RAPID COMMUNICATIONS IN MASS SPECTROMETRY
Rapid Commun. Mass Spectrom. 2009; **23**: 3753–3762
Published online in Wiley InterScience (www.interscience.wiley.com) DOI: 10.1002/rcm.4307

RCM

Removal of nitrite with sulfamic acid for nitrate N and O isotope analysis with the denitrifier method

Julie Granger* **and Daniel M. Sigman**
Department of Geosciences, Princeton University, Princeton, NJ 08544, USA

Received 5 June 2009; Revised 21 September 2009; Accepted 24 September 2009

In environmental water samples that contain both nitrate (NO_3^-) and nitrite (NO_2^-), isotopic analysis of nitrate alone by all currently available methods requires pretreatment to remove nitrite. Sulfamic acid addition, used previously for this purpose (Wu JP, Calvert SE, Wong CS. *Deep-Sea Research Part I – Oceanographic Research Papers* 1997; 44: 287), is shown here to be compatible with the denitrifier method for both N and O isotope analysis of nitrate. Sulfamic acid at a pH of ~1.7 reduces nitrite to N_2. Samples are then neutralized with base prior to isotope analysis, to alleviate the buffering demands of the bacterial media and as a precaution to prevent modification of nitrate during storage with the residual sulfamic acid at low pH. Under appropriate reaction conditions, nitrite is completely removed within minutes. Sulfamic acid treatment does not compromise the completeness of the conversion of nitrate into N_2O or the precision and accuracy of N and O isotope measurements by the denitrifier method. Nitrite concentrations upwards of 7 times the ambient nitrate can be removed without affecting the isotope composition of nitrate. The method is applied to analyses of the coupled N and O isotopes of nitrate and nitrite in waters of the Mexican Margin, to illustrate its efficacy and utility when employed either in the field upon sample collection or in the lab after months of frozen sample storage. Copyright © 2009 John Wiley & Sons, Ltd.

The advent of novel N_2O-based methodologies has led to an increasing number of reported measurements on the natural abundance N and O isotope ratios of nitrate (NO_3^-) in environmental samples. In particular, the 'denitrifier' method[1,2] boasts a low limit of detection and high precision for measurement of both the N and the O isotopes of nitrate in diverse natural waters, including freshwater and seawater. The basis of the method is the quantitative conversion of nitrate into N_2O gas by denitrifying bacteria that lack an active N_2O reductase. All the N atoms of nitrate, and one in six of the oxygen atoms, are recovered as the N_2O gas analyte, whose N and O isotope composition is measured by gas chromatography/isotope ratio mass spectrometry (GC/IRMS) with on-line cryo-trapping.

One limitation of the denitrifier method is that both the nitrate and the nitrite in a sample are converted into N_2O, such that there is no distinction between the isotopic compositions of either species. This is of limited concern for most oceanic samples, in which the nitrite concentration is negligible. Nevertheless, there are environments in which nitrite can comprise a significant portion of the total oxidized N species; these include some streams, rivers and lakes, waste treatment effluents, coastal and estuarine systems, sediment pore waters, and the ocean's oxygen-deficient zones. The presence of nitrite in such samples is problematic, not only because of its interference with nitrate in the context of the denitrifier method, but also because nitrite is chemically active, undergoing reactions after collection.[3] First, the oxygen atoms of nitrite are subject to chemical equilibration with the oxygen isotopes of water on relatively short time scales, such that the length of sample storage (including frozen samples) influences the O isotope composition of nitrite.[3] Second, nitrous acid in aqueous media readily decomposes to nitrogen oxides, which can escape in the gaseous phase or can re-oxidize to nitrite and nitrate in the aqueous phase.[4] The extent to which the decomposition of protonated nitrite alters the isotopic composition of nitrate during storage of environmental samples remains unclear. However, the rate of decomposition increases significantly with decreasing pH, augmenting the generation of nitrogen oxides and thus of secondary nitrite and nitrate.[5] In the past, this has demonstrably compromised oceanographic nitrate samples that were acidified as a means of preservation. As an example, as much as 10 μM nitrite in acidified seawater samples from the Peruvian Margin disappeared after a few months; moreover, nitrate concentration analyses indicated that roughly a third of this missing nitrite had been converted into nitrate (R. S. Robinson, unpublished results).

Current methods for nitrate N and O isotope analyses have been developed to quantify the isotopic composition of nitrite and to separate it from that of nitrate. The 'azide' method allows for measurements of the isotopic composition of nitrite discretely, and this component is then subtracted from the isotopic composition of nitrate plus nitrite to derive the isotopic composition of nitrate.[6] The isotopic composition of nitrite alone is obtained from direct reduction to an N_2O analyte with azide, whereas nitrate undergoes a

*Correspondence to: J. Granger, Department of Geosciences, Princeton University, Princeton, NJ 08544, USA.
E-mail: jgranger@princeton.edu

Copyright © 2009 John Wiley & Sons, Ltd.

two-step reduction; first the nitrate is reduced to nitrite by cadmium, then nitrite is reduced to N_2O with azide. Nitrite can be converted directly into N_2O by azide upon sample collection, thus ensuring the preservation of the original N and O isotope signature of nitrite in the N_2O analyte[3,6] (K. L. Casciotti, personal communication). However, the measurement of nitrate plus nitrite is performed later in the lab, such that any nitrite present along with nitrate may be subject to O exchange and decomposition during storage; measurements of the isotopic composition of nitrite, and possibly that of nitrate, may thus become compromised due to storage.

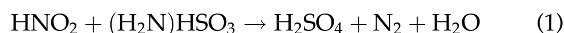
A bacterial procedure to measure the isotopic composition of nitrite has also been developed, wherein nitrite is converted into N_2O by a denitrifier strain that lacks both a nitrate reductase and a nitrous oxide reductase.[7] The isotopic composition of the remaining nitrate is then obtained from analysis of the spent sample with the traditional denitrifier method.[1,2] Here, again, the accuracy of the measurements may be compromised due to the dismutation of nitrite during sample storage, and it would not be practical to use this approach to remove nitrite in the field.

In previous work, we documented attempts to remove nitrite from aqueous samples using reduction to N_2O by hydroxylamine at neutral pH.[8] Hydroxylamine is non-toxic, and was thus deemed potentially compatible with the denitrifier method. While effective at removing nitrite, hydroxylamine remains active during the bacterial conversion of nitrate into N_2O and competes with the bacterial reaction. This yields incoherent N and O isotope measurements.

