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# Distinguishing Nitrous Oxide Production from Nitrification and Denitrification on the Basis of Isotopomer Abundances

R. L. Sutka,[1]* N. E. Ostrom,[1] P. H. Ostrom,[1] J. A. Breznak,[2] H. Gandhi,[1] A. J. Pitt,[1] and F. Li[3]

*Department of Zoology[1] and Department of Microbiology and Molecular Genetics,[2] Michigan State University, East Lansing, Michigan, and Department of Mathematics, Indiana University-Purdue University, Indianapolis, Indiana[3]*

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**The intramolecular distribution of nitrogen isotopes in $N_2O$ is an emerging tool for defining the relative importance of microbial sources of this greenhouse gas. The application of intramolecular isotopic distributions to evaluate the origins of $N_2O$, however, requires a foundation in laboratory experiments in which individual production pathways can be isolated. Here we evaluate the site preferences of $N_2O$ produced during hydroxylamine oxidation by ammonia oxidizers and by a methanotroph, ammonia oxidation by a nitrifier, nitrite reduction during nitrifier denitrification, and nitrate and nitrite reduction by denitrifiers. The site preferences produced during hydroxylamine oxidation were 33.5 ± 1.2‰, 32.5 ± 0.6‰, and 35.6 ± 1.4‰ for *Nitrosomonas europaea*, *Nitrosospira multiformis*, and *Methylosinus trichosporium*, respectively, indicating similar site preferences for methane and ammonia oxidizers. The site preference of $N_2O$ from ammonia oxidation by *N. europaea* (31.4 ± 4.2‰) was similar to that produced during hydroxylamine oxidation (33.5 ± 1.2‰) and distinct from that produced during nitrifier denitrification by *N. multiformis* (0.1 ± 1.7‰), indicating that isotopomers differentiate between nitrification and nitrifier denitrification. The site preferences of $N_2O$ produced during nitrite reduction by the denitrifiers *Pseudomonas chlororaphis* and *Pseudomonas aureofaciens* (−0.6 ± 1.9‰ and −0.5 ± 1.9‰, respectively) were similar to those during nitrate reduction (−0.5 ± 1.9‰ and −0.5 ± 0.6‰, respectively), indicating no influence of either substrate on site preference. Site preferences of ~33‰ and ~0‰ are characteristic of nitrification and denitrification, respectively, and provide a basis to quantitatively apportion $N_2O$.**

Over the past several decades, anthropogenic activity, primarily agriculture, has doubled the annual input of biologically reactive nitrogen into the environment (14). This surplus of reactive nitrogen has stimulated natural microbial activity, the largest source of the greenhouse gas nitrous oxide ($N_2O$) (17, 26). Ammonia- and methane-oxidizing organisms produce $N_2O$ during the oxidation of hydroxylamine ($NH_2OH$) to nitrite ($NO_2^-$). Ammonia-oxidizing bacteria also reduce $NO_2^-$ to $N_2O$ and $N_2$ under anoxic conditions by a process termed nitrifier denitrification (12, 22, 23). Nitrous oxide can also be produced and consumed by heterotrophic denitrifying organisms. In this case, $N_2O$ is produced and consumed by the stepwise reduction of nitrate ($NO_3^-$) to $N_2$ (33).

The relative importance of nitrification and denitrification in $N_2O$ production has proven difficult to determine. Previous attempts to differentiate nitrification- and denitrification-mediated $N_2O$ production in soils using stable isotope approaches (4, 20, 30, 31, 32) relied on the observation that the fractionation factor associated with $N_2O$ production by denitrifiers (2) is substantially less than that associated with nitrification (34). The assumption was that $N_2O$ with a high $\delta^{15}N$ value is indicative of denitrification, whereas a lower value reflects nitrification (4). However, this approach is problematic because the isotopic composition of the substrate (nitrate or ammonia) can vary temporally and spatially (20).

Yoshida and Toyoda (35) suggested that analyses of the intramolecular distributions of $^{15}N$ in $N_2O$ (isotopomers) could offer additional information to more tightly constrain sources and sinks of this greenhouse gas. Quantification of the relative abundances of $^{15}N$ in the central ($\alpha$) and terminal ($\beta$) N atoms of the linear $N_2O$ molecule relies on the fragmentation of $N_2O^+$ to $NO^+$ within the ion source of a mass spectrometer (5, 11, 29). The intramolecular distribution of $^{15}N$ is often expressed as the site preference ($SP = \delta^{15}N^{\alpha} - \delta^{15}N^{\beta}$) (28).

