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Isotopic Analysis of N and O in Nitrite and Nitrate by Sequential Selective Bacterial Reduction to N_2O

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Nitrite is an important intermediate species in the biogeochemical cycling of nitrogen, but its role in natural aquatic systems is poorly understood. Isotopic data can be used to study the sources and transformations of NO_2^- in the environment, but methods for independent isotopic analyses of NO_2^- in the presence of other N species are still new and evolving. This study demonstrates that isotopic analyses of N and O in NO_2^- can be done by treating whole freshwater or saltwater samples with the denitrifying bacterium *Stenotrophomonas nitritireducens*, which selectively reduces NO_2^- to N_2O for isotope ratio mass spectrometry. When calibrated with solutions containing NO_2^- with known isotopic compositions determined independently, reproducible $\delta^{15}N$ and $\delta^{18}O$ values were obtained at both natural-abundance levels (±0.2−0.5‰ for $\delta^{15}N$ and ±0.4−1.0‰ for $\delta^{18}O$) and moderately enriched ^{15}N tracer levels (±20−50‰ for $\delta^{15}N$ near 5000‰) for 5−20 nmol of NO_2^- (1−20 μmol/L in 1−5 mL aliquots). This method is highly selective for NO_2^- and was used for mixed samples containing both NO_2^- and NO_3^- with little or no measurable cross-contamination. In addition, mixed samples that were analyzed with *S. nitritireducens* were treated subsequently with *Pseudomonas aureofaciens* to reduce the NO_3^- in the absence of NO_2^-, providing isotopic analyses of NO_2^- and NO_3^- separately in the same aliquot. Sequential bacterial reduction methods like this one should be useful for a variety of isotopic studies aimed at understanding nitrogen cycling in aquatic environments. A test of these methods in an agricultural watershed in Indiana provides isotopic evidence for both nitrification and denitrification as sources of NO_2^- in a small stream.

Nitrite (NO_2^-) is a common constituent in soils, groundwaters, and surface waters, including the oceans. The behavior of NO_2^- is important because of its intermediate roles in the microbial oxidation and reduction of other aqueous nitrogen compounds and, in some cases, because of its potential toxicity in aquatic ecosystems. The isotopic composition of NO_2^- is linked to those of NO_3^-, N_2O, NH_4^+, and N_2 gas, the production or consumption of which can involve large isotope fractionations with respect to NO_2^-. Yet the isotopic composition of NO_2^- in the environment, and its effect on the isotopic compositions of other N species, are largely unknown because simple, sensitive, and precise methods for separation and isotopic analysis of NO_2^- from aqueous samples have been developed only very recently.

Nitrite isotopic analyses are difficult in many natural and experimental systems because NO_2^- typically is present in much lower concentration than NO_3^-. A useful method for extraction and isotopic analysis of NO_2^- in aqueous samples should satisfy the following criteria: (1) sensitive enough for NO_2^- concentrations of the order of 10^{-1} to 10^{1} μmol/L, (2) selective enough for NO_2^-/NO_3^- ratios of the order of 10^{-3} to 10^{-1}, and (3) precision of the isotope ratio measurement of the order of 0.1−1.0‰ for naturally occurring ratios. A method involving organic complexation and solvent extraction[1] has been used to process samples with low NO_2^- concentrations and low NO_2^-/NO_3^- ratios for N isotopic analysis. This method has yielded useful data in ^{15}N-enriched field tracer studies,[1−3] but it may not be as precise as needed for some isotope fractionation studies, and it cannot be used for O isotopic analysis. Anion chromatography can yield purified NO_2^- and NO_3^- with isotopic fidelity from samples with elevated NO_2^- concentrations[4] (this study; J. Hannon, U.S. Geological Survey, unpublished data), but it may be difficult to use for low NO_2^- concentrations, and it cannot be used for saline samples such as seawater. Casciotti et al.[5] describe experiments involving bacterial reduction of NO_2^- to N_2O with *Pseudomonas aureofaciens* for mass spectrometry, but that bacterium also reduces NO_3^- and therefore requires additional information to resolve the NO_2^- isotopic composition in mixed samples. Granger et al.[6] describe a method for removing NO_2^- from mixed samples to permit accurate isotopic analysis of NO_3^- using *P. aureofaciens*. McIlvin and Altabet[7] describe chemical reduction techniques to reduce NO_2^- selectively from mixed solutions to produce N_2O for isotopic analysis, giving useful results for N and O isotopes

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(1) Kator, H.; Morris, L. J.; Wetzel, R. L.; Koepfer, E. T. *Limnol. Oceanogr.* **1992**, *37*, 900−907.

(2) Smith, R. L.; Böhlke, J. K.; Garabedian, S. P.; Revesz, K. M.; Yoshinari, T. *Water Resour. Res.* **2004**, *40*, W07101. doi: 10.1029/2003WR002919. http://www.agu.org/journals/wr/.

(3) Lipschultz, F. *Deep−Sea Res., Part II* **2001**, *48*, 1897−1924.

(4) Böhlke, J. K.; Harvey, J. W.; Voytek, M. A. *Limnol. Oceanogr.* **2004**, *49*, 821−838.

(5) Casciotti, K. L.; Böhlke, J. K.; McIlvin, M. R.; Mroczkowski, S. J.; Hannon, J. E. *Anal. Chem.* **2007**, *79*, 2427−2436.

(6) Granger, J.; Sigman, D. M.; Prokopenko, M. G.; Lehman, M. F.; Tortell, P. D. *Limnol. Oceanogr.: Methods* **2006**, *4*, 205−212.

(7) McIlvin, M. R.; Altabet, M. A. *Anal. Chem.* **2005**, *77*, 5589−5595.

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after adjustments for N dilution by azide reagent, N isotope fractionation during reaction, and O exchanges with water.

The purpose of the current paper is to describe a bacterial reduction method for N and O isotopic analyses of aqueous NO_2^- that is simple, selective, sensitive, precise, and useful for both freshwater and saline samples such as seawater. This method is similar to the published bacterial reduction methods for isotopic analyses of NO_3^- and NO_2^- using *P. chlororaphis* or *P. aureofaciens*[5,8−10] but uses a different bacterium, *Stenotrophomonas nitritireducens*, that reduces NO_2^- to N_2O and does not reduce NO_3^-.[11] This bacterium was used previously to construct a NO_2^--specific microelectrode.[12] The *S. nitritireducens* method for NO_2^- can be used in tandem with the normal bacterial NO_3^- method to give sequential isotopic analyses of NO_2^- and NO_3^- in a single sample aliquot. In this paper, we document the performance of these techniques with samples containing isotopic reference materials for both high-precision fractionation studies and ^{15}N-enriched tracer studies. We also present preliminary data indicating multiple sources of NO_2^- in a small stream draining agricultural land in the midcontinent U.S.A.

