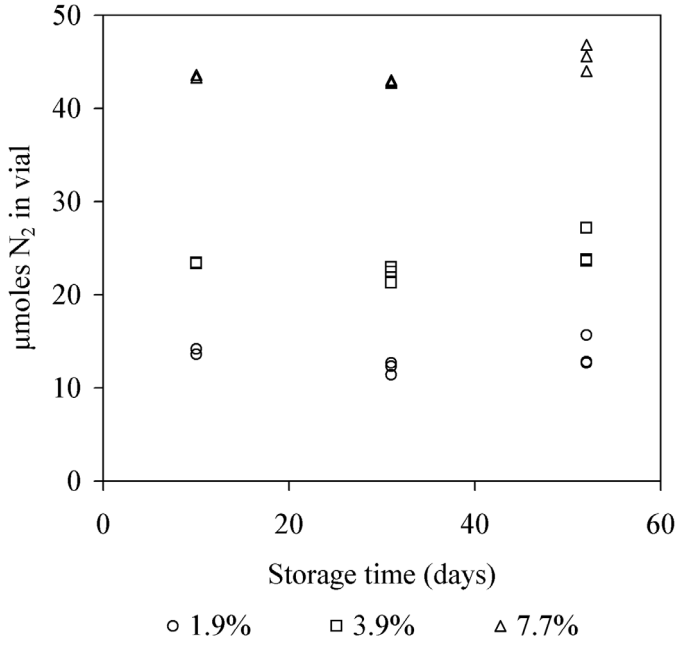

Fig. 3. Stability of N_2 content in Exetainer vials at three levels spanning those obtained in headspace equilibrations using 60 mL and 120 mL syringes (Table 1). Target % N_2 was achieved by diluting air with ultrapure He. Each storage time at each N_2 level has 5 to 6 replicates.

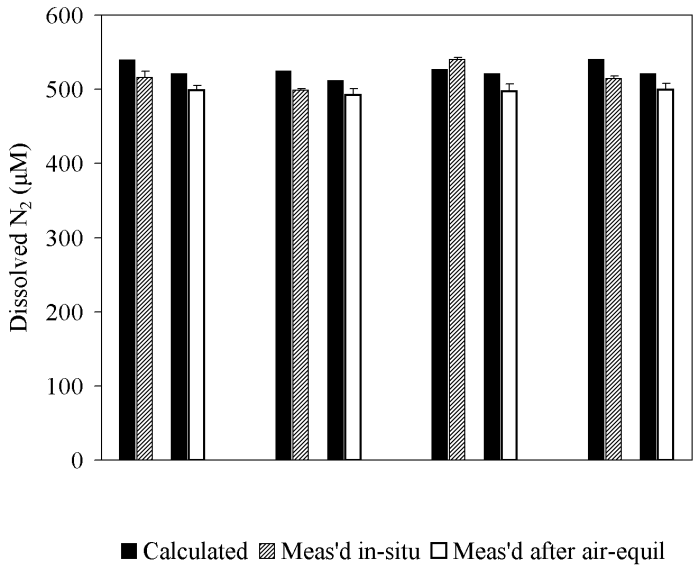

Fig. 4. Example of data from four streams in southwestern Michigan to demonstrate how the headspace equilibration methods employed here provide a reasonably accurate measurement of dissolved N_2 concentrations. Water from these streams was sampled and processed in the field, and subsamples of stream water were brought to the lab and allowed to equilibrate with air in shallow pans. Calculated N_2 concentrations assume atmospheric equilibrium and are based on the relationship between water temperature and solubility. Meas'd = measured using gas chromatography after headspace equilibration as described in the text. Error bars on measured means are standard deviations for triplicate measurements.

employed here provide a reasonably accurate measurement of dissolved N_2 concentrations (Fig. 4). Water from these streams was sampled and processed in the field, and the estimates of dissolved N_2 concentrations based on GC measurements of headspace N_2 are within 5% of calculated N_2 concentrations at atmospheric equilibrium (these shallow waters would be close to but not necessarily exactly at atmospheric equilibrium because they receive groundwater inputs, and they are subject to diel temperature changes). Subsamples of stream water were brought to the lab and allowed to equilibrate with air in shallow pans. Once again, good agreement was observed between calculated and measured N_2 concentrations.