Removal of nitrite with ascorbic acid prior to nitrate isotopic analysis proved viable.[8] The addition of ascorbic acid to nitrate samples at mildly acidic pH catalyzes the reduction of nitrite to nitric oxide (NO) gas, which is continually purged out of solution with an inert gas stream.[8] The ascorbate method has the advantage of being non-toxic and hence compatible with the denitrifier method under any imaginable ascorbate amendment. Ascorbate treatment has proven useful for the removal of very high concentrations ($\leq$2 mM) of nitrite in culture samples of denitrifiers to determine the N and O isotope effects associated with dissimilatory nitrate respiration,[9] as well as for isotope analyses of nitrate samples from sediment pore waters,[10] and from marine oxygen minimum zones.[11] However, with this protocol, it is difficult to categorically avoid NO oxidation to nitrate due to oxygen contamination,[8] especially when applied in the field. Thus, we have sought an alternate method that is more easily and robustly implemented to selectively remove nitrite from aqueous samples. Sulfamic acid ($(H_2N)HSO_3$) reduces nitrite to N_2 gas at acidic pH, and it has been used previously[12] to remove nitrite for nitrate N isotope analyses with the ammonia distillation method.[13] We had originally avoided using sulfamic acid to remove nitrite,[8] as it has antibiotic properties as a potent carbonic anhydrase inhibitor,[14] and this could interfere with the denitrifier method. We have, however, found that sulfamic acid, added at a concentration required to remove nitrite from environmental samples, does not demonstrably compromise nitrate conversion into nitrous oxide by the denitrifiers, nor does it affect the N or O isotopic composition of nitrate. We report observations that validate and inform the use of sulfamic acid to remove nitrite from environmental samples, in order to preserve the samples for subsequent N and O isotopic analysis of nitrate alone. We also explore the effect of the storage of frozen samples on nitrate isotope analyses for samples with significant initial nitrite, in the case where nitrite removal was conducted after the storage period. Based on our results, we recommend a simple protocol for the use of sulfamic acid, the reliability and convenience of which should prove useful in both lab and field settings.

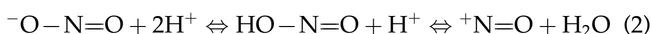
RATIONALE

Sulfamic acid is a moderately strong acid ($pK_a \sim 1.3$) that reacts with nitrous acid ($pK_a \sim 3.4$) to yield N_2 gas and sulfuric acid:[5,15]



$$HNO_2 + (H_2N)HSO_3 \rightarrow H_2SO_4 + N_2 + H_2O \quad (1)$$

The reaction involves nucleophilic attack by the primary amine group onto the nitrosonium cation (NO^+) that arises from the protonation of nitrous acid and is present in very low equilibrium concentrations. This is commonly referred to as a diotization reaction:[16]



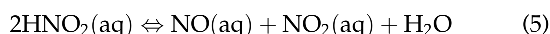
$$^-O-N{=}O + 2H^+ \Leftrightarrow HO-N{=}O + H^+ \Leftrightarrow {}^+N{=}O + H_2O \quad (2)$$

The resulting diazonium cation dissociates to N_2, sulfuric acid, and water. The reduction of nitrite to N_2 by sulfamic acid is a pH-dependent reaction with pseudo-first-order dependence on both nitrite and sulfamic acid. In general terms, at a fixed temperature and pH, the apparent reaction rate expression for nitrous acid reduction by sulfamic acid is as follows:[15]

$$v = k\,[HNO_2]\,[HSO_3NH_2] \quad (3)$$

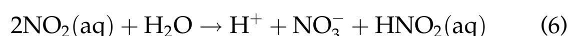
where the rate constant, k, is both temperature- and pH-dependent. Values of k increase with $[H^+]$ at low acidity, gradually changing to zero order dependence on $[H^+]$ as the acidity is increased ($<$ pH $\sim$1.3). This behavior is consistent with the sulfamate ion being the reactive species at lower acidity, whereas the leveling off is consistent with the protonation of the sulfamate ion (pK_a $\sim$1.3) to the less reactive sulfamic acid.[15]

The reduction of nitrous acid by sulfamate is relatively rapid at high acidity; however, when the pH is higher than 3 the reaction rate is reportedly comparable with the rate of nitrous acid decomposition into nitrogen oxides:[17,18]



$$2HNO_2(aq) \Leftrightarrow NO(aq) + NO_2(aq) + H_2O \quad (5)$$

The NO_2 produced during decomposition can readily undergo irreversible hydrolysis to yield nitric and nitrous acids:[17]



$$2NO_2(aq) + H_2O \rightarrow H^+ + NO_3^- + HNO_2(aq) \quad (6)$$

To minimize the extent of these secondary reactions during nitrite reduction, sulfamic acid can be added in relative excess to nitrite, and the pH of the reaction can be maintained near the pK_a of sulfamic acid, thus maximizing the reduction rate. It should be noted that carrying out the reaction at a pH

Copyright © 2009 John Wiley & Sons, Ltd.

DOI: 10.1002/rcm

much lower than the pK_a of sulfamic acid gives no increase in the reduction rate, and could further prove deleterious due to an increased likelihood of sulfamic acid reacting with nitric acid ($pK_a \sim -1.4$) to form N_2O:[19]

$$HNO_3 + NH_2SO_3H \rightarrow N_2O + H_2SO4 + H_2O \quad (7)$$

This would interfere with the accuracy of the subsequent nitrate isotope ratio measurements.

EXPERIMENTAL

Methodology

Sulfamic acid (≥99%) was purchased from Acros Organics (Morris Plains, NJ, USA). Hydrochloric acid solutions were made with analytical grade concentrated HCl. Sodium hydroxide for N determination was used throughout (3734-10; J.T. Baker, Phillipsburg, NJ, USA). Because NaOH is easily contaminated by atmospheric nitrogen oxides, the bottle of sodium hydroxide flakes was stored in a vacuum desiccator with dehydrated silica gel as well as permanganate pellets (Purafil® SP Media; Doraville, GA, USA) to adsorb contaminating NO_x species. All solutions were made with UV-oxidized 18.2 Mohm resistivity water.

Glassware was acid-washed then baked at 500°C prior to use. The sulfamic acid reagent was made by first dissolving the sulfamic acid crystals in water, after which the corresponding volume of HCl was added. Sulfamic acid solutions were stored in Wheaton glass bottles on the bench top and made fresh monthly. Because NaOH is particularly susceptible to contamination by nitrogen oxides, solutions were stored in acid-washed plastic bottles in sealed plastic bags along with the permanganate adsorbent to scavenge nitrogen oxides.

Nitrite was measured by reduction to nitric oxide (NO) in hot iodine solution followed by chemiluminescence detection of NO[20] on a Teledyne 200E chemiluminescence NO_x analyzer (Thousand Oaks, CA, USA). Nitrate plus nitrite were also measured by conversion into NO in hot vanadium solution followed by chemiluminescence detection.[21]

Nitrate N and O isotope ratios ($^{15}N/^{14}N$, and $^{18}O/^{16}O$, respectively) were measured with the denitrifier method.[1,2] Briefly, denitrifiers were cultured in standard Tryptic Soy Broth (Difco; Hunt Valley, MD, USA) amended with 10 mM nitrate, in stoppered glass bottles. We grew *P. chlororaphis*, which is used only for nitrate N isotope analyses, and *P. chlororaphis* f. sp. *aureofaciens* for coupled N and O analyses, as described previously.[1,2] Cells in the stationary phase were concentrated by centrifugation, resuspended in fresh, nitrate-free medium, and dispensed as 2 mL aliquots in stoppered glass vials, which were then purged with an inert gas for 5 h. Then, 20 nmol of nitrate sample solutions were injected into the purged vials and left to incubate overnight. The isotopic composition of N_2O was then analyzed by GC/IRMS (*m/z* 44, 45, 46) as described previously.[1,2]

Isotope ratios are expressed in delta (δ) notation in units of per mil (‰):

$$\delta^{15}N_{sample} = ((^{15}N/^{14}N)_{sample}/(^{15}N/^{14}N)_{reference} - 1) \times 1000,$$

and

$$\delta^{18}O_{sample} = ((^{18}O/^{16}O)_{sample}/(^{18}O/^{16}O)_{reference} - 1) \times 1000$$

The $^{15}N/^{14}N$ reference is N_2 in air, and the $^{18}O/^{16}O$ reference is Vienna Standard Mean Ocean Water (VSMOW). Individual analyses were referenced to injections of N_2O from a pure gas cylinder and then standardized through comparison with two nitrate reference materials: the international potassium nitrate reference material IAEA-N3 with an assigned $\delta^{15}N$ of +4.7 vs. atmospheric N_2[22] and a most recently reported $\delta^{18}O$ of +25.6‰ vs. VSMOW,[23] and USGS-34, with a $\delta^{15}N = -1.8$‰ and a $\delta^{18}O = -27.9$‰.[23] The size of the culture blank was estimated by running a prepared vial to which no sample had been added.