EXPERIMENTAL SECTION

The selective bacterial NO_2^- reduction method with *S. nitritireducens* was tested on solutions (mostly with freshwater, a few with seawater) containing NO_2^- salts with known N and O isotopic compositions, and it was tested on samples containing mixtures of NO_3^- and NO_2^- with contrasting isotopic compositions, including ^{15}N-enriched tracer samples. Experiments also were done to compare results from *S. nitritireducens* and *P. aureofaciens*. These included sequential analysis of mixed samples by *S. nitritireducens* followed by *P. aureofaciens* to determine the N and O isotopic compositions of NO_2^- and NO_3^- separately in the same sample.

Bacterial Reduction and Isotopic Analysis. The bacterium used in this study is *S. nitritireducens* (ATCC No. BAA-12).[11] The bacteria were grown on tryptic soy agar (3%) in slant tubes and stored (4 °C) for more than 1 year with no discernible degradation in quality. To prepare for a batch of analyses, bacteria were streaked onto tryptic soy agar plates prepared without NO_2^- and grown aerobically at 22 °C. After 4−5 days, individual colonies were transferred to sterile 50 mL centrifuge tubes with 35 mL of growth medium containing tryptic soy broth (15.0 g/L), $(NH_4)_2SO_4$ (0.25 g/L), and KH_2PO_4 (2.45 g/L). Tubes were capped with air headspace and agitated on a shaker table for 1−2 days at 22 °C. In contrast to the normal procedure for preparing *P. chlororaphis* and *P. aureofaciens* with NO_3^- in the growth medium, we found that *S. nitritireducens* grew best for this procedure aerobically without NO_2^- in the medium (presumably using O_2 as a terminal electron acceptor). Larger batches of bacteria also were grown in 160 mL serum bottles containing 130 mL of growth medium with air headspace. These batches appeared to grow best and last longest (at least 5 days) if the headspace was reaerated daily by admitting fresh air.

For each batch of analyses, the contents of four centrifuge tubes were combined (alternatively, the contents of one serum bottle), antifoaming agent was added (10−12 drops Sigma Antifoam B), and the contents were distributed directly, in 4 mL aliquots, into 32 20 mL vials. The vials were capped with aluminum-lined septum caps and purged with helium for 30 min to remove air and traces of N_2O. Water samples containing 20 μmol/L NO_2^- with pH ≈ 11−12 were injected in 1 mL aliquots into the vials containing bacteria and buffered medium and allowed to react for at least 1 h to convert NO_2^- to N_2O. For samples containing lower concentrations down to around 1 μmol/L, larger quantities up to about 5 mL were injected into the vials. Amounts of NO_2^- in samples and standards in each batch (typically 32 vials analyzed in 1 day) were matched to provide reliable calibration of the mass spectrometry. In an automated sequence, the N_2O in each vial was purged with He, collected cryogenically, purified by gas chromatography, and analyzed by continuous-flow isotope ratio mass spectrometry on N_2O using a headspace autosampler with a Thermo Finnigan GasBench II sample inlet system connected to a Thermo Finnigan Delta Plus mass spectrometer,[8,9] including modifications described by Coplen et al.[10] Each analysis consisted of four reference gas injections, followed by the sample peak (with GC retention time of approximately 650 s). Mass spectrometer responses to reference gas injections and sample peaks were monitored continuously at m/z 44, 45, and 46.

Data Reduction and Calibration. Integrated peak areas at m/z 44, 45, and 46 (in volt·s) were adjusted for background intensities and converted to peak area ratios at m/z 45/44 and 46/44. These ratios were converted to apparent values of $\delta^{15}N$ and $\delta^{18}O$ with respect to the reference gas by Thermo Finnigan ISODAT program, version 2.0, assuming mass-dependent variation of the O isotopes, yielding

$$\delta^{15}N_{i/rg} = [n(^{15}N)/n(^{14}N)]_i/[n(^{15}N)/n(^{14}N)]_{rg} - 1 \quad (1)$$

$$\delta^{18}O_{i/rg} = [n(^{18}O)/n(^{16}O)]_i/[n(^{18}O)/n(^{16}O)]_{rg} - 1 \quad (2)$$

where i refers to a sample, rg refers to the machine reference gas (tank N_2O), and $[n(^{15}N)/n(^{14}N)]$ is the isotope amount ratio (molar ratio) of N isotopes in a substance. Because δ values defined by eqs 1 and 2 are small, results are reported in parts per thousand (‰). The δ values were manipulated further by various stages of normalization (calibration) based on analyses of samples with independently known isotopic compositions, for example,

$$\delta_{i/STD} = \delta_{R1/STD} + [\delta_{i/rg} - \delta_{R1/rg}]_{meas}[\delta_{R2/STD} - \delta_{R1/STD}]/[\delta_{R2/rg} - \delta_{R1/rg}]_{meas} \quad (3)$$

where δ is either $\delta^{15}N$ or $\delta^{18}O$, R1 and R2 are secondary isotopic reference materials with different δ values determined independently, and STD refers to either atmospheric N_2 ($\delta^{15}N_{AIR/AIR} \equiv 0$) or Vienna Standard Mean Ocean Water ($\delta^{18}O_{VSMOW/VSMOW} \equiv 0$). Secondary reference materials used in the tests and calibrations include NO_3^- and NO_2^- salts that were dissolved in deionized

(8) Sigman, D. M.; Casciotti, K. L.; Andreani, M.; Barford, C.; Galanter, M.; Böhlke, J. K. *Anal. Chem.* **2001**, *73*, 4145−4153.
(9) Casciotti, K. L.; Sigman, D. M.; Hastings, M.; Böhlke, J. K.; Hilkert, A. *Anal. Chem.* **2002**, *74*, 4905−4912.
(10) Coplen, T. B.; Böhlke, J. K.; Casciotti, K. L. *Rapid Commun. Mass Spectrom.* **2004**, *18*, 245−250.
(11) Finkmann, W.; Altendorf, K.; Stackebrandt, E.; Lipski, A. *Int. J. Syst. Evol. Microbiol.* **2000**, *50*, 273−282.
(12) Nielsen, M.; Larsen, L. H.; Jetten, M. S. M.; Revsbech, N. P. *Appl. Environ. Microbiol.* **2004**, *70*, 6551−6558.

Table 1. Bulk Isotopic Data for Materials Used in This Study

material	sample ID[a]	δ^{15}N [AIR][b]	δ^{18}O [VSMOW][c]	ref
KNO_3	USGS34	−1.8	−27.9	14
KNO_3	IAEA-N3	+4.7	+25.6	14
KNO_3	RSIL-N11	+3.6	+26.7	14
KNO_3	USGS32	+180.0	+25.7	14
$NaNO_3$	RSIL-N21	+5625	+22.4	21
$NaNO_2$	RSIL-N7373	−79.6[d]	+4.5	5
$NaNO_2$	RSIL-N23	+3.7	+11.4	5
$NaNO_2$	RSIL-N10219	+2.8	+88.5	5
$NaNO_2$	RSIL-N30	+5256[d]	nd[e]	21
N_2O	RSIL-N51	+0.7	+42.9	5
N_2O	RSIL-R6 (bact. ref gas, rg)	−0.1	+42.4	5

[a] USGS and IAEA prefixes refer to internationally distributed reference materials; RSIL prefixes refer to local laboratory standards. [b] δ^{15}N of salts by offline conversion to N_2 and dual-inlet mass spectrometry. [c] δ^{18}O of salts by online conversion to CO and continuous-flow mass spectrometry. [d] Bulk δ^{15}N values of N7373 and N30 may be affected by minor amounts of NO_3^- with δ^{15}N closer to 0‰ (see text). [e] "nd" = no data.