Sutka et al. (27) demonstrated that $N_2O$ produced from $NH_2OH$ oxidation by *Nitrosomonas europaea* and *Methylococcus capsulatus* Bath had a higher SP than that produced by $NO_2^-$ reduction. In the present study, we evaluated the SPs of $N_2O$ produced during nitrification and denitrification more comprehensively. In addition to *N. europaea* and *M. capsulatus* Bath, we examined $N_2O$ production from $NH_2OH$ by the ammonia oxidizer *Nitrosospira multiformis* and the methane oxidizer *Methylosinus trichosporium*. We also examined the SPs of $N_2O$ produced during ammonia oxidation in batch cultures of *N. europaea* and compared them to the results from the $NH_2OH$ oxidation experiments. In order to understand culture conditions that might influence $N_2O$ production by $NO_2^-$ reduction rather than $NH_2OH$ oxidation, we evaluated the effects of the $NO_2^-$ concentration and the surface area available for oxygen diffusion in concentrated cell suspensions of *N. europaea*. Nitrous oxide production by denitrifying organisms that lack $N_2O$ reductase, namely, *Pseudomonas chlororaphis* and *Pseudomonas aureofaciens*, was also studied to compare the values to those obtained in studies of *Pseudomonas fluorescens* and *Pseudomonas denitrificans* by Toyoda et al. (29). These SP data provide an essential foundation to apportion the

* Corresponding author. Present address: Crewe Road, Wythenshawe, Manchester M23 9BE, United Kingdom. Phone: 44-161-902-2100. Fax: 44-161-902-2198. E-mail: Robin.Sutka@gvinstruments.co.uk.

production of $N_2O$ in field studies, such as those of Pérez et al. (20) and Yamulki et al. (32).

## MATERIALS AND METHODS

**Organisms and cultivation.** *Nitrosomonas europaea* (ATCC 19718) was maintained in ammonium mineral salts medium as described by Sutka et al. (27). The medium was autoclaved, cooled, adjusted to pH 7.5 to 7.7 with 5% (wt/vol) $K_2CO_3$, and inoculated with 5 ml of culture. Cultures were incubated at 25°C, and the pH was adjusted back to 7.5 to 7.7 as necessary (typically three to four times per week). *Nitrosospira multiformis* (ATCC 25196) was maintained in 75 ml of *Nitrosolobus* medium (ATCC medium 929) at 25°C, and the pH was adjusted to 7.5 to 7.7 with 5% (wt/vol) $K_2CO_3$. *Methylosinus trichosporium* (ATCC 49243) was maintained in modified nitrate mineral salts medium under a headspace of 30% (vol/vol) methane in air as described by Sutka et al. (27). *Pseudomonas aureofaciens* (ATCC 13985) and *Pseudomonas chlororaphis* (ATCC 43928) were maintained on tryptic soy agar (Difco, Detroit, MI) plates at 25°C. To prepare starter cultures for $N_2O$ production experiments, an isolated colony of *P. aureofaciens* or *P. chlororaphis* was used to inoculate 50 ml of citrate minimal medium (CMM) (1).