Mechanistic and procedural tests

Initial trials were conducted to determine the time required to reduce incremental concentrations of nitrite to N_2 with excess sulfamic acid, in both freshwater and seawater. Nitrite concentrations ranging from 25 to 500 μM were tested against correspondingly increasing sulfamic acid concentrations that ranged from 2 to 40 mM. The pH of the reactions was measured and, for the lower sulfamic acid additions of 2 to 10 mM, reagent solutions were adjusted with HCl so that the reactions proceeded at a pH between 1.6 and 1.8, near the pK_a of sulfamic acid, as is customarily used for diotization reactions[24] (Table 1). Individual reactions were initiated by adding sulfamic acid reagent to a given nitrite

Table 1. Apparent pseudo-first-order rate constants observed for nitrite reduction by excess sulfamic acid (k_{obs}) at a given pH and at a temperature of 20°C. The rate constant estimates correspond to the respective slopes of fitted least-squares regressions plotted in Fig. 2(B). fw = freshwater; sw = seawater; n.d. = not detectable

Solution	[sulfamic acid] (mM)	[HCl] (M)	reaction pH	$[NO_2^-]_{initial}$ (μM)	k_{obs} ($\times 10^{-2}$ s^{-1}) ± 1σ
fw	2	0.012	1.8	25	4.9 ± 0.1
fw	4	0.012	1.8	100	6.3 ± 0.2
fw	10	0.012	1.8	200	20.9 ± 0.4
fw	20	0	1.9	500	32.5 ± 2.7
fw	40	0	1.7	500	n.d.
sw	2	0.012	1.8	100	2.7 ± 0.1
sw	4	0.012	1.8	100	3.3 ± 0.1
sw	10	0.012	1.8	500	8.7 ± 0.4
sw	20	0	1.9	500	19.9 ± 1.0
sw	40	0	1.6	500	n.d.

Copyright © 2009 John Wiley & Sons, Ltd.

DOI: 10.1002/rcm

solution. Throughout the reaction, the concentration of unreacted nitrite along incremental time points was monitored by injecting 100 μL of reacting solution into hot iodine solution in line with a chemiluminescence NO_x detector (see Methodology section). For all the combinations of nitrite to sulfamic acid tested, the reduction rates proved very rapid, as nitrite was no longer detected after 3 min of reaction for all treatments (analyzed under conditions yielding a detection limit of 0.5 μM; Fig. 1(A)). At 40 mM sulfamic acid, the highest concentration of sulfamic acid tested, the reactions proceeded too rapidly to capture the drawdown of 500 μM nitrite in either seawater and freshwater: little to no nitrite was detected after an elapsed time of 10 s – the time required to draw and inject a 100 μL sample into the hot iodine solution for NO determination. Larger volume (1 mL) injections of reacted solutions into hot iodine, yielding a detection limit of ~50 nM, confirmed the universal absence of significant nitrite after 5 min of reaction (data not shown). Plots of the natural logarithm of the fraction of unreacted nitrite vs. time were linear, consistent with the expected first-order dependence of reaction rates on the concentration of nitrite when sulfamic acid is in excess (Fig. 1(B)). Rate constants for each of the reactions, k_{obs}, were derived from the respective slopes of fitted least-squares regressions (Table 1). As expected, higher concentrations of sulfamic acid gave way to faster reaction rates at roughly comparable pH and salinity. For instance, k_{obs} in freshwater was around $5 \times 10^{-2}\,s^{-1}$ with a 2 mM sulfamic acid addition, and upwards of $30 \times 10^{-2}\,s^{-1}$ with a 20 mM sulfamic acid addition. Reactions in freshwater proceeded more rapidly than the analogous reactions in seawater, from 1.6 to 2.4 times faster among the sulfamic acid reagent solutions tested, which is consistent with the previously observed sensitivity of diotization reactions to ionic strength.[24]

For comparison, the rate of decomposition of nitrous acid, as estimated from the published forward rate constant for nitrous acid decomposition (Eqn. (5): $k_{forward} = 13.4\,M^{-1}\,s^{-1}$ at 22°C[17]), is expected to be much slower than the reduction rates observed here, particularly at lower nitrous acid concentration (Fig. 2(A)). For example, the decomposition rate at 10 μM nitrous acid would be ~370 times slower than the corresponding rate of nitrite reduction by a 4 mM sulfamic acid reagent addition, and ~2400 times slower with 20 mM sulfamic acid. While the rate of decomposition becomes proportionally greater relative to reduction at higher nitrous acid concentrations – because decomposition has a second-order dependence with respect to nitrous acid – the rate of reduction by sulfamic acid would remain considerably greater than the corresponding rate of decomposition. Thus, at 100 μM nitrous acid, reduction by 20 mM sulfamic acid would still be 240 times faster than its decomposition. Assuming that (1) the hydrolysis of the NO_2 product of decomposition is the sole reaction that could generate nitrate under the experimental conditions (Eqn. (6)), and (2) that only up to one-third of the NO_2 generated would hydrolyze to nitrate (based on comparison of $k_{forward} = 8.4 \times 10^7\,M^{-1}\,s^{-1}$for Eqn. (6) vs. $k_{back} = 1.8 \times 10^8\,M^{-1}\,s^{-1}$ for Eqn. (5) at 22°C[17]), the concentration of nitrate expected to accrue from nitrous acid decomposition during the competing reduction by sulfamic acid would be relatively insignificant, except possibly at high ratios of initial nitrite to nitrate: Simulations of the decomposition of nitrous acid in competition with its reduction by sulfamic acid show that removal of 100 μM nitrite would yield nitrate concentrations of 0.4 μM or less, depending on the sulfamic acid concentration, while removal of 200 μM nitrite would yield 1.5 μM or less nitrate (Fig. 2(B)).