Table 2. Analyses of Selected Samples by Bacterial Reduction Methods (δ^{15}N) and Estimates of the Nitrate Contents of the Nitrite Salts[a,b]

	aur δ^{15}N	aur ±	nit δ^{15}N	nit ±	est NO_3^-/NO_2^- [c]	IC NO_3^-/NO_2^- [d]
			Nitrate			
N11	3.7	0.2	nd		nd	nd
USGS32	179.9	0.3	nd		nd	nd
N21	5625.0	34.0	nd		nd	nd
			Nitrite			
N10219	*2.0*[e]	0.1	*2.1*[e]	0.1	nd	0.0067
N23	3.7	0.1	3.7	0.1	nd	0.0016
N7373	−80.1	0.1	−81.2	0.1	0.014	0.0180
N30	5233.1	6.8	5367.7	8.6	0.025	0.0174

[a] All isotope data were calibrated (normalized) by fitting the nitrate data to the reference values in Table 1; results are given as mean ± standard deviation for multiple aliquots ($n = 3$ in each case). [b] "aur" indicates analysis done with *P. aureofaciens*; "nit" indicates analysis done with *S. nitritireducens*; "nd" = no data. [c] NO_3^-/NO_2^- ratio estimated (est) from the difference between apparent δ^{15}N values by the different bacteria, assuming $\delta^{15}N[NO_3^-] = 0$‰. [d] NO_3^-/NO_2^- ratio measured by ion chromatography (IC). [e] Calculated δ^{15}N values for N10219 are too low because of ^{17}O deficiency in this material (ref 5).

water (DIW) and analyzed as samples. Values of $\delta^{15}N_{i/AIR}$ and $\delta^{18}O_{i/VSMOW}$ in these materials (Table 1) were determined independently by dual-inlet isotope ratio mass spectrometry on N_2 (N2-DIIRMS) and by continuous-flow isotope ratio mass spectrometry on CO (CO-CFIRMS).[5,13,14] Reproducibilities of analytical data are expressed as mean ± standard deviation, not including errors in the reference data.

RESULTS AND DISCUSSION

The selective bacterial NO_2^- reduction method with *S. nitritireducens* was tested first on solutions containing NO_2^- with varying isotopic compositions (Tables 1 and 2). Results for δ^{15}N and δ^{18}O demonstrate the reproducibility of the method for NO_2^- (Tables 2 and 3). The NO_2^- solutions then were combined with NO_3^- solutions to prove the selectivity of *S. nitritireducens* in mixed samples (Table 3). Mixed solutions were treated and analyzed sequentially with *S. nitritireducens* and *P. aureofaciens* to demonstrate the feasibility of selective bacterial treatments for isotopic analysis of both NO_2^- and NO_3^- in single samples (Figure 1). Applications of these methods in a small stream in an agricultural watershed illustrate some of the important types of information to be gained, as well as some of the complications involved in the interpretation of isotopes in natural systems.

Efficiency and Selectivity of NO_2^- Reduction by *S. nitritireducens*. For solutions of NO_2^- in DIW, analyzed in batches of uniform sample size (5, 10, or 20 nmol of NO_2^-), the ratios of N_2O gas produced (peak areas at m/z 44 in volt·s) to NO_2^- reacted (μmol in sample aliquot) typically were uniform in each batch to within about ±1−3%, indicating constant relative yields of N_2O from NO_2^- using *S. nitritireducens*. Furthermore, we obtained similar average yields from three different types of measurements in a single batch: relative to the yield of N_2O from NO_3^- by *P. aureofaciens* (100% ± 2%, $n = 6$), the yield of N_2O from NO_2^- by *P. aureofaciens* was 101 ± 2 ($n = 9$) and the yield of N_2O from NO_2^- by *S. nitritireducens* was 95% ± 1% ($n = 9$). In another batch, the relative yield of N_2O from NO_2^- by *S. nitritireducens* was 96 ± 2 ($n = 8$). Although the absolute amounts of N_2O were not determined independently because of larger uncertainty and variability in volumes, flow rates, and electronic parameters in the sample handling and analysis system, we interpret these mixed method results to indicate that NO_3^- and NO_2^- were converted to N_2O almost completely (95−100%, with respect to N), as reported previously for NO_3^- with *P. aureofaciens*.[8] Discrepancies of a few percent may be caused in part by minor NO_3^- contamination of some of the NO_2^- reference solutions (see the Nitrogen Isotopes in Nitrite Solutions section). Nielsen et al.[12] report approximately linear response for a biosensor based on NO_2^- conversion to N_2O by *S. nitritireducens* for NO_2^- concentrations ranging from about 1 to 1000 μmol/L. Based on results summarized above, we routinely have used the relative yields of N_2O from samples and reference solutions with known volumes to measure NO_2^- concentrations in samples, with estimated overall uncertainties of around ±5−10%.

Blanks consisting of N_2O or NO_2^- in the analytical system, as well as cross-contamination by partial reduction of NO_3^-, could cause problems for the method because of the small amount of NO_2^- in a typical sample aliquot (5−20 nmol) and because NO_2^- concentrations and NO_2^-/NO_3^- ratios typically are low in environmental samples. To test for various sources of contamination, all sets of analyses included vials containing (1) bacteria inocula and growth medium alone (no additional water or NO_x) and (2) bacteria inocula and growth medium with pure NO_3^- solutions (no NO_2^-). Some sets also included bacteria inocula and growth medium with pure DIW added (no NO_x). Analyses of vials containing bacteria and growth medium alone almost always yielded ≤0.02 nmol of N_2O-N, which is near the peak detection limit of the mass spectrometer. Analyses of vials with added DIW with or without NO_3^- had an average yield of 0.05 ± 0.04 nmol,

(13) Böhlke, J. K.; Coplen, T. B. In *Reference and Intercomparison Materials for Stable Isotopes of Light Elements*; IAEA TECDOC 825; International Atomic Energy Agency: Vienna, 1995; pp 51−66.
(14) Böhlke, J. K.; Mroczkowski, S. J.; Coplen, T. B. *Rapid Commun. Mass Spectrom.* **2003**, *17*, 1835−1846.

