

A method for nitrite removal in nitrate N and O isotope analyses

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Abstract

We describe a method to remove nitrite from seawater and freshwater samples to determine the nitrogen (N) and oxygen (O) isotopic composition of nitrate in aqueous samples ($^{15}\text{N}/^{14}\text{N}$ and $^{18}\text{O}/^{16}\text{O}$, respectively). This method is simple, inexpensive, and effective in the removal of nitrite without compromising the natural N and O isotopic composition of nitrate. Moreover, the method is nontoxic and compatible with the “denitrifier method” for nitrate N and O isotope analysis. Nitrite is removed from solution by reduction to nitric oxide (NO) gas using ascorbic acid at a pH of ~ 3.5 . The NO produced is continually degassed with an inert gas during the reaction, preventing O_2 from reacting with NO to form new nitrate. Nitrate N and O isotope ratios were measured with the denitrifier method. The precision of isotope measurements of nitrate by the denitrifier method after nitrite removal averaged $\pm 0.34\text{‰}$ for $^{15}\text{N}/^{14}\text{N}$ and $\pm 0.39\text{‰}$ for $^{18}\text{O}/^{16}\text{O}$.

Introduction

Current methods to determine nitrate (NO_3^-) nitrogen (N) and oxygen (O) isotope ratios ($^{15}\text{N}/^{14}\text{N}$ and $^{18}\text{O}/^{16}\text{O}$) in aqueous samples do not distinguish between the isotopic composition of nitrate versus that of nitrite in a given sample. In the case of the ammonia distillation (Cline and Kaplan 1975) and ammonia diffusion (Sigman et al. 1997) methods for nitrate N isotope analysis, both nitrate and nitrite are converted to ammonia, then the N isotopic composition of extracted ammonia is measured. Similarly, the denitrifier method for coupled nitrate N and O isotopic analysis (Casciotti et al. 2002; Sigman et al. 2001) does not distinguish between the respective signals imparted by nitrate and nitrite, as the strains of denitrifiers used convert both nitrate and nitrite to the nitrous oxide (N_2O) gas analyte. Finally, anion exchange and pyrolysis of samples for N and O isotope analysis of nitrate in freshwater samples (Amberger and Schmidt 1987; Revesz et al. 1998; Silva et al. 2000) also includes the nitrite in a sample.

Acknowledgments

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Separation of nitrate from nitrite is particularly important when measuring the O isotopes with the denitrifier method, as the $\delta^{18}\text{O}$ of the N_2O product depends on whether nitrate or nitrite is the original substrate (Casciotti et al. 2002). The use of azide to quantitatively convert nitrite to N_2O allows for the analysis of nitrite without interference from nitrate, and this method (when combined with nitrate reduction using spongy cadmium) also allows for isotopic analysis of both nitrate and nitrite (McIlvin and Altabet 2005). Thus, the nitrate isotopes could be derived by difference using this method, but the error associated with this approach will be great when nitrite occurs at high concentration.

Nitrate samples collected in the ocean are generally devoid of nitrite, such that interference from nitrite is of no concern. Notable exceptions include oxygen minimum zones, where nitrite accumulates at mid-depths due to denitrification. Concentrations of nitrite in regions such as the Arabian Sea, the Eastern Tropical North Pacific, and the Peru Upwelling can be upwards of $13\ \mu\text{M}$, whereas nitrate concentrations in these waters are in the range of 10 to $30\ \mu\text{M}$. Nitrite also typically accumulates at the bottom of the ocean's euphotic zone from oxidation of regenerated ammonia (Ward 1987; Ward et al. 1989). Eutrophic freshwater systems characterized by high biological oxygen demand can also have significant concentrations of coincident nitrate and nitrite, as can sediment pore waters. Other instances where nitrite interferes with measurements of nitrate isotopic composition include cultures of denitrifying bacteria, where nitrite often accumulates in large amounts as nitrate is being consumed. Accurate estimates of N and O iso-

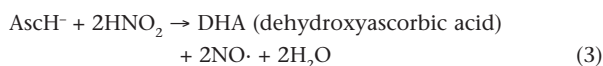
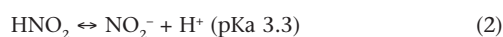
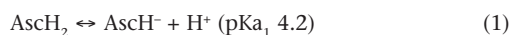
topic fractionation imparted on nitrate by denitrification therefore require that interference from nitrite be eliminated.