**Preparation of cell suspensions for $NH_2OH$ oxidation experiments.** Cell suspensions of *N. europaea* ($6.2 \times 10^7$ to $7.0 \times 10^7$ cells/ml) and *N. multiformis* ($2.3 \times 10^7$ to $2.5 \times 10^7$ cells/ml) were prepared by combining three cultures (75-ml liquid volume) grown to late exponential phase. The cultures used for cell suspensions were tested for heterotrophic contamination by inoculating 3 ml of culture separately into 5 ml of nutrient broth (Difco) and tryptic soy broth (Difco) in 25-ml screw-cap tubes. In all cases, turbidity was not detected in the tubes after 30 days of incubation, indicating an absence of detectable contamination in the ammonia oxidizer cultures. Cell suspensions of *M. trichosporium* ($7.4 \times 10^7$ to $1.9 \times 10^8$ cells/ml) were prepared from three cultures (50-ml liquid volume) grown to the late exponential phase of growth. *N. europaea*, *N. multiformis*, and *M. trichosporium* cells were concentrated by centrifugation at 10,000 $\times$ *g* for 10 min at 5°C. The cells were subsequently resuspended in 20 ml of 0.1 M potassium phosphate buffer (pH 7.5) and then reconcentrated by centrifugation. This process was completed twice to remove ammonium ($NH_4^+$), $NO_2^-$, and $NH_2OH$. The final cell pellet was resuspended in 10 ml of 0.01 M potassium phosphate buffer. Experimental cultures to test the amount of $N_2O$ produced from $NH_2OH$ oxidation were prepared in 25-ml anaerobic culture tubes (Bellco, Vineland, NJ) by adding 2 ml of cell suspension and 300 μl of 0.01 M of $NH_2OH$ solution and stoppering the tubes under air.

**Preparation of cultures for ammonia oxidation experiments.** Nitrous oxide production with $NH_4^+$ as the substrate was investigated in batch cultures of *N. europaea* ($1.9 \times 10^7$ to $3.8 \times 10^7$ cells/ml after 6 days of growth). The experimental cultures consisted of 25 ml of ammonium mineral salts medium, as described by Sutka et al. (27), in 165-ml serum bottles stoppered under air. An additional aliquot of 5 ml of $O_2$ was added to the headspace of the serum bottles to ensure oxic conditions throughout the course of incubation. The serum bottles were inoculated with 0.5 ml of *N. europaea* stock culture and incubated for 6 days on a rotating arm to facilitate equilibration of the liquid phase with the headspace gases. Headspace samples were obtained at the end of the 6-day incubation period and analyzed immediately.

**Preparation of cell suspensions for $NO_2^-$ and $NO_3^-$ reduction experiments.** Cells of *N. multiformis* for $NO_2^-$ reduction experiments were concentrated and resuspended in potassium phosphate buffer as described for the $NH_2OH$ oxidation experiments. The cultures were constructed by adding 2 ml of cell suspension ($2.3 \times 10^7$ to $2.5 \times 10^7$ cells/ml), 300 μl of a 0.01 M $NaNO_2$ solution, and 100 μl of a 0.01 M $NH_2OH$ solution. The headspace was purged for 5 min with $N_2$ to hasten the onset of anoxia, and then the tubes were stoppered.

Cultures for cell suspensions of *P. chlororaphis* ($1.2 \times 10^9$ to $4.9 \times 10^9$ cells/ml) and *P. aureofaciens* ($4.4 \times 10^9$ cells/ml) were prepared by inoculating 0.1 ml of each seed culture into 50 ml of CMM with 10 mM $NO_3^-$ in a 160-ml serum bottle purged with $N_2$. After the cultures were grown to late exponential phase, two 50-ml cultures were combined and concentrated by centrifugation at 10,000 $\times$ *g* for 10 min at 5°C. Cells were resuspended in 20 ml of CMM without $NO_3^-$. The experimental cultures were prepared by adding 2 ml of the cell suspension to a 25-ml serum tube and purging the headspace for 5 min with $N_2$. For the $NO_3^-$ and $NO_2^-$ reduction experiments, 300 μl of a 0.01 M $NaNO_3^-$ or $NaNO_2$ solution, respectively, was added to the cell suspension. The experiment to calculate isotopic enrichment factors for $NO_3^-$ reduction to $N_2O$ by *P. chlororaphis* and *P. aureofaciens* was completed in 12-ml Exetainer vials (Labco, United Kingdom) with 2 ml of cell suspension and 300 ml of 0.01 M $NaNO_3^-$.