Table 3. Representative Nitrogen and Oxygen Isotope Data for Single- and Mixed-Reagent Tests with *S. nitritireducens*[a]

ID	N_2O $\delta^{15}N$ *i*/rg	±	*n*	N_2O $\delta^{15}N$ *i*/AIR	NO_2^- $\delta^{15}N$ *i*/AIR	N_2O $\delta^{18}O$ *i*/rg	±	*n*	N_2O $\delta^{18}O$ *i*/VSMOW	NO_2^- $\delta^{18}O$ *i*/VSMOW	cross contam f_{NO_3}[b]	cross contam nmol-N[b]
					20 μmol/L Nitrite (Set 1):							
N7373	−80.0	0.1	6	−80.1	**−81.2**	−26.7	0.2	6	14.6	4.4		
N23	3.5	0.1	6	3.4	**3.7**	−20.3	0.4	6	21.2	**11.4**		
N10219	*2.0*[c]	*0.2*	*6*	1.9	*2.2*	50.5	0.4	6	95.0	**88.5**		
N30	5238	20	6	5237	5326	144.0	2.0	6	192.5	190.4		
scale factors[d]					*1.017*					*1.090*		
					20 μmol/L Nitrite (Set 2):							
N7373	−79.8	0.1	5	−79.9	**−81.2**	−26.5	0.3	5	14.8	4.7		
N23	3.6	0.1	5	3.5	**3.7**	−20.2	0.2	5	21.3	**11.4**		
N10219	*2.2*[c]	*0.1*	*2*	2.1	*2.3*	51.4	2.6	5	95.9	**88.5**		
N30	5287	6	6	5287	5385	143.3	3.3	6	191.8	187.6		
scale factors[d]					*1.018*					*1.077*		
					20 μmol/L Nitrite + 2000 μmol/L Nitrate (Set 1):							
N23 + N21	30.6	9.6	4	30.5	31.2	−19.2	0.6	4	22.4	12.6	0.0050	0.10
N30 + N11	5224	9	4	5223	5312	143.8	1.8	4	192.3	190.2	0.0026	0.05
					20 μmol/L Nitrite + 2000 μmol/L Nitrate (Set 2):							
N23 + N21	5.2	0.2	4	5.1	5.3	−19.6	0.6	4	22.0	12.1	0.0003	0.006
N30 + N11	5274	9	4	5273	5372	148.0	1.2	4	196.6	192.5	0.0024	0.05

[a] All samples were analyzed as 1 mL aliquots in two separate batches (set 1 and set 2) with the anion concentrations listed (in DIW). Raw data for N_2O measured against the machine reference gas ($\delta_{i/rg}$) are given as mean ± standard deviation for multiple aliquots (*n*). These data were normalized by using eq 3 (with AIR or VSMOW substituted for STD) with the independently measured values of $\delta_{rg/AIR}$ (Table 1) to obtain $\delta_{i/AIR}$ for the N_2O. These data were normalized further with the assumed values of $\delta_{R/AIR}$ for two NO_2^- reference materials (N23 and N7373, in bold, from Table 2) to obtain $\delta_{i/AIR}$ for all of the other samples. [b] N_2O formed by reduction of NO_3^- ("cross-contam") was calculated from analyses of mixed samples by using eq 4 and expressed as the fraction of the total N_2O in the sample that was derived from NO_3^- (f_{NO_3}) and as the quantity of N_2O-N in the sample that was derived from NO_3^- (nmol-N). [c] Calculated $\delta^{15}N$ values for N10219 are too low because of ^{17}O deficiency in this material (ref 5). [d] Scale factors are values of the quotient $[\delta_{R2/STD} - \delta_{R1/STD}]/[\delta_{R2/rg} - \delta_{R1/rg}]_{meas}$ in eq 3.

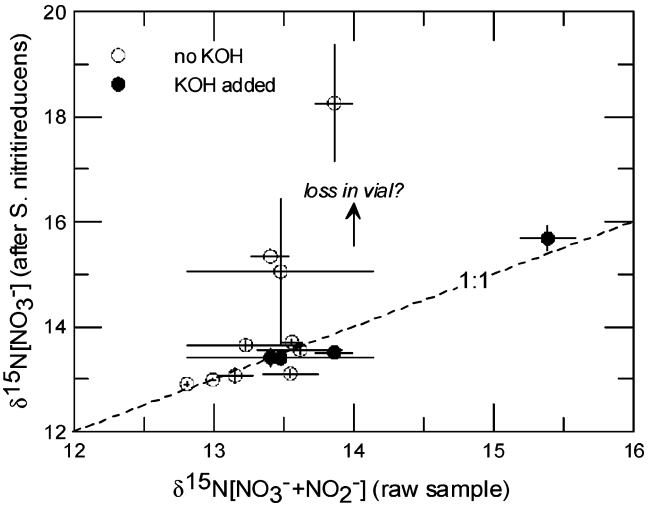

Figure 1. Comparison of nitrate isotopic analyses by *P. aureofaciens* before and after removal of NO_2^- by *S. nitritireducens*. Samples are from a small stream in Indiana (see Figures 2 and 3). Approximate agreement (near the 1:1 line) is consistent with the low NO_2^-/NO_3^- ratios of the samples. Discrepancies for some samples are attributed to partial loss of NO_3^- with isotopic fractionation after treatment with *S. nitritireducens* and before treatment with *P. aureofaciens* when KOH was not added between treatments. Error bars represent ±1 standard deviation for analyses of different aliquots treated similarly.

or 0.04 ± 0.04 μmol/L of N_2O-N ($n = 17$), with no consistent relation to the amount of H_2O (1−5 mL) or to the presence or absence of NO_3^-. The overall magnitudes of these different types of N_2O "blanks" are small compared to the normal sample sizes of 5 to 20 nmol, and the NO_3^- tests confirm that this bacterial culture was highly selective for NO_2^- (see also the Analysis of Mixed Samples with Nitrite + Nitrate section).

In some of the early experiments, the yields from NO_3^- samples and from mixed NO_3^- plus NO_2^- samples indicated substantial reduction of NO_3^-. These failures are tentatively attributed to cross-contamination of bacterial strains (*S. nitritireducens* and *P. aureofaciens*) when the same fixed multiport apparatus was used on different days to purge sample vials after introduction of different bacteria. This problem seems largely to have been solved by careful cleaning of the purging apparatus and by dedicating different purging ports to the different bacteria. In addition, some laboratory DIW sources yielded anomalously high blanks, presumably because of NO_2^- in the DIW.