Table 1. Dissolved N_2 concentrations at atmospheric equilibrium and resultant headspace partial pressures for air-equilibrated sample water, extracted in syringes as described in the text

	10°C	25°C
N_2 Bunsen coefficient (β_T, L L^{-1} atm^{-1})	0.01881	0.01442
N_2 partial pressure (atm) in water-saturated air	0.7713	0.7564
N_2 concentration in water at atmospheric equilibrium (µM)	647	486
N_2 partial pressure (atm) in headspace, V_{liq} = 0.041 L and V_{gas} = 0.019 L	0.03009	0.02282
N_2 µmol in headspace	25.4	19.3
N_2 partial pressure (atm) in headspace, V_{liq} = 0.121 L and V_{gas} = 0.019 L	0.08251	0.06361
N_2 µmoles in headspace	69.9	53.9

V_{liq} and V_{gas} are the volumes of liquid and gas in the syringe, corrected for the 1.0 mL of dead space in the stopcock. Bunsen solubility coefficients were calculated from Wiess (1970). Calculations assume negligible salinity, P = 1 atm, and an atmospheric N_2 partial pressure of 0.7808 atm. Atmospheric partial pressure was corrected for the vapor pressure of water-saturated air (0.012 atm at 10ºC and 0.031 atm at 25ºC) above a water surface. At least 75% of the headspace gas can be injected into the Exetainer; typically 17 of 19 mL were injected.

Comparison with a traditional method for measurement of $\delta^{15}N$ in N_2—The method we describe here was directly compared with the traditional vacuum-flask method (Emerson et al. 1999). To prepare a set of samples spanning the range we encountered in the LINX project, we added a mixture of nitrate as ^{15}N (5%) and ^{14}N (95%) to attain a concentration of 10 mg N/L in shallow water overlying pond sediment in a tray. After 24 h, the overlying water was collected and analyzed to determine that the $\delta^{15}N$ of the dissolved N_2 had reached 2500‰. Subsamples of this water were then diluted in a 140-mL syringe with air-equilibrated water ($\delta^{15}N$ of dissolved N_2 ~0.8‰) to prepare a series of samples with varying ^{15}N enrichment in the dissolved N_2. Each diluted sample was then split for analysis by the two methods; 70 mL were added to an evacuated glass vacuum flask and the remaining 70 mL were extracted into a 20-mL He headspace followed by injection into an Exetainer as described above. The results showed excellent agreement over a range of

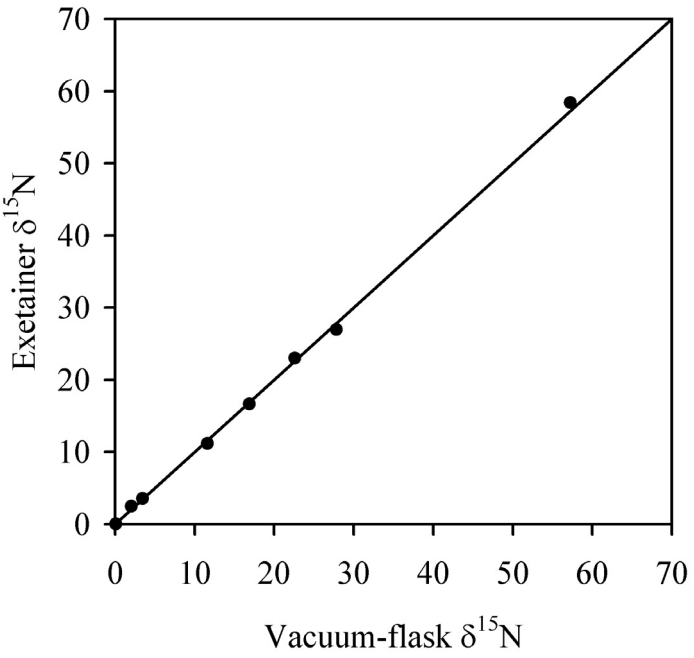

Fig. 5. Comparison of methods for measurement of $\delta^{15}N$ of dissolved N_2. The method described here ("Exetainer $\delta^{15}N$") yielded results that agree closely with results obtained using the vacuum-flask method of Emerson et al. (1999). The 1:1 line is depicted; a regression line fit to these data has a slope of 1.01.