A number of published methods allow for the removal of nitrite without affecting nitrate concentrations. Binding of nitrite to sulfamic acid has been used previously to measure N isotope ratios of nitrate (Wu et al. 1997). However, sulfamic acid is a potent antibiotic and thus incompatible with the denitrifier method. Binding of nitrite to iodide (Garside 1982), a method commonly used to detect low concentrations of nitrite, was also deemed problematic to measure nitrate isotopes, in part because high concentrations of iodide are likely to be toxic to bacteria, and also because the method requires very low pH, which can lead to oxygen atom exchange between nitrate and water (Bunton et al. 1952). We initially used hydroxylamine to remove nitrite from our samples, the product being N₂O (Bothner-By and Friedman 1952). Though this reagent is nontoxic, any hydroxylamine remaining in solution competed with denitrifiers for the nitrite being produced from the sample nitrate, disturbing the isotopic relationships between a nitrate sample and its product N₂O.

We have developed an ascorbate-based method to remove nitrite from both freshwater and seawater samples that imparts no change in the concentration or the N and O isotopic composition of coincident nitrate. The procedure relies on the capacity of ascorbate to reduce nitrite to nitric oxide (NO) gas in mildly acidic solution at room temperature. The method is nontoxic to the bacteria used in the denitrifier method of Sigman et al. (2001) and Casciotti et al. (2002) and is cost-effective. The method presented here allows for precise and accurate quantification of nitrate N and O isotopic ratios in aqueous samples where nitrite concentrations are significant.

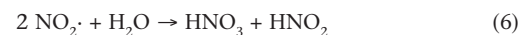
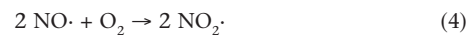
Materials and procedures

Reduction of nitrous acid by ascorbate—The pK_{a1} of ascorbic acid (AsCH₂) is 4.2, around which pH the concentration of nitrous acid (HNO₂) becomes sufficiently high to engender its spontaneous reduction by ascorbate (AsCH⁻), generating NO (nitric oxide) gas (Kanda and Taira 2003):



Whereas ascorbate readily reduces nitrous acid, it does not reduce nitrate. Thus, nitrite can be selectively removed from aqueous samples with an ascorbic acid addition sufficient to bring the solution down to a pH of around 3.5.

However, diatomic oxygen can readily react with the NO free radical and oxidize it to nitrogen dioxide (NO₂) (Equation 4). The latter can then react with the ascorbate anion to form nitrite (Equation 5) or with water to form both nitrite and nitrate (Equation 6):



To avoid these reactions, nitric oxide gas is removed continually throughout the reaction by bubbling the solution with an inert gas, maintaining a low oxygen concentration in the sample and sweeping away product NO before it reacts with any oxygen that is present.

Methodology—Nitrate and nitrite concentrations were measured by conversion to NO followed by chemiluminescence detection (Braman and Hendrix 1989) on an Antek 1750 nitrate/nitrite analyzer. Our limit of detection was ≤ 20 nM nitrate or nitrite. Nitrite was also quantified colorimetrically by reaction with Greiss reagents (sulfanilamide and *N*-1-naphthylethylenediamine [NNED]) and measurement of absorbance at 543 nm.

Before use, glassware and plasticware were acid washed (10% HCl) and rinsed with milli-Q water (Millipore). The culture medium samples analyzed in this study consisted of synthetic ocean water (Price et al. 1988/89) made from milli-Q water. This mixture did not contain any detectable nitrate or nitrite. Freshwater samples refer to milli-Q water that was not amended with salts.

A nitrate consumption experiment with the denitrifying bacterium *Pseudomonas aureofaciens* (ATCC 13985, recently reclassified as a strain of *P. chlororaphis*) was used to test nitrite removal by the present method. The strain was grown in artificial seawater medium with ~260 μM initial nitrate according to a procedure outlined previously (Granger and Ward 2003).

The ¹⁵N/¹⁴N and ¹⁸O/¹⁶O of nitrate and/or nitrite were determined following the denitrifier method (Casciotti et al. 2002; Sigman et al. 2001). Isotope ratios are reported using delta (δ) notation in units of per mil (‰):

$$\delta^{15}\text{N}_{\text{sample}} = [({}^{15}\text{N}/{}^{14}\text{N})_{\text{sample}}/({}^{15}\text{N}/{}^{14}\text{N})_{\text{reference}} - 1] \times 1000 \quad (9)$$

$$\delta^{18}\text{O}_{\text{sample}} = [({}^{18}\text{O}/{}^{16}\text{O})_{\text{sample}}/({}^{18}\text{O}/{}^{16}\text{O})_{\text{reference}} - 1] \times 1000 \quad (10)$$