Nitrogen Isotopes in Nitrite Solutions. The *S. nitritireducens* method was tested by analyzing DIW solutions containing dissolved $NaNO_2$ reagents with bulk $\delta^{15}N$ values ranging from about −80‰ to +5256‰ (Tables 2 and 3). In addition, a sample of NO_2^--free natural seawater (SW) was spiked with NO_2^- (N23) and analyzed against a DIW solution of N23 with the following results: $\delta^{15}N(DIW) = +3.7‰ \pm 0.1‰$; $\delta^{15}N(SW) = +3.6‰ \pm 0.1‰$. Typical reproducibilities of normalized $\delta^{15}N$ values determined with *S. nitritireducens* in these reference solutions are of the order of ±0.2−0.5‰ for $\delta^{15}N$ around 0‰ and ±20−50‰ for $\delta^{15}N$ around 5000‰, for 5−20 nmol NO_2^- (1−20 μmol/L in 1−5 mL aliquots). Results are consistent with quantitative transfer of N from NO_2^- to N_2O with little or no fractionation, interference, or blank effect. Apparent $\delta^{15}N$ values derived from N_2O produced by *S. nitritireducens* were similar to those derived from N_2O produced by *P. aureofaciens*, but with some minor exceptions at the high and low values. For N7373 and N30, the $\delta^{15}N$ values derived from *P. aureofaciens* reduction ($NO_2^- + NO_3^-$) were slightly closer to each other (closer to 0‰) than the $\delta^{15}N$ values

derived from *S. nitritireducens* reduction (NO_2^- only) (Table 2). These differences appear to be related to small amounts of NO_3^- contamination in the NO_2^- reference salts. If the $\delta^{15}N$ values of the NO_3^- contamination are close to 0‰, then the amounts of NO_3^- needed to cause the differences between the different bacterial reduction results for N7373 and N30 are of the order of 1−2% of the amounts of NO_2^- on a molar basis. These are approximately the same as the amounts of NO_3^- measured in the NO_2^- salts by ion chromatography (Table 2). Therefore, the $\delta^{15}N$ values measured with *S. nitritireducens* may be more accurate for the NO_2^- in the reference salts than the values measured with *P. aureofaciens* or the CO-CFIRMS bulk analyses in Table 1. Assuming this to be the case, the $\delta^{15}N$ scale factors needed to normalize the *S. nitritireducens* data to reference values typically range from about 1.01 to 1.04, similar to the range that is observed routinely for *P. aureofaciens* in this laboratory.

Oxygen Isotopes in Nitrite Solutions. The *S. nitritireducens* method was tested by analyzing DIW solutions containing dissolved $NaNO_2$ reagents with known $\delta^{18}O$ values ranging from +4.5‰ to +88.5‰ (Table 1). Overall reproducibilities of normalized $\delta^{18}O$ values determined with *S. nitritireducens* in these reference solutions are of the order of ±0.4−1.0‰ for 5−20 nmol NO_2^- (1−20 μmol/L in 1−5 mL aliquots). Results indicate substantial but reproducible fractionation of the O isotopes during partial transfer of O from NO_2^- to N_2O. For example, for N23, the average measured $\delta^{18}O$ value of the N_2O (+21.3‰ ± 0.3‰ with respect to VSMOW) was 9.9‰ higher than that of the initial NO_2^- (+11.4‰). This offset is similar in magnitude to the offset of 11.7‰ reported for N_2O produced from N23 by *P. aureofaciens*.[5] The apparent fractionation effect of NO_2^- reduction is substantially less than that of NO_3^- reduction by *P. aureofaciens* ($\delta^{18}O_{N_2O} - \delta^{18}O_{NO_3^-} \approx +38‰$), because of the difference in the number of reaction steps and number of O atoms lost during reduction to N_2O.[5] In addition, the $\delta^{18}O$ scale factors required to normalize the data for reference salts (e.g., 1.09 and 1.08 in set 1 and set 2, respectively, in Table 3) indicate that partial O isotope exchange occurred between H_2O and NO_2^- (or other NO_x species) within the sample vials during conversion of the NO_2^- to N_2O by *S. nitritireducens*. Although the amount of O exchange apparently differed from batch to batch (day to day) from about 1.05 to 1.3 in other experiments, the differences between individual samples within a given batch were much smaller. Reasons for variations in the amount of O exchange could include differences in populations and reaction rates in different batches of bacteria, which were homogenized within each sample set, or other batch-specific environmental variables. Similar amounts of exchange were reported for inorganic chemical conversion of NO_2^- to N_2O,[7] indicating that O isotopic exchange between NO_2^- and H_2O may be largely a function of reaction rate (time) and pH (abundance of protonated nitrous acid species).[5,7] As a preliminary test of the bacterial method in seawater, a sample of NO_2^- -free natural seawater from south Florida was spiked with NO_2^- (N23) and analyzed against a DIW solution of N23 with the following results: $\delta^{18}O(DIW) = +11.4‰ \pm 0.1‰$; $\delta^{18}O(SW) = +10.7‰ \pm 0.2‰$, possibly indicating a small difference in the rate of isotopic exchange between freshwater and seawater.[5,7]

Analysis of Mixed Samples with Nitrite + Nitrate. To quantify the amount and reproducibility of unwanted N_2O production from NO_3^- in mixed samples, experiments were conducted with mixtures containing either (1) ^{15}N-enriched NO_2^- (N30) and normal NO_3^- (N11) or (2) ^{15}N-enriched NO_3^- (N21) and normal NO_2^- (N23), in both cases with NO_3^- concentrations 100 times higher than the NO_2^- concentrations. Two representative sets of analyses indicate that the amount of cross-contamination was small and reproducible within each set but somewhat different between sets (Table 3). In set 1, when NO_2^- had $\delta^{15}N = +3.7‰$ and NO_3^- had $\delta^{15}N = +5625‰$, the apparent $\delta^{15}N$ value of the NO_2^- by the *S. nitritireducens* method was elevated slightly to about +31‰ ± 10‰. Conversely, when NO_2^- had $\delta^{15}N = +5368‰$ and NO_3^- had $\delta^{15}N = +3.6‰$, the apparent $\delta^{15}N$ value of the NO_2^- by the *S. nitritireducens* method was lower in the mixed sample by about 14‰. For each pair of samples, the apparent fraction of the total N_2O-N contributed from NO_3^- ($f_{NO_3^-}$) was calculated by mass balance:

$$f_{NO_3^-} = (x^{15}N_{meas} - x^{15}N_{NO_2^-})/(x^{15}N_{NO_3^-} - x^{15}N_{NO_2^-}) \quad (4)$$

where $x^{15}N_{meas}$ refers to the normalized apparent amount fraction (mol fraction) of ^{15}N in a given mixture measured by the *S. nitritireducens* method and $x^{15}N_{NO_3^-}$ and $x^{15}N_{NO_2^-}$ refer to the independently determined $x^{15}N$ values of the individual reagents in the mixture (derived from $\delta^{15}N$ values in Tables 1 and 2). Results from the set 1 mixtures indicate that the amount of N_2O-N that was produced from NO_3^- was equivalent to 0.003−0.005 times the amount of N_2O-N produced from NO_2^-, or 0.00003−0.00005 times the amount of NO_3^- present in the samples, or about 0.05−0.1 nmol per vial (Table 3). In set 2, similar calculations indicate even smaller cross-contamination effects: the amount of N_2O-N produced from NO_3^- was 0.000003−0.00003 times the amount of NO_3^- present, or around 0.006−0.05 nmol, which is near the limit of detection even with the ^{15}N tracer. These results confirm the excellent selectivity of this bacterial culture for NO_2^- even in the presence of much larger concentrations of NO_3^-.