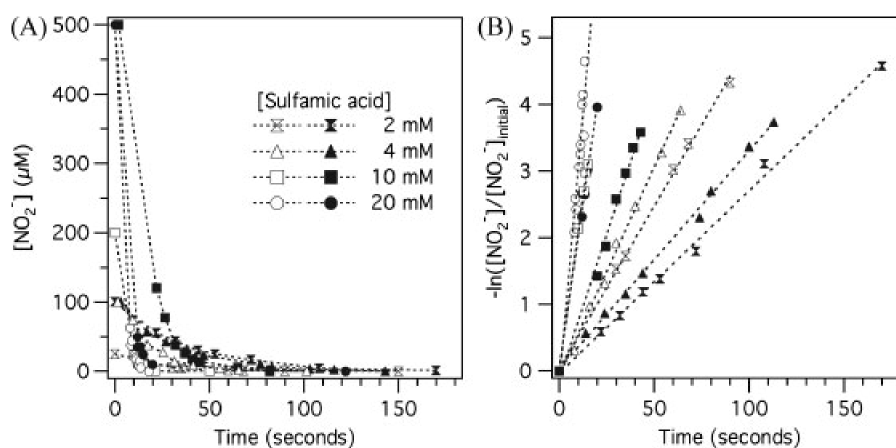


Figure 1. (A) Disappearance of incremental nitrite concentrations in freshwater ('fw'; open symbols) and seawater ('sw'; closed symbols) solutions as a function of time after the addition of discrete sulfamic acid reagent concentrations at 20°C (as per Table 1). Each time point measurement for a given treatment originates from a discrete sample reaction. (B) Linear plots of the -ln of the fraction of nitrite remaining in solution as a function of time. Slopes of fitted least-squares linear regressions correspond to the observed peudo-first-order rate constants ($k_{obs} \pm$ standard error of the slope, summarized in Table 1, where the reaction rate is modeled as $k_{obs}[NO_2^-]$) of the reactions for a given sulfamic acid concentration and at the corresponding pH and temperature.

Copyright © 2009 John Wiley & Sons, Ltd.

DOI: 10.1002/rcm

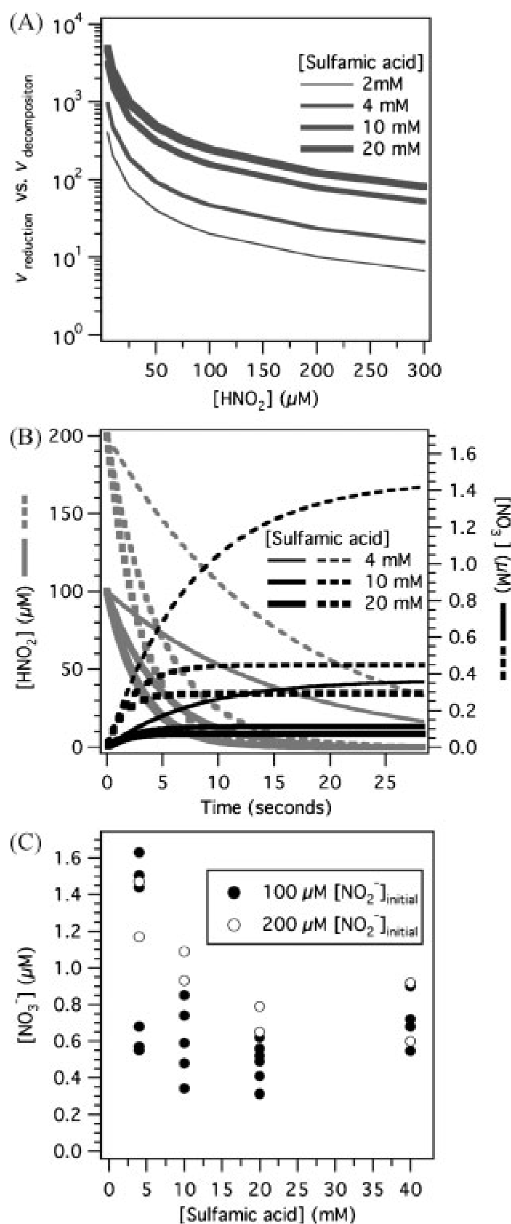


Figure 2. (A) Ratio of the reduction rate of nitrous acid vs. the corresponding rate of nitrous acid decomposition ($v_{\text{reduction}}$ vs. $v_{\text{decomposition}}$) as a function of the nitrous acid concentration at 20°C in freshwater at different sulfamic acid additions. Reduction rates expected for nitrous acid concentrations between 1 and 300 μM were computed from the observed pseudo-first-order rate constants for nitrite removal by sulfamic acid (k_{obs}) summarized in Table 1. The corresponding rates of decomposition of nitrous acid were computed from the published[17] forward second-order rate constant for Eqn. (5) at 20°C in deionized water ($k_f = 13.4\,M^{-1}\,s^{-1}$). (B) The production of nitrate expected from the decomposition of nitrous acid and hydrolysis of the NO_2 product, in competition with the reduction of nitrous acid by sulfamic acid. The concentrations of nitrous acid and nitrate were simulated from k_{obs} in freshwater (Table 1) and from the corresponding forward and reverse rate constants for Eqns. (5) and (6)[17] at incremental time steps of 0.01 s along the reaction path. (C) Concentration of nitrate detected in spent reactions in freshwater after nitrite removal by discrete sulfamic acid reagent additions. Reaction pH as in Table 1.

To ensure that the production of nitrate during nitrite removal by sulfamic acid is indeed negligible, the presence of nitrate was verified following nitrite removal by sulfamic acid. Reactions were initiated by adding from 4 to 40 mM sulfamic acid to 100 and 200 μM fresh nitrite solutions in freshwater. After a minimum of 5 min, the absence of nitrite in reacted solutions was ascertained by injecting 1 mL of sample into hot iodine followed by chemiluminescence detection of NO. Subsequently, the presence of nitrate was verified with analogous injection into hot vanadium. Nitrate was detected in all spent reactions (Fig. 2(B)). The concentrations were variable among replicate experiments, but within the same range among sulfamic acid treatments of 10 to 40 mM sulfamic acid: nitrate was between 0.3 and 0.9 μM for 100 μM nitrite removed, and between 0.6 and 1.1 μM nitrate for 200 μM removed. Nitrate concentrations were more variable in the 4 mM sulfamic acid additions, ranging from 0.5 to 1.6 μM. Thus, nitrate can apparently be produced during nitrous acid reduction to N_2 by sulfamic acid, at least in the 4 mM sulfamic acid treatment. However, the fact that nitrate concentrations were generally higher than predicted in Fig. 2(B), and that these were roughly similar among the 10 to 40 mM sulfamic acid treatments rather than proportionally lower at higher sulfamic acid concentrations, suggests that some of the nitrate detected may be a contaminant in the nitrite stock or that there are other nitrate-producing reactions for which we are not accounting. Notwithstanding, the concentrations of nitrate detected in the reacted solutions could conceivably compromise the isotopic composition of nitrate samples in the low-μM range.

Sulfamic acid reagent at the concentrations tested above was added to the international nitrate isotope reference IAEA-N3 dissolved in freshwater and to seawater samples collected on the eastern shelf of the Bering Sea to determine: (1) whether the bacterial strains used in the denitrifier method tolerate sulfamic acid at these concentrations, and (2) whether sulfamic acid additions, in and of themselves, compromise the isotopic composition of nitrate. Nitrate solutions ranging in concentration from 5 to 15 μM were treated with 2 up to 40 mM sulfamic acid reagent additions. The reactions proceeded for at least 5 min, at which point the samples were neutralized with 2 N NaOH, pending isotope analysis.