**$N_2O$ concentration and isotopic analysis.** Headspace samples were obtained from the cell suspensions with gas-tight syringes (Hamilton, Reno, NV). Typical headspace sample sizes ranged from 100 to 2,000 μl. Prior to sampling, an equal volume of air (for $NH_2OH$ oxidation experiments) or $N_2$ (for $NO_2^-$ and $NO_3^-$ reduction experiments) was injected into the headspace to maintain atmospheric pressure in the culture tubes. Multiple gas samples were obtained from the same tube, and $N_2O$ concentrations were corrected to take into account the dilution due to the addition of air or $N_2$. Samples were immediately analyzed on a Trace Gas system interfaced with a multicollector IsoPrime mass spectrometer (GV Instruments, United Kingdom) as described by Sutka et al. (27). The isotopic composition of $^{15}N$ and $^{18}O$ in $N_2O$ is expressed in δ notation with respect to the air and Vienna Standard Mean Ocean Water (VSMOW) standards, as follows: $\delta = [(R_{sample}/R_{standard}) - 1] \times 1{,}000$, where $R_{sample} = {}^{15}N/{}^{14}N$ and $^{18}O/^{16}O$ for the sample and $R_{standard} = {}^{15}N/{}^{14}N$ and $^{18}O/^{16}O$ for the standard.

The isotopic composition of $^{15}N$ at the β position was calculated after measurement of the $\delta\text{-}{}^{15}N^{bulk}$ and $\delta\text{-}{}^{15}N^{\alpha}$, as follows: $\delta\text{-}{}^{15}N^{bulk} = (\delta^{15}N^{\alpha} + \delta^{15}N^{\beta})/2$.

Isotope values were corrected for the presence of $^{17}O$ and rearrangement within the ion source by the approach indicated by Toyoda and Yoshida (28). The $\delta^{15}N$, $\delta^{15}N^{\alpha}$, and $\delta^{18}O$ values for the in-house $N_2O$ reference are 1.6, 14.9, and 41.7‰, respectively.

**Relative importance of $NO_2^-$ reduction and $NH_2OH$ oxidation to $N_2O$ production.** The relative importance of $NH_2OH$ oxidation and $NO_2^-$ reduction can be influenced by the $O_2$ and $NO_2^-$ concentrations. In our experiments, we varied the surface area available for oxygen diffusion to either limit or promote oxygen availability in the liquid phase of concentrated *N. europaea* cell suspensions. A 1% (wt/wt) mixture of >98% $^{15}N$-enriched $NaNO_2^-$ (Cambridge Isotope Laboratories, Andover, MA) and $NaNO_2^-$ at its natural abundance level (1.5‰) was diluted to make a working solution with a final concentration of 0.01 M $NO_2^-$ and a nitrogen isotopic composition of 0.99 ± 0.02 atom% ($n$ = 3). The liquid surface area available for oxygen diffusion was varied by incubating 25-ml stoppered tubes either horizontally (ratio of liquid surface area to total liquid volume [S/V], 1.2 $cm^{-1}$) or vertically (S/V,of 12.6 $cm^{-1}$). The experimental cultures for the two incubation conditions consisted of 2 ml of concentrated *N. europaea* cell suspension amended with 300 μl of $NH_2OH$ and 100 μl of $^{15}N$-enriched $NO_2^-$. The experimental cultures were incubated statically for 120 min, and the headspace gas was then sampled and analyzed immediately for the concentration and $\delta^{15}N$ value of $N_2O$. The fraction of $N_2O$ derived from the $^{15}N$-enriched $NO_2^-$ versus $NH_2OH$ oxidation was calculated based on isotope mass balance.

In experiments to examine the effect of $NO_2^-$ concentration on the fraction of $N_2O$ derived from $NO_2^-$ reduction versus $NH_2OH$ oxidation, concentrated *N. europaea* cell suspensions were prepared similarly to those used in the surface area experiments. In this case, the concentration of $^{15}N$-enriched $NO_2^-$ varied from 0.05 mM to 0.6 mM, and 100 μl of 0.01 M $NH_2OH$ was added to each cell suspension. All incubations were conducted with a high S/V ratio (12.6 $cm^{-1}$) for 90 min. Headspace gas samples were obtained at the end of the incubation period and immediately analyzed to obtain the concentration and $\delta^{15}N$ value of $N_2O$.