The tracer experiments indicate that some of the N_2O "blank" may consist of residual N_2O left in the system from the previous analysis. For example, in set 2, a vial containing only bacteria and growth medium had 0.02 nmol N_2O-N with a negative $\delta^{15}N$ value not much different from that of the preceding sample of N10219. Similarly, the first aliquot of N23 following tracer sample N30 had a $\delta^{15}N$ value about 5‰ higher than the subsequent samples, indicating a "memory effect" consistent with 0.02 nmol of residual N_2O-N from the previous sample. This memory effect, as well as the documented cross-contamination from NO_3^- reduction, would not be noticeable normally in the absence of isotopic tracers, nor would they introduce substantial errors for most isotope tracer applications, but they are potential sources of error for analyses of nontracer NO_2^- in the presence of tracer NO_3^-.

Sequential Bacterial Method for Nitrite and Nitrate. Given the excellent selectivity of the *S. nitritireducens* method for NO_2^- isotopes, it would be especially useful to be able to analyze the same sample aliquots subsequently for NO_3^- isotopes after the NO_2^- was removed. This possibility was tested by analyzing both artificial and natural mixed samples containing NO_2^- and NO_3^-, first with *S. nitritireducens*, then with *P. aureofaciens*. The amount of sample treated with *P. aureofaciens* was adjusted in each case

Table 4. Nitrogen Isotope Analyses of Nitrate and Nitrite from Tracer Tests in Benthic Chambers[a,b]

time h[c]	NO_3^- μmol/L	NO_2^- μmol/L	NH_4^+ μmol/L	NH_4^+ $\delta^{15}N$	whole aur NO_3^- and NO_2^- $\delta^{15}N$	±	*n*	whole nit NO_2^- $\delta^{15}N$	±	*n*	spe aur NO_3^- $\delta^{15}N$	±	*n*	spe aur NO_2^- $\delta^{15}N$	±	*n*	spe nit NO_2^- $\delta^{15}N$	±	*n*
Injected with ^{15}N-Enriched NO_2^-																			
0	205	73	0	nd	3670	9	4	14243	265	3	118	1	2	14021	152	3	13639		1
9	171	55	0	nd	3243	28	2	13899	253	3	264	2	2	13751	127	3	13750		1
21	129	36	0	nd	3064	23	2	13467	320	3	570	3	2	13398	14	2	13313		1
Injected with ^{15}N-Enriched NH_4^+																			
0	183	1.2	120	11932	36	0	2	504	23	3	26	0	3	389	0	3	394	3	2
9	168	1.4	92	11220	129	0	2	1959	63	2	116	0	3	1601	2	3	1586	49	2
21	118	1.3	53	10207	235	0	2	2809	68	3	209	5	3	2368	19	3	2347	65	2
Injected with ^{15}N-Enriched NO_3^-																			
0	1328	1.0	0	nd	10790	54	2	3788	50	2	nd			nd			nd		
9	1061	1.2	0	nd	10717	33	2	5550		1	nd			nd			nd		
20	936	1.5	0	nd	10623	30	2	7462	116	2	nd			nd			nd		

[a] Normalized results are given as mean ± standard deviation for multiple aliquots (*n*). [b] "whole" indicates whole filtered-water samples analyzed without pre-separation of N species; "spe" (solid-phase extraction) indicates NO_3^- and NO_2^- separated by ion chromatography prior to analysis; "aur" indicates analysis done with *P. aureofaciens*; "nit" indicates analysis done with *S. nitritireducens*, "nd" = no data. [c] Approximate time after the first sample was taken from the homogenized chamber (some reaction occurred prior to the first sampling event).

to account for the difference between the concentrations of NO_2^- and NO_3^-, so the amounts of N_2O produced from NO_3^- in all samples and reference solutions were similar, but no other intervening treatments were done. For mixed samples containing ^{15}N tracers, the values of $\delta^{15}N[NO_3^-]$ derived from these sequential analyses were similar to those of the individual salts, indicating that the sequential bacterial method for NO_2^- and NO_3^- isotopes was successful. This result also was confirmed for $\delta^{15}N$ and $\delta^{18}O$ in streamwater samples containing both NO_2^- and NO_3^- (see below). In a few of the streamwater samples, the apparent yields of NO_3^- were low and the measured $\delta^{15}N[NO_3^-]$ values were high in comparison to independent bulk analyses of $\delta^{15}N[NO_3^- + NO_2^-]$ (Figure 1). These results are consistent with partial reduction of the NO_3^- in these samples after the NO_2^- isotopic analysis with *S. nitritireducens* and before NO_3^- isotopic analysis with *P. aureofaciens* (typically 1−2 days). This problem did not occur with synthetic solutions, and it is tentatively attributed to the presence of denitrifiers other than *S. nitritireducens*, either from lab contamination or from the original streamwater, that became active when exposed to the pH-buffered growth medium containing *S. nitritireducens*. This problem apparently was solved by adding KOH to the vials, after reaction with *S. nitritireducens*, just before the beginning of the automated mass spectrometer run on a batch of samples (leaving only about 1−2 h of exposure to pH-buffered medium, rather than 1−2 days, before introduction of *P. aureofaciens*). Streamwater samples preserved with KOH at the time of collection and again between the different bacterial treatments yielded excellent isotopic results from the sequential bacterial reduction method.

Sampling and Preservation of Dissolved Nitrite Samples. Casciotti et al.[5] discuss some of the conditions necessary for preservation and handling of aqueous NO_2^- samples for isotopic analysis. Experience in the current study is consistent with their data indicating that NO_2^- will exchange O with H_2O within days to weeks if the pH is less than 11. Freezing apparently is not a reliable substitute for high pH. In the current study, all reference materials and samples were prepared and stored at pH 11−12 by adding KOH. In a test involving samples of streamwater collected over a diel cycle, aliquots that were stored at room temperature at pH 11−12 for more than 1 year exhibited systematic variations in $\delta^{18}O$ ranging from about +12‰ to +18‰ that may be attributed to diel variations in the biogeochemical processes in the stream at the time of collection (see below). In contrast, aliquots of the same samples that were stored frozen and without pH adjustment had relatively constant $\delta^{18}O$ of +9.4‰ ± 0.9‰ when analyzed. The data from the frozen samples are approximately consistent with O isotope equilibration between NO_2^- and water with $\delta^{18}O = -6.9‰$ at low temperature[5] and are interpreted to indicate O isotope exchange during storage.

It is important to emphasize, however, that high pH can interfere with the bacterial reduction of NO_2^- (or NO_3^-) to N_2O during the isotopic analysis. This generally was not a problem when 1−2 mL of sample containing 10−20 μmol/L of NO_2^- is injected into 4 mL of bacterial growth medium, presumably because dilution and the buffering capacity of the growth medium are sufficient to keep the pH in the sample vial at acceptable levels for the bacteria during the reaction. Larger sample aliquots with high pH (e.g., 5 mL of sample with pH > 12 in 4 mL of growth medium) apparently overwhelmed the buffering capacity of the system and prevented bacterial NO_2^- reduction to N_2O in some cases. For freshwaters from small streams with NO_2^- concentrations of around 1−4 μmol/L, preservation at around pH 11.5 appears to provide conditions favorable for both storage and analysis. Attempts to adjust higher pH values downward by HCl titration prior to analysis caused rapid O isotopic exchange and were not successful. Presumably, larger amounts of growth medium buffer could be used for more dilute samples or higher pH samples if necessary.