where the ¹⁵N/¹⁴N reference is N₂ in air and the ¹⁸O/¹⁶O reference is Vienna standard mean ocean water (VSMOW). Referencing to air and VSMOW was through comparison to the international potassium nitrate reference material IAEA-N3, with an assigned δ¹⁵N of +4.7‰ (Gonfiantini et al. 1995) and reported δ¹⁸O of +22.7 to +25.6‰ (Böhlke et al. 1997; Böhlke et al. 2003; Revesz et al. 1997; Silva et al. 2000). We adopted a δ¹⁸O of 25.6‰ (Böhlke et al. 2003), but without consequence, since only isotope ratio differences are reported in this study. Unless indicated otherwise, the N and O isotopic ratios represent the mean of any replicate measurements; measurements of roughly 10% of the samples were duplicated.

N and O isotope ratios of nitrate measured for the nitrate consumption experiment with *P. aureofaciens* were fitted to the Rayleigh isotope fractionation model to determine the isotope

effect, ϵ (Mariotti et al. 1981), according to the following approximate Rayleigh linearization:

$$\delta^{15}\text{N (or } \delta^{18}\text{O)}_{\text{reactant}} = \delta^{15}\text{N (or } \delta^{18}\text{O)}_{\text{initial}} - \epsilon (\ln f) \quad (11)$$

where nitrate is the reactant and f is the fraction of the initial nitrate pool that remains.

Procedure—The protocol for removal of nitrite from aqueous samples involves the reaction of nitrite (as nitrous acid) with ascorbic acid (as ascorbate) to form NO gas that is continuously removed by bubbling with an inert gas (N_2 , Ar, He). The concentration of nitrate in the samples containing nitrite was first measured on the nitrate/nitrite analyzer after trapping nitrite with sulfanilamide and NNED (Greiss reagents). The samples were then transferred into serum vials. Either 10 or 20 mL sample was aliquoted into 20- or 30-mL vials, respectively. The vials were capped with (gray) butyl or silicone septa and secured with an aluminum crimp seal. The weight of each capped sample vial was then recorded in order to account for evaporation of water incurred by bubbling.

Sealed serum vials containing samples that originated from dense cultures of denitrifying bacteria were gently heated on a hotplate for 10 min to temperatures slightly below 100 °C to sterilize the mixture. This step prevented bacteria from reacting in the samples. This heating step appeared to cause no change in the isotopic composition of nitrate (data not shown).

Oxygen was then stripped from the samples by gently bubbling the vials with an inert gas for 2 h at room temperature. The gas inflow consisted of a 1.5-inch 24-G needle (Becton-Dickinson) perforating the septum and immersed in the sample, and the outflow was a 0.5-inch 26-G needle emerging from the headspace in the vial.

A 1.0 M solution of ascorbic acid was prepared daily with milli-Q water in a 10-mL serum vial that was capped and sealed like the samples. Each preparation of ascorbic acid was tested for nitrate or nitrite contamination with the nitrate/nitrite analyzer. Ascorbic acid solutions from different stock salts were consistently found to be devoid of either contaminant. The sealed ascorbic acid solution was then bubbled for 2 h concomitantly with the samples to strip it of dissolved (and headspace) oxygen.

After gas purging, an aliquot of the ascorbic acid solution was added to each sample to a final concentration of 10 mM (which was found to be optimal for nitrite removal, as described below). The solution was transferred using an acid-rinsed gas-tight syringe, taking care not to contaminate the samples with oxygen. While extracting the ascorbic acid, the inflow of inert gas remained immersed in this solution, and the outflow needle was removed to prevent oxygen entry into the solution as it was syringed from the vial.

Bubbling of each sample was continuous while injecting the ascorbic acid. The ascorbic acid solution was injected into the samples' headspace as a spray, to minimize the dissolution of any contaminating oxygen in the samples. Once the ascorbic acid was added, the samples were left to bubble from 3 h

to overnight, depending on the initial nitrite concentration (see below). Continuous bubbling allowed for generated NO gas to escape from the vials, thus shifting the equilibrium of the reaction to NO production. Moreover, the positive pressure imparted by continuous bubbling of the samples ensured that no oxygen leaked into the samples and that NO was efficiently purged away after being produced (as neither butyl nor silicone septa are completely gas-tight), thus preventing formation of nitrite or nitrate from NO.