The bacterial nitrate conversion into N_2O was complete for all concentrations of sulfamic acid, as estimated from ion current yields, which were the same between control and sulfamic-treated samples, whether in freshwater or seawater (data not shown). The nitrate isotope ratios for samples treated with sulfamic acid were indistinguishable from those of untreated samples, in freshwater or in seawater, and whether nitrate was converted into N_2O by *P. chlororaphis* ($\delta^{15}N$ measurements only) or by *P. aureofaciens* (Table 2). Thus, sulfamic acid reagent in the proportions tested here neither measurably interfered with the bacterial conversion of nitrate into N_2O nor compromised the original isotopic composition of nitrate.

The above nitrate solutions were amended with 2 to 200 μM nitrite and then treated with a proportional range of sulfamic acid reagent concentrations, from 2 to 40 mM. These

Copyright © 2009 John Wiley & Sons, Ltd.

DOI: 10.1002/rcm

Table 2. The effect of incremental additions of sulfamic acid reagent on the N and O isotope composition of nitrate in freshwater standards (IAEA-N3) and in seawater samples from the Bering Sea shelf. Nitrate isotope ratios were measured with the denitrifier method using the bacterial strains *P. aureofaciens* (N and O isotopes) and *P. chlororaphis* (N isotopes only). n = number of experimental replicates

	[sulfamic acid][a] (mM)	n	$\delta^{15}N \pm 1\sigma$ (‰)	$\delta^{18}O \pm 1\sigma$ (‰)
5 μM IAEA-N3	0	3	**4.7 ± 0.2**	**25.6 ± 0.1**
	2	3	4.8 ± 0.1	25.4 ± 0.1
	4	3	4.7 ± 0.1	25.7 ± 0.3
	10	3	4.7 ± 0.2	25.6 ± 0.2
	20	3	4.6 ± 0.2	25.6 ± 0.3
	40	3	4.8 ± 0.2	25.5 ± 0.2
5 μM IAEA-N3	0	3	**4.7 ± 0.1**	
	4	2	4.8 ± 0.1	
	10	2	4.7 ± 0.1	
	20	2	4.9 ± 0.2	
11 μM Bering shelf (sample 07-21.06)	0	2	**7.6 ± 0.1**	**3.5 ± 0.1**
	2	4	7.8 ± 0.2	3.8 ± 0.2
	4	2	7.8 ± 0.1	3.5 ± 0.1
	10	2	7.7 ± 0.1	3.6 ± 0.1
	20	2	7.6 ± 0.1	3.5 ± 0.2
15 μM Bering shelf (sample 07-26.01)	0	3	**6.7 ± 0.3**	
	4	2	6.6 ± 0.1	
	10	2	6.4 ± 0.1	
	20	2	6.6 ± 0.1	

[a] Sulfamic acid added in concert with HCl in the proportions outlined in Table 1.

combinations of nitrate to nitrite were tested to mimic potentially co-occurring concentrations observed in various environments, as well as to determine the potential limits of the method in terms of the amount of nitrite that can be reduced without compromising the isotope composition of nitrate.

The resulting nitrate isotope ratios indicate that nitrite concentrations up to 7-fold the ambient nitrate can be removed by sulfamic acid without detectably compromising the isotopic composition measured for nitrate (Table 3). Removal of 25 μM nitrite from a 5 μM IAEA-N3 nitrate standard yielded the same nitrate isotope ratios as nitrite-free standards. Experiments conducted with higher initial nitrate concentrations of IAEA-N3 standards and of Bering Sea samples (10 to 16 μM) showed analogous patterns, revealing that nitrite concentrations up to 7-fold the ambient nitrate resulted in no detectable difference in the nitrate isotope ratios. However, nitrite concentrations greater than 10-fold the ambient nitrate concentration resulted in more enriched nitrate N isotope ratios than in the control samples (between 0.5 and 1.3‰ greater). Similarly, the corresponding O isotope composition was also detectably compromised; however, only at initial nitrite concentrations of 20-fold and above was the $\delta^{18}O$ low relative to control solutions (by as much as 2.8‰). These patterns were coherent among freshwater and seawater samples and whether nitrate isotope measurements used *P. aurofaciens* or *P. chlororaphis*. Variations in the concentration of sulfamic acid also resulted in some apparent qualitative differences, wherein the 4 mM sulfamic acid treatments yielded more variable and generally larger nitrate isotope ratios (for initial nitrite concentrations of 100 μM and above) than analogous reactions with more concentrated sulfamic acid reagent additions. This trend appears consistent with the observation of more variable and elevated nitrate concentrations measured in spent reactions with 4 mM sulfamic acid reagent additions relative to higher reagent concentrations (see Fig. 2(B)). Taken together, these results demonstrate that sulfamic acid is an effective reagent to remove nitrite concentrations up to 7-fold the ambient nitrate concentration for subsequent measurement of the N and O isotope composition of nitrate with the denitrifier method.

Nitrate samples from the Mexican Margin

We tested nitrite removal with sulfamic acid on seawater samples collected at a station in the Gulf of California during the GoCAL-4 cruise aboard the New Horizon in July 2008 (20°32′N, 106°30′W). This station lies in a region of slow thermocline circulation, intense upwelling and high surface productivity, which leads to significant water-column denitrification and consequent accumulation of nitrite.[25] From each sampling depth, two bottles of sample water were collected. Immediately after collection, one replicate was amended to achieve 4 mM sulfamic acid, shaken, left to react for at least 10 min, then neutralized with NaOH. Both treated and untreated samples were stored frozen. The N and O isotope ratios of nitrate and nitrite in the untreated and treated samples were measured in the laboratory 9 months later. Shortly prior to isotope analysis, subsamples of some of the untreated samples were also treated with sulfamic acid and neutralized in the lab, and were measured concomitantly with untreated and field-treated samples to determine whether the nitrate isotope ratios of lab-amended samples differed from those treated with sulfamic acid 9 months earlier, immediately upon sample collection.

As observed in previous studies in this region,[26] a significant nitrate deficit relative to phosphate[27] is evident

Copyright © 2009 John Wiley & Sons, Ltd.

DOI: 10.1002/rcm

Table 3. The N and O isotope compositions of nitrate in freshwater standards (IAEA-N3) and in seawater samples from the Bering Sea shelf after the removal of incremental nitrite concentrations with discrete sulfamic acid reagent additions. Nitrate isotope ratios were measured with the denitrifier method using the bacterial strains *P. aureofaciens* (N and O isotopes) and *P. chlororaphis* (N isotopes only). n = number of experimental replicates