NITRITE ISOTOPIC ANALYSES AND NITROGEN CYCLING IN A SMALL STREAM

The *S. nitritireducens* method was applied to samples from a small stream to seek isotopic evidence for the source of NO_2^-, which ranges in concentration from about 1 to 4 μmol/L. The samples are from a second-order reach of Sugar Creek (depth ≈ 15 cm, flow ≈ 18 L/s), which drains agricultural land in western

Indiana.[4,15−17] A variety of experiments have been conducted in this stream to investigate processes affecting N, C, and O transport and cycling.[4,15−18] As a field test of the bacterial method, we analyzed NO_2^- and NO_3^- isotopes at natural-abundance levels in a diel sample set and at ^{15}N tracer levels in samples from benthic chamber experiments.

Benthic Chamber Experiments with Tracer ^{15}N. The benthic chamber experiments included tracer additions of $^{15}NO_3^-$, $^{15}NH_4^+$, and $^{15}NO_2^-$, followed by isotopic analyses of the N species to determine rates of denitrification and nitrification.[18] Isotopic analyses of NO_2^- from some of these tracer experiments are compared in Table 4. For samples with low NO_2^- concentrations (1−2 μmol/L), the $\delta^{15}N[NO_2^-]$ values determined by the *S. nitritireducens* method on whole samples (no prior treatment other than preservation) are significantly higher than those determined in NO_2^- separated from the same samples by ion chromatography. For NO_2^- isolated by ion chromatography, the $\delta^{15}N[NO_2^-]$ values were similar when analyzed by *S. nitritireducens* and *P. aureofaciens*, indicating that the chromatography was successful in separating NO_2^- from NO_3^-. The differences between the results for whole samples and NO_2^- separates, therefore, may be caused by minor amounts of nontracer NO_2^- introduced during the chromatography, possibly from the resin or from the DIW used to rinse and elute the columns. This effect is noticeable for samples with low NO_2^- concentrations (<2−4 μmol/L) but disappears for samples with higher NO_2^- concentrations, for which all three methods gave similar results. It is concluded that the *S. nitritireducens* data for the whole samples are the most accurate for samples with low NO_2^- concentrations.

Multistep modeling of denitrification in the benthic chamber systems indicates that the $\delta^{15}N[NO_2^-]$ values should have been closer to the $\delta^{15}N[NO_3^-]$ values by the end of the experiment if denitrification was the only source of the NO_2^-.[18,19] In a chamber labeled with $^{15}NO_3^-$, values of $\delta^{15}N[NO_2^-]$ increased rapidly but did not reach the $\delta^{15}N$ values of the coexisting NO_3^-. Therefore, the benthic chamber isotope tracer data indicate that much of the NO_2^- was formed by reduction of the tracer NO_3^-, but a substantial fraction of the NO_2^- (perhaps as much as 20−30%) may have had another source such as nitrification. A nitrification source of NO_2^- is indicated also by increasing $\delta^{15}N[NO_2^-]$ values in a benthic chamber labeled with $^{15}NH_4^+$ (Table 4).[18] A similar interpretation was given for $\delta^{15}N[NO_2^-]$ data from a reach-scale $^{15}NO_3^-$ tracer experiment in Sugar Creek, when less than half of the NO_2^- appeared to be related to denitrification of the tracer NO_3^-.[4]

Diel Variations in Natural Isotopic Abundances. A diel sample series from Sugar Creek exhibited substantial variations in the concentration and isotopic composition of NO_2^-, which could be caused by diel variations in the rates of NO_2^- production or consumption, possibly in relation to other environmental variables such as light, temperature, and dissolved O_2 (Figures 2 and 3). The concentration of NO_2^- varied between about 1.4 and

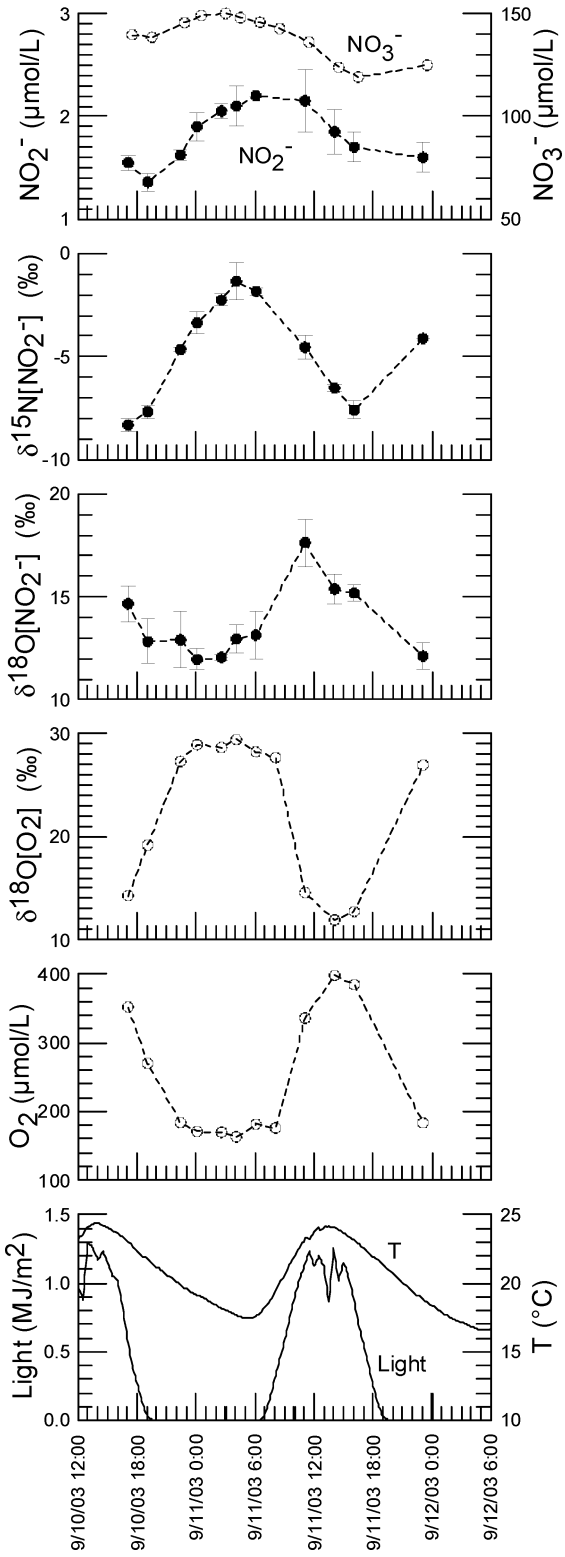

Figure 2. Diel variations in chemical and physical parameters in Sugar Creek, Indiana, September 10−12, 2003. Error bars represent ± standard deviation for analyses of NO_2^- concentrations and isotopic compositions with *S. nitritireducens* (solid symbols). Temperature, light, and dissolved O_2 data (concentrations and $\delta^{18}O$ values) are from Tobias et al. (ref 17).