**Abiological $N_2O$ production.** Control experiments were conducted to evaluate the abiological production of $N_2O$ from $NH_2OH$ and $NO_2^-$. In the first experiment, 2 ml of 0.1 M potassium phosphate buffer and 300 μl of a 0.01 M $NH_2OH$ solution were added to a 25-ml stoppered serum tube with an air headspace. The concentration of $N_2O$ attributed to abiological reactions involving $NH_2OH$ after 8 h of incubation at 25°C was 0.4 μM. A second experiment was constructed with 2 ml of 0.1 M phosphate buffer, 300 μl of a 0.01 M $NaNO_2^-$ solution, and 100 μl of 0.01 M $NH_2OH$ in a 25-ml test tube with a headspace of $N_2$. The headspace concentration of $N_2O$ was 0.8 μM in the second experimental control after 8 h of incubation. A killed-cell experiment was conducted with 2 ml of *N. europaea* cell suspension that had been autoclaved for 20 min at 120°C and 22 $lb/in^2$. The killed-cell suspension was amended with 300 μl of a 0.01 M $NH_2OH$ solution with an air headspace. The $N_2O$ concentration was 0.1 μM in the killed-cell control after 8 h of incubation.

**Statistical analysis.** A general linear mixed model with $N_2O$ as the covariate was used to determine if there was a trend between SP and $N_2O$ concentrations. We found no trend and used repeated-measure analysis of variance (RMANOVA) with a general linear mixed model to investigate differences in SP associated with taxa and reaction pathways within nitrifiers and denitrifiers. Specifically, we asked (i) if there was a difference in SP of $N_2O$ produced by individual taxa during $NH_2OH$ oxidation, (ii) if $N_2O$ produced by $NH_2OH$ oxidation and $NO_2^-$ reduction by nitrifiers had different SPs, (iii) if the SP of $N_2O$ produced by $NO_3^-$ reduction differed from that produced by $NO_2^-$ reduction for the same denitrifier taxon, and (iv) if there was a difference in SP of $N_2O$ produced from $NO_3^-$ and $NO_2^-$ by denitrifiers. For RMANOVA, taxon was the fixed effect, time was the repeated measure, and replicate cultures were the subject units. A similar RMANOVA was used to determine if $\delta^{15}N$ and $\delta^{18}O$

TABLE 1. Concentration, $\delta^{15}N$, $\delta^{18}O$, and site preference of $N_2O$ produced during $NH_2OH$ oxidation by concentrated cell suspensions of *N. europaea, N. multiformis*, and *M. trichosporium*

| Organism and replicate[a] | Time elapsed (min) | [$N_2O$] (μM) | $\delta^{15}N$–$N_2O$ (‰) | $\delta^{18}O$–$N_2O$ (‰) | Site preference (‰) |
|---|---|---|---|---|---|
| *Nitrosomonas europaea* | | | | | |
| A | 304 | 10.8 | −5.5 | 35.8 | 34.5 |
| A | 390 | 10.5 | −5.5 | 35.5 | 34.6 |
| A | 442 | 10.9 | −4.8 | 37.0 | 31.1 |
| B | 135 | 12.6 | 3.8 | 40.8 | 33.1 |
| B | 315 | 15.2 | 4.6 | 42.5 | 31.9 |
| B | 395 | 16.6 | 5.1 | 42.3 | 32.1 |
| C | 289 | 6.8 | 0.6 | 38.4 | 33.7 |
| C | 429 | 6.8 | −1.6 | 38.3 | 37.5 |
| C | 507 | 6.7 | 0.9 | 38.5 | 33.1 |
| Avg | | | −0.3 | 38.8 | 33.5 |
| SD | | | 4.9 | 2.9 | 1.2 |
| *Nitrosospira multiformis* | | | | | |
| A | 260 | 7.5 | −3.6 | 35.6 | 33.1 |
| A | 369 | 8.8 | −3.9 | 35.5 | 32.1 |
| A | 422 | 9.3 | −3.5 | 36.1 | 34.2 |
| B | 215 | 7.5 | 1.3 | 40.0 | 31.4 |
| B | 270 | 8.5 | 0.9 | 39.7 | 30.7 |
| B | 315 | 8.8 | 0.9 | 39.9 | 33.6 |
| C | 411 | 8.6 | 1.7 | 38.0 | 32.7 |
| C | 490 | 9.9 | 1.6 | 41.2 | 31.8 |
| C | 573 | 10.1 | 1.6 | 41.8 | 32.8 |
| Avg | | | −0.3 | 38.6 | 32.5 |
| SD | | | 2.9 | 2.5 | 0.6 |
| *Methylosinus trichosporium* | | | | | |
| A | 165 | 5.7 | 1.4 | 35.1 | 37.3 |
| A | 185 | 5.7 | 1.4 | 35.4 | 35.0 |
| A | 205 | 6.3 | 1.3 | 35.6 | 37.5 |
| B | 95 | 12.7 | 5.2 | 39.2 | 34.9 |
| B | 155 | 19.5 | 5.0 | 40.4 | 33.3 |
| B | 195 | 23.3 | 4.7 | 41.1 | 35.1 |
| B | 290 | 30.8 | 4.2 | 42.6 | 35.0 |
| B | 330 | 30.9 | 4.2 | 41.8 | 35.0 |
| B | 450 | 38 | 3.9 | 45.7 | 34.4 |
| Avg | | | 3.4 | 39.7 | 35.6 |
| SD | | | 1.9 | 4.0 | 1.4 |