	$[NO_2^-]_{initial}$ (μM)	[sulfamic acid][a] (mM)	n	$\delta^{15}N \pm 1\sigma$ (‰)	$\delta^{18}O \pm 1\sigma$ (‰)
5 μM IAEA-N3	0	0	3	**4.7 ± 0.1**	**25.6 ± 0.1**
	25	10	3	4.8 ± 0.3	25.5 ± 0.3
	50	10	3	5.2 ± 0.4	25.5 ± 0.3
	75	10	3	5.2 ± 0.1	25.4 ± 0.3
	100	4	3	6.0 ± 0.4	22.8 ± 0.4
	100	10	3	5.2 ± 0.1	24.5 ± 0.5
	100	20	3	5.6 ± 0.6	23.9 ± 0.6
	200	4	3	6.0 ± 0.2	25.0 ± 2.1
	200	10	3	5.6 ± 0.1	24.1 ± 0.1
	200	20	3	5.9 ± 0.1	23.9 ± 0.5
5 μM IAEA-N3	0	0	3	**4.7 ± 0.1**	
	100	4	2	8.5 ± 1.0	
	100	10	2	5.3 ± 0.0	
	100	20	2	5.4 ± 0.2	
10 μM IAEA-N3	0	0	4	**4.7 ± 0.0**	**25.6 ± 0.1**
	50	10	2	4.8 ± 0.1	25.6 ± 0.0
	50	20	2	4.9 ± 0.3	25.5 ± 0.2
	100	10	2	5.0 ± 0.0	25.6 ± 0.2
	100	20	2	5.1 ± 0.2	25.6 ± 0.1
	200	10	2	4.9 ± 0.1	25.1 ± 0.2
	200	20	2	5.1 ± 0.0	25.5 ± 0.3
	200	40	2	4.8 ± 0.2	25.4 ± 0.2
11 μM Bering shelf (sample 07-21.06)	0	0	2	**7.6 ± 0.1**	**3.5 ± 0.1**
	5	2	2	7.8 ± 0.3	3.6 ± 0.2
	10	2	2	7.4 ± 0.5	3.4 ± 0.3
	10	4	2	7.6 ± 0.0	3.3 ± 0.2
	20	2	2	7.6 ± 0.2	3.6 ± 0.2
	20	4	2	7.5 ± 0.2	3.5 ± 0.2
16 μM Bering shelf (sample 07-21.03)	0	0	3	**7.0 ± 0.1**	**3.2 ± 0.1**
	50	10	2	6.8 ± 0.3	3.3 ± 0.2
	50	20	2	7.0 ± 0.1	3.1 ± 0.1
	100	10	2	7.0 ± 0.1	3.1 ± 0.1
	100	20	2	7.0 ± 0.0	2.8 ± 0.2
	200	10	2	7.2 ± 0.1	2.6 ± 0.5
	200	20	2	7.2 ± 0.1	2.7 ± 0.3
	200	40	2	7.3 ± 0.1	2.8 ± 0.1
15 μM Bering shelf (sample 07-26.01)	0	0	3	**6.7 ± 0.3**	
	100	4	3	6.6 ± 0.1	
	100	10	3	6.6 ± 0.3	
	100	20	3	6.6 ± 0.0	
nitrite	40	0	2	**−0.1 ± 0.5**	**−16.3 ± 0.8**[b]

[a] Sulfamic acid added in concert with HCl in the proportions outlined in Table 1.
[b] Corrected from raw isotope ratio measurements assuming that nitrate was the sole analyte.[3]

at mid-depths (−20 μM at ~300 m), coincident with the heart of the oxygen minimum (Fig. 3(A); O_2 data not shown). A peak in accumulated nitrite of 4 μM sits in the upper portion of the oxygen minimum at 150 m, and the nitrite concentration is significant down to 500 m, below which depth nitrite is not detected. Measurements of the N and O isotope ratios of nitrate and nitrite (untreated samples) at depths of 70 to 1000 m reveal a broad range in $\delta^{15}N_{NO3+NO2}$ and $\delta^{18}O_{NO3+NO2}$, spanning 7.9‰ in deep water to a maximum of 22.0‰ (vs. air) for N, and 4.2‰ at depth to 17.3‰ (vs. SMOW) for O (Figs. 3(B) and 3(C)). Maxima in both $\delta^{15}N_{NO3+NO2}$ and $\delta^{18}O_{NO3+NO2}$ coincide with the highest extent of the nitrate deficit. It should be recognized that the $\delta^{18}O_{NO3+NO2}$ values given here are corrected from the raw isotope ratio measurements assuming that the sum of nitrate and nitrite is nitrate alone; the data could be converted into an actual approximation of the $\delta^{18}O_{NO3+NO2}$ for the sum of nitrate and nitrite, using the relevant correction factors for the two N pools.[11] Samples treated with sulfamic acid in the field and in the laboratory correspondingly reveal that the $\delta^{15}N_{NO3}$ and $\delta^{18}O_{NO3}$ for the depths at which nitrite was removed are significantly more positive than the corresponding $\delta^{15}N_{NO3+NO2}$ and $\delta^{18}O_{NO3+NO2}$ values, with $\delta^{15}N_{NO3}$ reaching 24.6‰ vs. air and $\delta^{18}O_{NO3}$ reaching 20.4‰ vs. SMOW (Figs. 3(B) and 3(C)). Maxima in both $\delta^{15}N_{NO3}$ and $\delta^{18}O_{NO3}$ remain coincident with the largest extent of the nitrate deficit. The differences in $\delta^{15}N_{NO3+NO2}$ and $\delta^{18}O_{NO3+NO2}$ from the corresponding $\delta^{15}N_{NO3}$ and $\delta^{18}O_{NO3}$ values are similar to those reported previously for a profile at a station located further south in the Eastern Tropical North Pacific, where nitrite was removed with ascorbate.[11] Thus, the $\delta^{15}N$ and $\delta^{18}O$ values are significantly higher once nitrite is removed,

Copyright © 2009 John Wiley & Sons, Ltd.