For each run, a single sample was split into duplicates, and one of these was amended with 50 μL per 10 mL of 10% atom H_2^{18}O (Medical Isotopes), yielding a sample with water having a $\delta^{18}\text{O}$ of approximately 300‰. A significant positive deviation in the $\delta^{18}\text{O}$ of nitrate in the enriched sample compared to its unamended duplicate would indicate the formation of nitrate from nitric oxide during nitrite removal, due to contamination with oxygen during the reaction. Also, the possibility of O exchange in nitrate mediated by the acidic pH values generated by the ascorbic acid would be tested by the addition of $^{18}\text{O}\text{-H}_2\text{O}$, although this side-reaction was of minor concern as it should be extremely slow at a pH of 3.5 (Bunton et al. 1952).

To ensure complete nitrite removal in the samples by ascorbate at the end of the bubbling phase, an aliquot of each sample was injected into the nitrate/nitrite analyzer (whose vanadium solution rested in a methanol/ice bath to detect nitrite but not nitrate). Samples in which nitrite was still present were immediately further degassed to prevent contamination with oxygen. Samples devoid of nitrite were then weighed individually to account for volume loss during bubbling.

The concentration of nitrate in each sample was measured on the nitrate/nitrite analyzer. Although the concentration of nitrate measured was generally accurate once we accounted for any loss of volume from purging, our initial measurements of nitrate while trapping nitrite proved more reliable. Approximately 4 μL of a 10 M solution of sodium hydroxide (NaOH) was then added to 10 mL of sample, restoring the pH to 7 or higher. Although NaOH of the purest available quality was used, prepared stock solutions of NaOH were consistently found to be contaminated with at least $\sim 0.1 \mu\text{M}$ nitrate/nitrite (corresponding to an addition of 0.4 nM nitrate/nitrite to the samples). Blank samples (milli-Q + nitrate + no base; milli-Q + nitrate + base) were thus run to account for this; however, NaOH addition was not found to perceptibly alter the isotopic composition of nitrate. The neutralization step with NaOH ensures that any nitrite generated by the denitrifiers during isotope analysis will not be converted to NO by ascorbate, but rather converted to N_2O solely by the bacteria. Trial analyses without the NaOH suggested that this step was not necessary, presumably because the sample additions were too small to affect the pH of the bacterial growth medium. However, we cannot rule out an effect under all conditions. The neutralized samples were then transferred to acid-washed polypropylene bottles and frozen awaiting isotope analysis.