[a] Replicates (A, B, or C) represent experiments conducted on separate days with different cultures sampled over time.

values for $N_2O$ produced by $NH_2OH$ oxidation differed among taxa. All analyses were performed using SAS, version 8.0 (SAS Institute).

## RESULTS

**$NH_2OH$ oxidation.** The headspace $N_2O$ concentrations from $NH_2OH$ oxidation by *N. europaea*, *N. multiformis*, and *M. trichosporium* increased during time course experiments (Table 1). The average $\delta^{15}N$ values of $N_2O$ produced by $NH_2OH$ oxidation by *N. europaea* (−0.3 ± 4.9‰), *N. multiformis* (−0.3 ± 2.9‰), and *M. trichosporium* (3.4 ± 1.9‰) were similar, as were the average $\delta^{18}O$ values of 38.8 ± 2.9‰ for *N. europaea*, 38.6 ± 2.5‰ for *N. multiformis*, and 39.7 ± 4.0‰ for *M. trichosporium*. We define an apparent fractionation for branched reactions as follows: $\delta^{15}N_{(substrate)} - \delta^{15}N_{(product)}$, or $\Delta^{15}N$. The $\Delta^{15}N$ associated with $N_2O$ production from $NH_2OH$ oxidation was −2.0‰ for *N. europaea* and *N. multiformis* and −5.7‰ for *M. trichosporium* ($\delta^{15}N$ of $NH_2OH$ = −2.3‰). The average SPs of $N_2O$ produced during $NH_2OH$ oxidation were high for *N. multiformis* (32.5 ± 0.6‰), *N. europaea* (33.5 ± 1.2‰), and *M. trichosporium* (35.6 ± 1.4‰). The site preference for *N. europaea* did not differ from that for *N. multiformis* ($P = 0.198$) or *M. trichosporium* ($P = 0.166$). The significant difference observed between *M. trichosporium* and *N. multiformis* ($P = 0.025$) was related in part to the low standard deviation associated with the *N. multiformis* data.

TABLE 2. Concentration, $\delta^{15}N$, $\delta^{18}O$, and site preference of $N_2O$ produced during ammonia oxidation by cultures of *Nitrosomonas europaea* for four independent experiments

| $N_2O$ concn (μM) | $\delta^{15}N$–$N_2O$ (‰) | $\delta^{18}O$–$N_2O$ (‰) | Site preference (‰) |
|---|---|---|---|
| 2.4 | −46.6 | 22.2 | 36.8 |
| 1.8 | −46.9 | 22.6 | 27.5 |
| 3.4 | −46.2 | 24.9 | 28.6 |
| 3.7 | −46.1 | 24.4 | 32.8 |
| Avg | −46.5 | 23.5 | 31.4 |
| SD | 0.4 | 1.3 | 4.2 |

**Ammonia oxidation.** The $N_2O$ concentrations in the headspaces of *N. europaea* batch cultures with ammonia as the substrate were 1.8 to 3.7 μM after 6 days of incubation (Table 2). The $\delta^{15}N$ and $\delta^{18}O$ values for $N_2O$ were −46.5 ± 0.4‰ and 23.5 ± 1.3‰, respectively (Table 2). The $\Delta^{15}N$ associated with the production of $N_2O$ during ammonia oxidation was −46.9‰ [$\delta^{15}N$ of $(NH_4)_2SO_4$ = 0.4‰]. There was no difference between the SP of the $N_2O$ produced from ammonia oxidation by *N. europaea* (31.4 ± 4.2‰; Table 2) and that in the $NH_2OH$ oxidation experiments ($P = 0.334$) (Table 1).