DOI: 10.1002/rcm

RCM

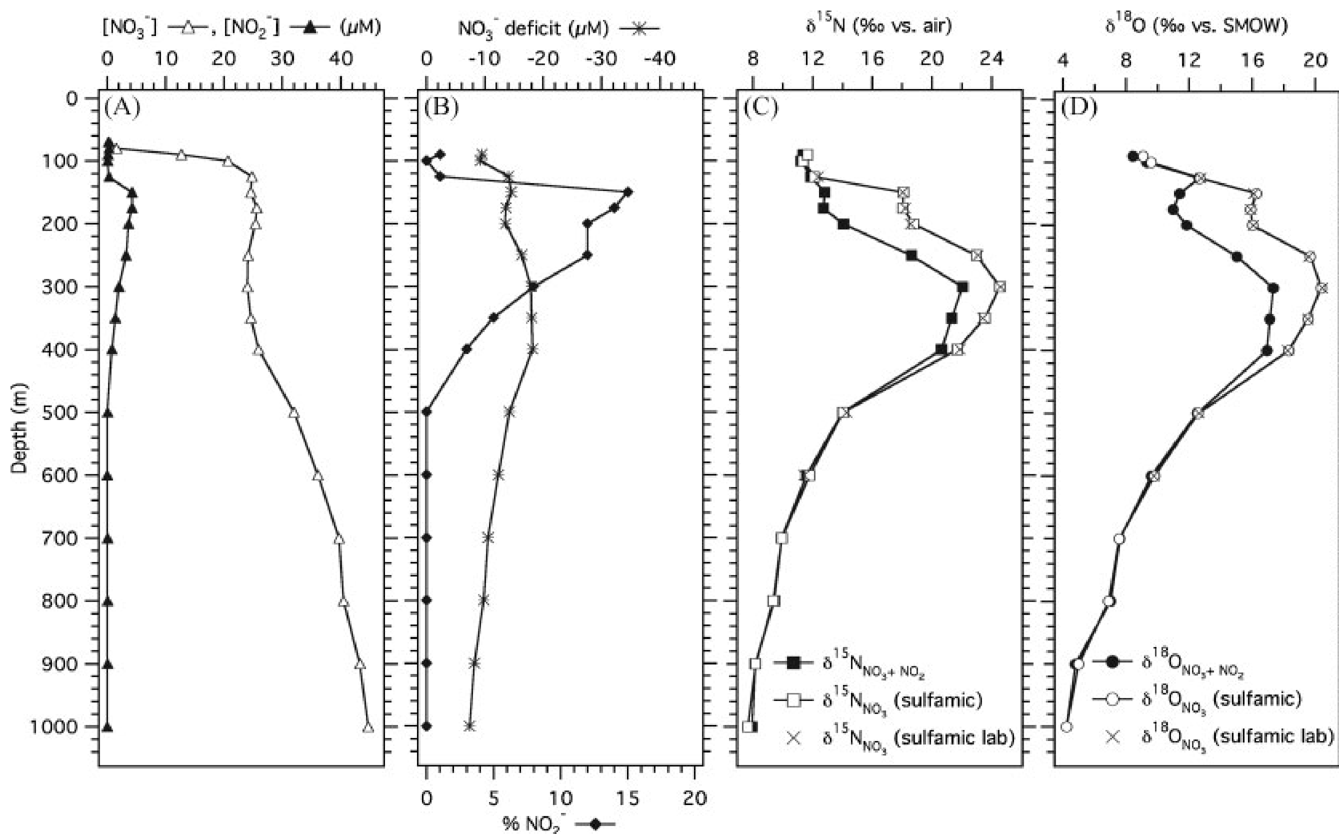


Figure 3. Depth distributions of chemical properties from a station in the Gulf of California (20°32′N, 106°30′W) sampled in July 2008 during the GoCAL-4 cruise aboard the New Horizon. (A) Concentrations of nitrate and nitrite (μM). (B) The nitrate deficit relative to phosphate (plotted as negative values, reverse axis; NO_3^- deficit (μM) = $[NO_3^- + NO_2^-] - 16 \times [PO_4^{3-}] + 2.9$[27]) and the percentage of nitrite relative to nitrate plus nitrite (% $NO_2^- = 100 \times [NO_2^-]/([NO_3^-] + NO_2^-]$). (C) The $\delta^{15}N_{NO3+NO2}$ and $\delta^{15}N_{NO3}$ treated with sulfamic acid (4 mM) ship-board and the $\delta^{15}N_{NO3}$ of a subset of samples treated with sulfamic acid (4 mM) in the lab 9 months after sample collection. Measurement error (1σ) was ≤0.4‰ vs. air for three to five replicate analyses. (D) The $\delta^{18}O_{NO3+NO2}$ and $\delta^{18}O_{NO3}$ treated with sulfamic acid reagent (4 mM) ship-board and the $\delta^{18}O_{NO3}$ of a subset of samples treated with sulfamic acid reagent (4 mM) in the lab 9 months after sample collection. The plotted $\delta^{18}O$ values are the average of two to four analyses and 1σ ≤0.4‰.

roughly 5‰ for both N and O isotopic compositions at the depth of maximum nitrite accumulation, which indicates the importance of considering the contribution of nitrite to the N and O isotope composition of nitrate samples. In addition, the relationship of $\delta^{15}N_{NO3}$ to $\delta^{18}O_{NO3}$ in this profile is broadly similar to the relationship observed in oxygen-deficient zones devoid of detectable nitrite, in which the $\delta^{15}N_{NO3}$ and $\delta^{18}O_{NO3}$ values are elevated roughly proportionally due to significant discrimination against heavy nitrate N and O isotopologues during denitrification.[26,28] A more complete analysis will be published elsewhere.

Overall, treatment with sulfamic acid yields $\delta^{15}N_{NO3}$ and $\delta^{18}O_{NO3}$ values that are consistent with expected trends. The data also demonstrate that treatment with sulfamic acid does not alter the $\delta^{15}N_{NO3}$ and $\delta^{18}O_{NO3}$ when the samples are stored frozen at neutral pH: samples that were devoid of detectable nitrite showed no difference in their respective isotopic composition whether amended with sulfamic acid or not (Figs. 3(B) and 3(C)). Moreover, the $\delta^{15}N_{NO3}$ and $\delta^{18}O_{NO3}$ values of samples amended in the field vs. those ~9 months later in the laboratory were remarkably similar. Over a period of 9 months, while the oxygen isotopic composition of nitrite undoubtedly changed,[3] the $\delta^{15}N_{NO3}$ and $\delta^{18}O_{NO3}$ values were not detectably modified by the presence of nitrite during storage. Therefore, when amending samples with sulfamic acid in the field is impractical, removing nitrite at some later (albeit, unspecified) time appears adequate to obtain accurate isotope ratios of nitrate. However, we note that the concentration of nitrite in the Gulf of California samples was significantly less than that of nitrate (<20%; Fig. 3(B)). We cannot rule out the possibility that higher proportions of nitrite could yield erroneous nitrate isotope measurements after prolonged storage, that is, if any nitrite dismutates to nitrate.

One could conceivably compute the isotopic composition of nitrite from the concentration-weighted difference of the nitrate isotope ratios minus those of nitrate plus nitrite. However, the concentration of nitrite is typically low compared with that of ambient nitrate, and the error in the concentration and isotope ratio measurements both propagate into the uncertainties of the $\delta^{15}N_{NO2}$ and $\delta^{18}O_{NO2}$ estimates.[11] Moreover, attempting to obtain the $\delta^{18}O_{NO2}$ through differencing presents additional difficulties. First, there is a lower fractional oxygen loss to water for nitrite reduction than for nitrate reduction during the bacterial conversion (i.e. a branching isotope effect of ~25 to 30‰[1,3]), which results in artificially depleted $\delta^{18}O_{NO2}$ values when corrected against nitrate standards. Second, a difference in

Copyright © 2009 John Wiley & Sons, Ltd.

the $\delta^{18}O$ calibration slope has been reported for isotopic analysis of nitrate vs. nitrite, yielding error in the estimated $\delta^{18}O_{NO2}$ if corrected against nitrate standards.[3] Even if the $\delta^{18}O_{NO2}$ is accurately estimated through differencing, it would probably reflect the equilibration of the oxygen isotopes of nitrite with ambient water during storage, overprinting the original $\delta^{18}O_{NO2}$.[3,11] Thus, only direct measurements of the isotopic composition of nitrite, obtained shortly after sample collection, are likely to be of value.[11] For $\delta^{15}N_{NO2}$, when at-sea reaction is not feasible, the differencing approach may be worthwhile in some cases.

Adaptations for routine use with field samples

We optimized the reagent concentrations in order to minimize the dilution of samples with reagent. Based on our experiments, 4 mM sulfamic acid additions appeared adequate to remove $\leq$20 μM nitrite; above this, we recommend adding 10 mM sulfamic acid for up to 100 μM initial nitrite, and 20 mM sulfamic acid above 100 μM initial nitrite. The 4 mM reagent addition can be obtained by adding 10 μL per mL of 4% w/v sulfamic acid ($\sim$0.4 M) dissolved in 10% v/v HCl (1.2 N). Sulfamic acid should first be dissolved in water and the HCl added after its complete dissolution in order to maximize the solubility of the sulfamic acid crystals. A 10 mM sulfamic acid addition is obtained by adding 20 μL per mL of 5% w/v sulfamic acid in 5% v/v HCl – the moderate aqueous solubility of sulfamic acid sets this upper limit to the concentration of sulfamic acid that is easily dissolved in reagent solutions. Finally, a 20 mM addition consists of adding 40 μL per mL of a 5% w/v sulfamic acid solution (with no HCl). The samples are then neutralized with a corresponding addition of 2 M NaOH solution (ranging from $\sim$5 to 15 μL/mL depending on the sulfamic acid treatment). While reaction times of less than 5 min proved adequate to remove nitrite at $\sim$20°C in the laboratory, longer reaction times should be allotted for the treatment of freshly collected field samples at lower temperatures. The rate of nitrite reduction by sulfamic acid at 0°C is reportedly 2.5 times slower than that at 25°C.[18] We suggest allowing samples to react with sulfamic acid for at least 10 to 20 min prior to neutralizing with NaOH to ensure complete reduction of nitrite. Following neutralization, samples should be stored frozen.