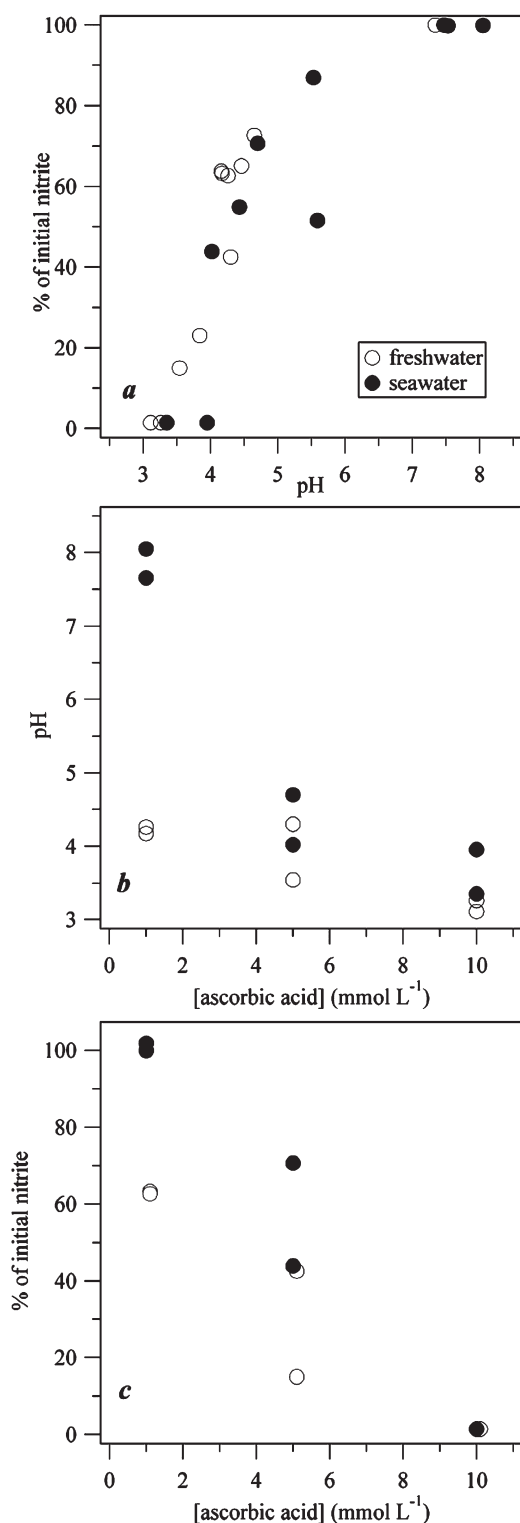


Fig. 1. Nitrite removal from freshwater and seawater samples with ascorbic acid. Vials containing 10 mL water and 300 μ M nitrite were amended with a range of ascorbic acid additions (0 to 10 mM) and purged with helium gas for 4 h. (A) Percentage of initial nitrite (300 μ M) remaining as a function of pH. (B) Change in the pH of seawater and freshwater samples with increasing concentrations of ascorbic acid. (C) Percentage of initial nitrite remaining as a function of ascorbic acid concentration.

Assessment and discussion

To determine the amount of ascorbic acid necessary to remove nitrite, seawater and freshwater samples amended with 300 μ M nitrite were titrated with increasing concentrations of ascorbic acid. As illustrated in Figure 1A, the amount of nitrite removed by ascorbate in solution after 4 h of purging increased with decreasing solution pH. The amount of nitrite removed was greatest around a pH of 3.5; explicably, this lies between the pKa values of nitrous acid and ascorbic acid, wherein nitrous acid and the ascorbate anion coexist in sufficiently large concentrations to react with one another. The decrease in pH of the solutions was itself a direct function of the amount of ascorbic acid added to the solution (Figure 1B). In both freshwater and seawater samples, 10 mM ascorbic acid reduced the pH of the solution sufficiently to allow for complete nitrite removal (Figure 1C).

The time dependence of the reaction was monitored to determine the length of time necessary to completely remove nitrite and any resulting NO from the samples (Figure 2). Replicate seawater samples were amended with 1 mM nitrite and subsampled at time intervals after the addition of ascorbic acid at a final concentration of 10 mM. The subsamples were extracted with a syringe and transferred immediately to Greiss reagents for colorimetric analysis. In this experiment, detection of nitrite with the Greiss reaction was preferable to detection using the nitrate/nitrite analyzer because the latter would also have detected nitric oxide present in solution. As illustrated in Figure 2, more than 90% of the nitrite was lost within 2 h of ascorbic acid addition. However, only after 5 h were both nitrite and nitric oxide no longer detectable in the sample solution and sample headspace (≤ 20 nM with the nitrate/

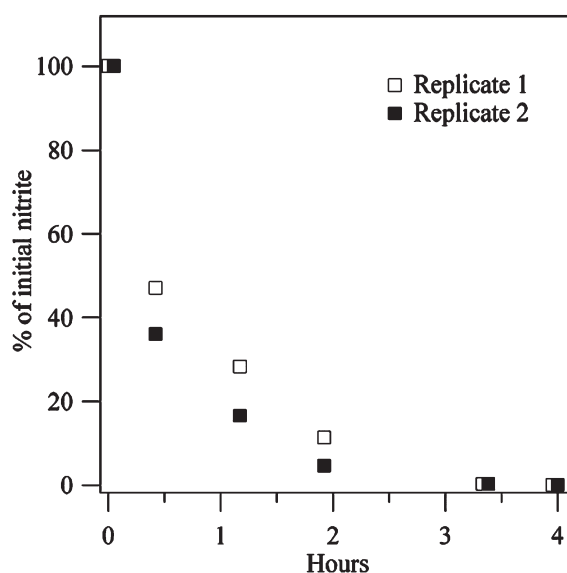


Fig. 2. Time dependence of nitrite removal by ascorbic acid. Nitrite solution (1 mM) in replicate 10-mL seawater samples amended with 10 mM ascorbic acid and bubbled with N₂ gas.