(15) Antweiler, R. C.; Smith, R. L.; Voytek, M. A.; Böhlke, J. K.; Richards, K. D. *Water-quality data from two agricultural drainage basins in northwestern Indiana and northeastern Illinois: I. Lagrangian and synoptic data, 1999–2002*; U.S. Geological Survey Open-File Report 2004-1317; U.S. Geological Survey: Boulder, CO, 2005.

(16) Smith, L. K.; Voytek, M. A.; Böhlke, J. K.; Harvey, J. W. *Ecol. Appl.* **2006**, *16*, 2191−2207.

(17) Tobias, C. R.; Böhlke, J. K.; Harvey, J. W. *Limnol. Oceanogr.*, in press.

(18) Smith, R. L.; Böhlke, J. K.; Repert, D. A.; Hart, C. P. *EOS, Trans. Am. Geophys. Union* **2005**, *86*, JA229.

(19) Smith, R. L.; Böhlke, J. K.; Repert, D. A.; Hart, C. P. Manuscript in preparation.

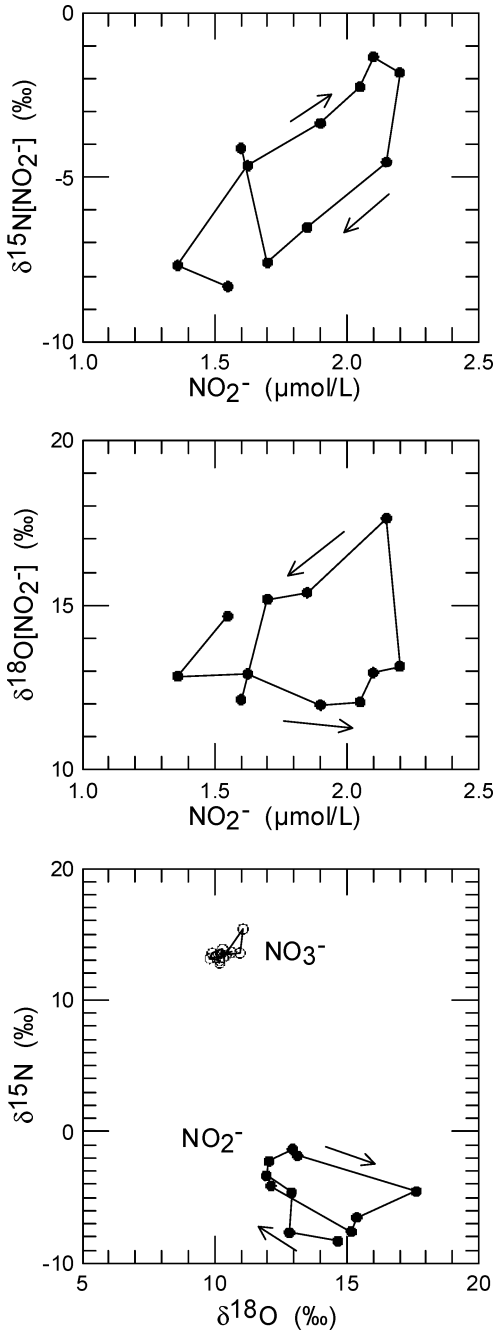

Figure 3. Isotopic composition of NO_2^- and NO_3^- in Sugar Creek, Indiana, September 10−12, 2003. Arrows indicate the direction of time (see Figure 2), highlighting apparent hysteresis-like effects in the diel cycles for NO_2^-.

2.2 μmol/L, whereas the concentration of NO_3^- varied between about 120 and 150 μmol/L. These samples were analyzed by the sequential bacterial method with *S. nitritireducens* followed by *P. aureofaciens*, and they were also analyzed by using *P. aureofaciens* alone (Figure 1). Values of $\delta^{15}N[NO_3^-]$ and $\delta^{18}O[NO_3^-]$ were similar in both cases (around +14‰ and +10‰, respectively), reflecting the fact that NO_2^- was only a small fraction of the total $NO_3^- + NO_2^-$. For NO_2^-, the measured $\delta^{15}N$ values ranged from about −8‰ to −1‰ and were highest at night, whereas the $\delta^{18}O$ values ranged from about +12‰ to +18‰ and were lowest at night.

Limited experimental data indicate that NO_2^- in isotopic equilibrium with H_2O in these streamwaters might have $\delta^{18}O \approx +7‰$ (14‰ higher than that of the coexisting H_2O at −7‰),[5] whereas NO_2^- formed by denitrification in the absence of exchange might have $\delta^{18}O$ as high as +35‰ (25‰ higher than that of the NO_3^- at +10‰).[5] Nitrite formed by nitrification could have a range of $\delta^{18}O$ values depending on the conditions of nitrification; for example, around +7‰ if it equilibrated with H_2O after it formed, around +2‰ to +12‰ if formed from equal amounts of unfractionated O from H_2O and O from dissolved O_2 with $\delta^{18}O$ ranging from +11‰ to +30‰ in a diel cycle (Figure 2), in the absence of exchange with H_2O,[20] or lower than those values if the O from H_2O or O_2 was fractionated isotopically during the reaction or if other intermediate species equilibrated with H_2O with smaller equilibrium isotope fractionation factors than NO_2^-.

The measured $\delta^{18}O[NO_2^-]$ values indicate that the NO_2^- was not in isotopic equilibrium with the H_2O. The isotope data could be consistent with the NO_2^- having formed to varying degrees by a combination of NH_4^+ oxidation and NO_3^- reduction, as indicated by the benthic chamber tracer data (Table 4).[18] In this case, the diel variations of $\delta^{18}O[NO_2^-]$, $\delta^{15}N[NO_2^-]$, and NO_2^- concentration could be related to accumulation of NO_2^- by an increase in the ratio of production (nitrification, denitrification) to consumption (denitrification) at night, followed by net loss of NO_2^- during the day (possibly related to increased overall denitrification or other changes in both production and consumption). Clearly more work would be needed to document and understand these sorts of variations, which are not related in a simple way to each other and appear to exhibit hysteresis effects (Figure 3). Nevertheless, our preliminary data illustrate the potential usefulness of the sequential bacterial method (*S. nitritireducens* followed by *P. aureofaciens*) for independent isotopic analysis of NO_2^- and NO_3^- for such studies.

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(20) Andersson, K. K.; Hooper, A. B. *FEBS Lett.* **1983**, *164*, 236−240.
(21) Böhlke, J. K.; Hannon, J. E.; Mroczkowski, S. J. Unpublished data obtained in 2006.