FURTHER COMMENTS AND RECOMMENDATIONS

While this method is relatively simple, it nevertheless calls for care in its use. Primarily, both the pH of the reaction and the pH of the samples following neutralization must be within an appropriate range in order to yield precise isotope measurements. The optimal pH required to reduce nitrite with sulfamic acid is $\geq$1.3, but appreciably lower than 3, above which the reaction rate is significantly slower and unfavorably similar to the competing rate of decomposition of nitrous acid to nitrogen oxides.[18] As currently designed, the proposed reagent additions should yield an adequate reaction pH in natural samples. However, samples generated from laboratory cultures containing a pH buffer may require adjustment of the HCl in the sulfamic acid reagent solution in order to achieve an adequate reaction pH. Similarly, one should ensure that neutralization with NaOH returns the sample pH to very near neutral. An overly low or high sample pH can compromise the isotope measurements, because addition of such samples to the bacterial concentrate can overpower the buffering capacity of the bacterial medium, which otherwise maintains a pH of $\sim$7.2. Moreover, above a pH of 9, seawater samples become turbid (probably from precipitation of magnesium hydroxide), and deleterious effects on the isotope measurements have been observed. It is thus advisable to test the pH of all steps with dummy samples prior to amending valuable batches of samples with the reagents.

An additional source of error is the potential for contamination of the reagents with nitrate or nitrite, although we have not encountered such problems ourselves. We suggest amending nitrate isotope standards with sulfamic acid and NaOH reagents concurrently with a given batch of samples to ensure that no significant contaminant originates from the solutions, or to correct for potential contamination.

We observed that brief acidification with the sulfamic acid solution followed by neutralization does *not* extinguish all biological activity in samples, at least in samples originating from lab plankton cultures. We thus advise that all samples be stored frozen following treatment with sulfamic acid and base.

Conceivably, field samples could be stored at room temperature after being acidified with sulfamic acid reagent, and then re-neutralized later in the laboratory; however, it is unclear whether the isotopic composition of nitrate remains unchanged during extended storage with sulfamic acid at low pH. Prior to further tests being carried out, we advise neutralizing the samples shortly after amending with the sulfamic acid solution and storing these samples frozen.

Acknowledgements

We are grateful to Brian Popp and Julie Fliegler for collecting the samples in the Gulf of California and amending them with sulfamic acid on board, as well as for useful comments on the manuscript from Brian Popp. Fred Prahl provided the nutrient data presented in Fig. 2. Comments by two anonymous reviewers helped improve the original manuscript significantly. This work was funded by US NSF grant OPP-0612198 (BEST Program) to D.M.S.

REFERENCES

1. Casciotti KL, Sigman DM, Hastings MG, Böhlke JK, Hilkert A. *Anal. Chem.* 2002; **74**: 4905.
2. Sigman DM, Casciotti KL, Andreani M, Barford C, Galanter M, Böhlke JK. *Anal. Chem.* 2001; **73**: 4145.
3. Casciotti KL, Böhlke JK, McIlvin MR, Mroczkowski SJ, Hannon JE. *Anal. Chem.* 2007; **79**: 2427.
4. Baumgarten P. *Erbe H. Berichte Der Deutschen Chemischen Gesellschaft* 1938; **71**: 2603.
5. Brasted RC. *Anal. Chem.* 1952; **24**: 1111.
6. McIlvin MR, Altabet MA. *Anal. Chem.* 2005; **77**: 5589.
7. Böhlke JK, Smith RL, Hannon JE. *Anal. Chem.* 2007; **79**: 5888.
8. Granger J, Sigman DM, Prokopenko MG, Lehmann MF, Tortell PD. *Limnol. Oceanogr. Methods* 2006; **4**: 205.
9. Granger J, Sigman DM, Lehmann MF, Tortell PD. *Limnol. Oceanogr.* 2008; **53**: 2533.
10. Prokopenko MG, Sigman DM, Berelson W, Hammond DE, Barnett B, Chong L. *Proc. Natl. Acad. Sci. USA* 2009; in review.

Copyright © 2009 John Wiley & Sons, Ltd.

RCM

11. Casciotti KL, McIlvin MR. *Mar. Chem.* 2007; **107**: 184.
12. Wu JP, Calvert SE, Wong CS. *Deep-Sea Research Part I – Oceanographic Research Papers* 1997; **44**: 287.
13. Velinsky DJ, Pennock JR, Sharp JH, Cifuentes LA, Fogel ML. *Mar. Chem.* 1989; **26**: 351.
14. Scozzafava A, Banciu MD, Popescu A, Supuran CT. *J. Enzyme Inhibition* 2000; **15**: 443.
15. Hughes MN. *J. Chem. Soc. A - Inorganic Physical Theoretical* 1967; 902.
16. Ridd JH. *Quart. Rev.* 1961; **15**: 418.
17. Park JY, Lee YN. *J. Phys. Chem.* 1988; **92**: 6294.
18. Mahfud L, Ronze D, Wehrer A, Zoulalian A. *Chem. Eng. J.* 1998; **70**: 85.
19. Hughes MN, Lusty JR, Wallis HL. *J. Chem. Soc. Dalton Trans.* 1978; 530.
20. Garside C. *Marine Chemistry* 1982; **11**: 159.
21. Braman RS, Hendrix SA. *Anal. Chem.* 1989; **61**: 2715.
22. Gonfiantini R, Stichler W, Rosanski K. *Standards and Intercomparison Materials Distributed by the IAEA for Stable Isotope Measurements*, International Atomic Energy Agency: Vienna, 1995.
23. Böhlke JK, Mroczkowski SJ, Coplen TB. *Rapid Commun. Mass Spectrom.* 2003; **17**: 1835.
24. Norwitz G, Keliher PN. *Analyst* 1985; **110**: 689.
25. Cline JD, Richards FA. *Limnol. Oceanogr.* 1972; **17**: 885.
26. Sigman DM, Granger J, DiFiore PJ, Lehmann MM, Ho R, Cane G, van Geen A. *Global Biogeochem. Cycles* 2005; **19**: 14.
27. Gruber N, Sarmiento JL. *Global Biogeochem. Cycles* 1997; **11**: 235.
28. Sigman DM, Robinson R, Knapp AN, van Geen A, McCorkle DC, Brandes JA, Thunell RC. *Geochem. Geophys. Geosystems* 2003; **4**: 20.

Copyright © 2009 John Wiley & Sons, Ltd.

DOI: 10.1002/rcm