**Nitrifier denitrification.** The concentration of $N_2O$ in the headspace of *Nitrosospira multiformis* cell suspensions incubated with $NO_2^-$ increased in each replicate (Table 3). The average $\delta^{15}N$ and $\delta^{18}O$ values for the $N_2O$ produced were −22.9 ± 0.6‰ and 10.8 ± 0.5‰, respectively. The average SP for this experiment (0.1 ± 1.7‰) was significantly different from those produced from $NH_2OH$ by *N. multiformis*, *N. europaea*, and *M. trichosporium* ($P < 0.001$ for all taxa).

**Denitrification.** Nitrous oxide was produced by *P. chlororaphis* and *P. aureofaciens* with $NO_2^-$ and $NO_3^-$ as electron acceptors (Table 4). The SPs of $N_2O$ produced by *P. chlororaphis* and *P. aureofaciens* from $NO_3^-$ were −0.5 ± 1.9‰ and −0.5 ± 0.6‰, respectively, and those of $N_2O$ produced from $NO_2^-$ were −0.6 ± 1.9‰ and −0.5 ± 1.9‰, respectively. There was no significant influence of the inorganic nitrogen substrate on SP values for either taxon ($P = 0.087$ and 0.099, for *P. chlororaphis* and *P. aureofaciens*, respectively), indicating that the SP of $N_2O$ produced during denitrification is independent of the substrate (Table 4). The isotope enrichment factor for a unidirectional reaction relating the isotopic composition of the product to the substrate ($\varepsilon_{p/s}$) is described as follows (16): $\delta^{15}N_{(product)} = \delta^{15}N_{(substrate)} - \varepsilon_{p/s}[(f \times \ln f)/(1 - f)]$, where $f$ =

TABLE 3. Concentration, $\delta^{15}N$, $\delta^{18}O$, and site preference of $N_2O$ produced during $NO_2^-$ reduction in concentrated cell suspensions of *Nitrosospira multiformis*

| Replicate | Time elapsed (min) | $N_2O$ concn ($\mu M$) | $\delta^{15}N$–$N_2O$ (‰) | $\delta^{18}O$–$N_2O$ (‰) | Site preference (‰) |
|---|---|---|---|---|---|
| A | 773 | 4.2 | −23.6 | 10.7 | −3.8 |
| A | 854 | 4.4 | −23.5 | 10.9 | 0.5 |
| A | 874 | 5.1 | −23.7 | 10.1 | 0.0 |
| A | 984 | 6.4 | −23.5 | 9.8 | −4.0 |
| B | 335 | 3.2 | −23.4 | 11.5 | 1.2 |
| B | 479 | 7.9 | −22.0 | 10.5 | 1.1 |
| B | 541 | 8.2 | −21.7 | 10.5 | 1.5 |
| C | 210 | 6.2 | −24.2 | 11.5 | 0.3 |
| C | 280 | 8.5 | −23.9 | 11.4 | 0.6 |
| C | 390 | 14.1 | −23.1 | 11.5 | 1.9 |
| C | 460 | 17.4 | −21.7 | 10.9 | 1.6 |
| C | 530 | 20.8 | −21.5 | 11.4 | 0.3 |
| Avg | | | −22.9 | 10.8 | 0.1 |
| SD | | | 0.6 | 0.5 | 1.7 |