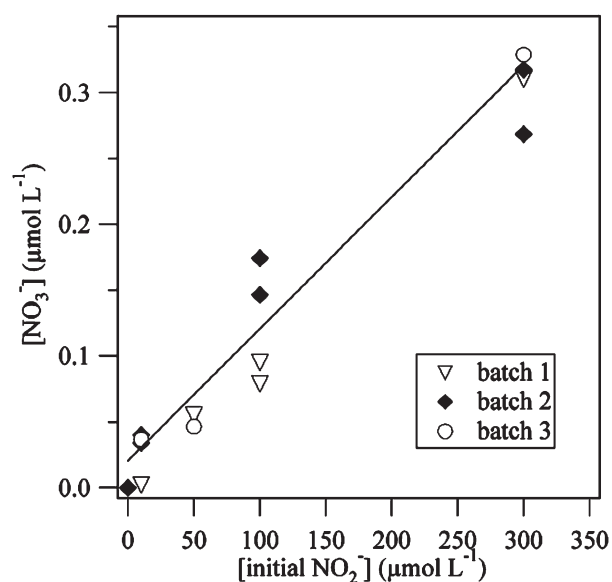


Fig. 3. The concentration of nitrate remaining in seawater after the removal of incremental amounts of nitrite. Ten-milliliter samples containing from 0 to 300 μM nitrite were amended with 10 mM ascorbic acid and bubbled with N_2 gas overnight in 3 separate experiments. Symbols represent the separate removal experiments.

nitrite analyzer), as the rate of reaction seemingly decreased with time. Based on these results, we suggest bubbling the samples for at least 3 h after ascorbic acid addition for nitrite concentrations $\leq 10 \mu\text{M}$, and for 5 h or more for concentrations exceeding 300 μM nitrite. In many instances, we allow our samples to bubble overnight for the sake of convenience.

To ascertain that nitrate was not formed during nitrite removal, we carried out the reaction in nitrate-free seawater amended with a range of nitrite concentrations, from 0 to 300 μM . Figure 3 illustrates that a small amount of nitrate was detected in the samples after nitrite was removed, except in the control sample to which no nitrite was added. This could possibly indicate that some nitrate was formed in the reaction. However, the concentration of nitrate remaining in the samples was similar among runs for a given nitrite concentration, and it increased roughly linearly with initial nitrite. This suggests that the nitrate is likely a contaminant of the nitrite stock, amounting to a 0.1% molar ratio in the stock. We posit

that detectable formation of nitrate due to oxygen contamination would have yielded less consistent nitrate concentrations than those observed here. As a positive control, ascorbic acid was added to seawater samples containing 100 μM nitrite, and these samples were left uncapped on the bench top overnight. Though no nitrite remained in the samples the following day, variable amounts of nitrate had formed, ranging from 6 to 20 μM (data not shown).

Shown in Table 1, an increasing range of nitrite additions was removed from a 10- μM nitrate solution to verify that the isotopic composition of nitrate was not altered by nitrite removal. No clear isotopic differences were detectable at nitrite concentrations up to 50 μM .

The isotopic composition of standard additions of IAEA-N3 potassium nitrate to seawater, from which a more narrow range of nitrite concentrations were removed, is summarized in Table 2. The isotopic composition of nitrate in the treatment samples showed slightly lower $\delta^{15}\text{N}$ and $\delta^{18}\text{O}$ values than standard, with negative deviations in the treatments averaging 0.2‰ and 0.3‰ for $\delta^{15}\text{N}$ and $\delta^{18}\text{O}$, respectively. The magnitude of individual deviations was not proportional to the concentration of nitrite removed from samples.

A number of samples were collected from various growing cultures of denitrifying bacteria (Granger et al. 2004a). Individual samples were split into duplicates, from which nitrite was removed and nitrate isotopes were analyzed in separate runs (Table 3). Duplicate measurements were, in most cases, similar for both N and O isotopes. The reproducibility of the measurements is quite good considering that both nitrate and nitrite spanned a considerable range of concentrations in these samples, from a few micromoles per liter to upwards of 300 μM . The mean precision based on 15 duplicate analyses was 0.34‰ for $\delta^{15}\text{N}$ and 0.39‰ for $\delta^{18}\text{O}$. These estimates include variability in vial preparation, nitrite removal, blank variability, and isotope analyses. Also shown in Table 3 are nitrate isotopic measurements of duplicate samples where ^{18}O -enriched water was added to one of the duplicates before nitrite removal. Again, the $\delta^{18}\text{O}$ values measured among duplicates were nearly identical, indicating no measurable nitrate formation in these samples.

One uncertainty that remains regarding our protocol and all isotope work on nitrite-bearing samples involves the possi-

Table 1. N and O isotopic composition of nitrate (or nitrite) after nitrite was removed from seawater with ascorbic acid while bubbling with N_2 gas.