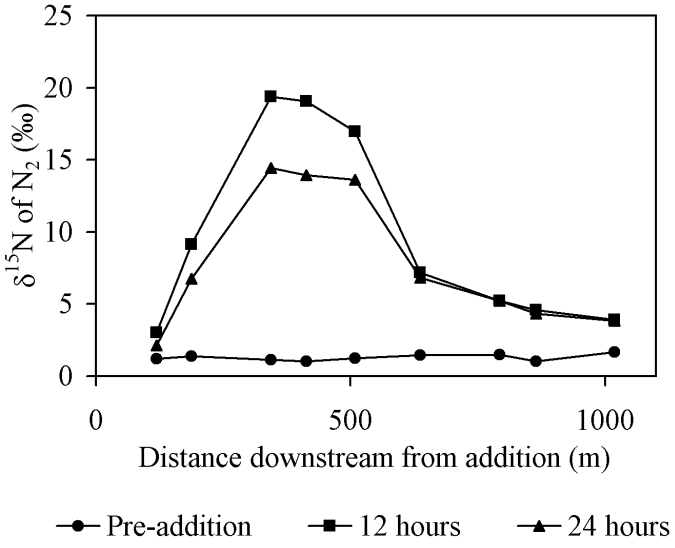

Fig. 6. Example of LINX data obtained using the methods described here. "Pre-addition" refers to the transect sampled before the addition of $^{15}NO_3^-$ at 0 meters; transects were subsequently sampled at 12 and 24 h after the addition commenced.

$\delta^{15}N$ from near natural abundance to ~58‰ (Fig. 5). The regression slope of 1.01 is very close to the 1:1 line depicted in the figure. This agreement suggests that accuracy and precision of the two methods are comparable.

Stable isotope measurements in a LINX experiment—Longitudinal patterns in $\delta^{15}N$ of N_2 measured during a LINX experiment in Michigan show the adequacy of the methods described here (Fig. 6). Prior to the $^{15}NO_3^-$ addition, all isotope values for N_2 along the transect showed $\delta^{15}N$ values consistently near the expected value of ~0.8 ‰. As expected based on theory (Mulholland et al. 2004), during the $^{15}NO_3^-$ addition, $\delta^{15}N$ of dissolved N_2 peaked midway along the transect, reflecting the balance between production of ^{15}N-labeled N_2 by denitrification and its loss by air-water gas exchange. Further downstream the $\delta^{15}N$ of the NO_3^- dropped as the added $^{15}NO_3^-$ was largely removed from the water and the ^{15}N in the N_2 was increasingly diluted by gas exchange. An increase in discharge due to rainfall before the 24 h sampling may have diluted the tracer ^{15}N in the stream and produced the lower values we observed then.

Comments and recommendations

The methods described here have been found to be appropriate for the LINX experiments and should be useful for other studies that conduct ^{15}N tracer additions in aquatic environments. In the course of performing 72 experiments in 8 biomes, many researchers have tested the protocol and found it to be easily performed and to provide accuracy that is superior to results from more automated protocols. The precautions against air contamination may not be critical, but ensure that contamination from air is minimal. The final step—measurement of $\delta^{15}N$ in N_2—must be carefully evaluated for its precision and accuracy.

Dissolved N_2 in small streams and other shallow surface waters is often close to atmospheric equilibrium for concentrations and close to 0.8‰ in $\delta^{15}N$ (perhaps varying by ± 1‰). Waters that are more isolated from the atmosphere can depart considerably from atmospheric equilibrium and can potentially deviate in $\delta^{15}N$ as well. For example, the partial pressure of N_2 in sediment porewaters of freshwater environments is often reduced because of CH_4 production and ebullition (Chanton et al. 1989). Contamination during sample handling and storage due to bulk air entry or diffusive exchange of N_2 with air would shift $\delta^{15}N$ values closer to that of air (0‰). Measurement of the amount of N_2 in the samples provides a basis to evaluate whether or not air contamination has occurred that otherwise might not be evident on the basis of $\delta^{15}N$.

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