Nitrate, μM	Nitrite, μM	Ascorbic acid, mM	<i>n</i>	$\delta^{15}\text{N}$, ‰	$\delta^{18}\text{O}$, ‰
10	—	—	2	1.63 ± 0.01	24.55 ± 0.49
10	—	10	2	1.77 ± 0.20	23.99 ± 0.40
10	10	10	2	1.71 ± 0.21	23.21 ± 0.39
10	50	10	2	1.95 ± 0.18	23.52 ± 0.28
-	100	—	2	0.94 ± 0.20	$-14.17 \pm 2.64^*$

Data are mean \pm SD. * $\delta^{18}\text{O}$ that would be assigned if analyte were assumed to be nitrate.

Table 2. N and O isotopic composition of international potassium nitrate reference material IAEA-N3 (solutions in deionized water) after nitrite was removed with ascorbic acid while purging with helium gas.

Nitrate, μM	Nitrite, μM	Ascorbic acid, mM	<i>n</i>	$\delta^{15}\text{N}$, ‰	$\delta^{18}\text{O}$, ‰
20	0	0	3	4.60 ± 0.05	25.77 ± 0.12
20	2.7	10	3	4.39 ± 0.04	25.35 ± 0.15
20	5.4	10	3	4.52 ± 0.16	25.29 ± 0.30
20	10.8	10	3	4.51 ± 0.19	25.30 ± 0.11

IAEA-N3 was assigned $\delta^{15}\text{N}$ $4.7 \pm 0.05\text{‰}$ (Gonfiantini et al. 1995) and reported $\delta^{18}\text{O}$ $25.6 \pm 0.03\text{‰}$ (Böhlke et al. 2003). Data are mean \pm SD.

bility of nitrite loss and conversion (in particular, to nitrate) during sample storage (Dore et al. 1996). We and others have observed that nitrite is partially converted to nitrate in acidified samples during storage (M.A. Altabet, personal communication, 2005), and the same may occur in frozen samples (freezing being the preservation method for all samples analyzed here). It is possible that this underlies some of the cases of poor external replication reported in Table 3. Preservation and storage tests on dissolved nitrite are badly needed and are underway in another laboratory (K.L. Casciotti, personal communication, 2005). Until these questions are resolved, we recommend that the nitrite removal step be performed as soon as possible after sample collection.

We tested our method on samples collected from the nitrate consumption experiment with the denitrifier *P. aureofaciens*. As illustrated in Figure 4A, nitrate was respired during growth, and nitrite was concomitantly produced in nearly stoichiometric amounts to the nitrate consumed. Nitrite, which reached concentrations of more than 240 μM , was removed from the samples, and the N and O isotope ratios of nitrate were then mea-

sured. Figure 4A shows that, as nitrate concentration decreased, both the $^{15}\text{N}/^{14}\text{N}$ and $^{18}\text{O}/^{16}\text{O}$ of the nitrate increased owing to isotope fractionation imparted by the uptake and reduction of nitrate. On a Rayleigh plot (Figure 4B), the nitrate isotope ratios define an approximately straight line against the natural logarithm of the fraction of nitrate consumed, yielding slopes approximating respective isotope effects (ϵ) of 23.5‰ for N and 22.0‰ for O. The N isotope effect observed here falls in the range expected from previous observations (Barford et al. 1999). Oxygen isotope effects on nitrate during denitrification have not been reported previously. However, the similarity in the magnitudes of the N and O isotope effects of *P. aureofaciens* is consistent with our previously reported 1:1 relationship between nitrate N and O isotope fractionation in nitrate assimilation by marine eukaryotic algae (Granger et al. 2004b).

Comments and recommendations

Presented here is a simple, nontoxic, and cost-effective method to remove nitrite, allowing for accurate quantification of the nitrate N and O isotopic composition of samples with

Table 3. Nitrate $\delta^{15}\text{N}$ and $\delta^{18}\text{O}$ measured for duplicate nitrite removals of individual samples from denitrifying bacterial cultures.

Sample	Nitrate, μM	Nitrite, μM	$\delta^{15}\text{N}$, ‰			$\delta^{18}\text{O}$, ‰		
			1	2	Difference, ‰	1	2	Difference, ‰
28.3B	3	283	21.68	20.42	1.26	41.23	41.81	0.57
40.5A	53	270	6.53	6.80	0.28	29.86	30.12	0.26
40.5B	132	118	5.97	5.80*	0.17	28.96	28.79*	0.17
39.7A	144	146	20.91	21.87	0.97	43.70	44.56	0.86
39.5A	187	63	3.73	3.72*	0.01	26.80	26.83*	0.02
36.7B	244	246	13.23	13.60	0.37	34.94	35.52	0.58
38.3B	252	38	6.93	6.23	0.70	29.35	29.17	0.18
39.5B	262	28	6.21	5.50	0.71	29.18	30.08	0.90
40.1A	322	0	1.24	1.29*	0.05	24.64	24.64*	0.01
37.3B	348	142	9.35	9.50	0.15	32.00	32.41	0.41
37.2B	367	123	8.94	8.95	0.01	32.17	31.60	0.57
36.5B	374	139	6.42	6.51	0.10	28.66	28.42	0.24
36.5A	425	65	4.24	4.13	0.11	27.25	26.93	0.32
37.1B	515	3	1.34	1.25	0.09	24.45	24.71	0.26
37.1A	524	0	1.30	1.21	0.09	24.45	24.25	0.20
Mean					0.34			0.39
SD					0.37			0.26

*Replicates amended with ^{18}O -enriched water.

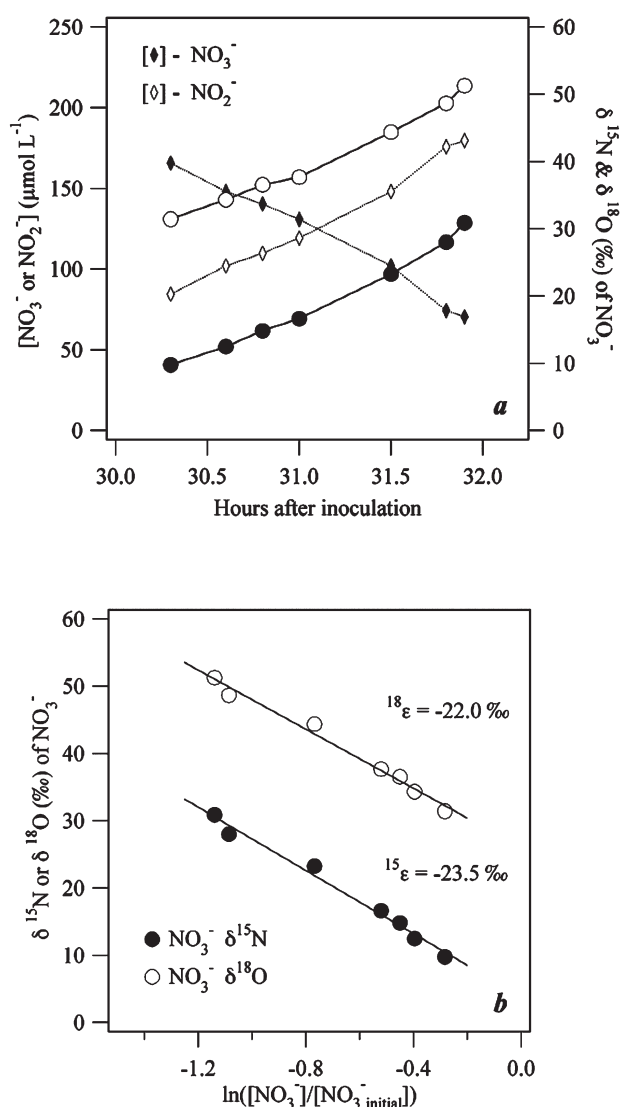


Fig. 4. N and O isotope enrichment of nitrate during the growth of *P. aureofaciens*. (A) Changes in the concentration of nitrate and nitrite as a function of time, as well as concomitant increases in the $\delta^{15}\text{N}$ (●) and $\delta^{18}\text{O}$ of nitrate (○). (B) The $\delta^{15}\text{N}$ and $\delta^{18}\text{O}$ of nitrate plotted against the natural logarithm of the fraction of nitrate consumed. The slopes of the fitted regressions approximate the respective isotope effects, ϵ , for N and O imparted by the dissimilatory reduction of nitrate by *P. aureofaciens*.

co-occurring nitrite. One concern is the risk of exposure to oxygen during nitrite removal, where the NO generated from nitrite reduction can be oxidized to nitrate. This can be monitored with ^{18}O -labeled water addition or simply by replication of the nitrite removal step for individual samples, allowing for detection of outlying measurements. This method is allowing for examination of the isotopic composition of nitrate associated with its consumption by denitrifying bacteria in culture (Granger et al. 2004a), in which nitrite often accumulates in large amounts as nitrate is consumed. Moreover, this method

can be used to remove nitrite from natural samples, allowing for accurate measurements of the N and O isotopic composition of nitrate in oxygen-deficient and eutrophic waters.

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