$[NO_2^-]/[NO_2^-]_{initial}$. The slope of the relationship of $-(f \times \ln f)/(1 - f)$ to $\delta^{15}N$, $\delta^{15}N^{\alpha}$, or the SP of accumulated $N_2O$ from $NO_2^-$ is equated with $\varepsilon_{p/s}$ during nitrifier denitrification (16). The values for $\varepsilon_{p/s}$ for $\delta^{15}N$ and $\delta^{15}N^{\alpha}$ associated with *P. chlororaphis* were 12.7‰ and 12.9‰, respectively (Fig. 1A). For *P. aureofaciens*, $\varepsilon_{p}/_{s}$ for $N_2O$ production from $NO_3^-$ reduction is 36.7‰, with a value of 37.4‰ for $\delta^{15}N$ and $\delta^{15}N^{\alpha}$, respectively (Fig. 1B). The SP for $N_2O$ did not change appreciably during the course of the reaction. This is demonstrated by the slope, which cannot be distinguished from zero based on our analytical precision (slope = 0.3 and 1.3 for *P. chlororaphis* and *P. aureofaciens*, respectively) (Fig. 1).

TABLE 4. Concentration and site preference of $N_2O$ produced by concentrated cell suspensions of *P. chlororaphis* and *P. aureofaciens* in time course samples with $NO_2^-$ and $NO_3^-$ as substrates

| Organism and substrate | Sampling point | Time elapsed (min) | $[N_2O]$ ($\mu M$) | Site preference (‰) |
|---|---|---|---|---|
| *Pseudomonas chlororaphis* | | | | |
| $NO_3^-$ | 1 | 55 | 21.1 | 2.5 |
| | 2 | 75 | 40.5 | 3.7 |
| | 3 | 95 | 57.2 | −0.3 |
| Avg | | | | −0.5 |
| SD | | | | 1.9 |
| $NO_2^-$ | 1 | 50 | 6.6 | −2.5 |
| | 2 | 110 | 12.2 | 0.9 |
| | 3 | 190 | 22.6 | −1.9 |
| | 4 | 230 | 29.3 | 1.2 |
| Avg | | | | −0.6 |
| SD | | | | 1.9 |
| *Pseudomonas aureofaciens* | | | | |
| $NO_3^-$ | 1 | 234 | 7.1 | −1.2 |
| | 2 | 461 | 16.5 | −0.2 |
| | 3 | 554 | 25.1 | 0.0 |
| Avg | | | | −0.5 |
| SD | | | | 0.6 |
| $NO_2^-$ | 1 | 186 | 6.2 | −1.8 |
| | 2 | 233 | 16.8 | 0.4 |
| | 3 | 268 | 21.8 | 1.8 |
| | 4 | 324 | 25.8 | −2.2 |
| Avg | | | | −0.5 |
| SD | | | | 1.9 |

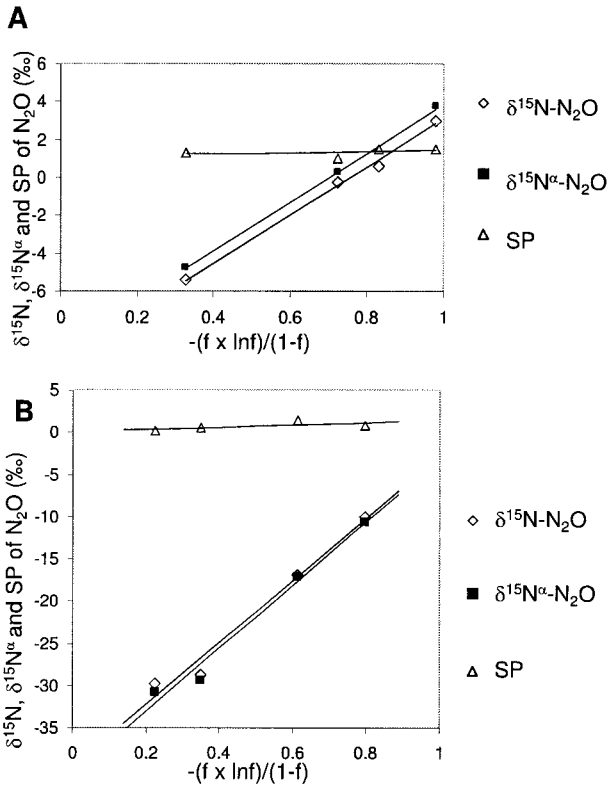


FIG. 1. $\delta^{15}N$, $\delta^{15}N^{\alpha}$, and SPs of $N_2O$ produced in concentrated cell suspensions of *P. chlororaphis* (A) and *P. aureofaciens* (B), with $NO_3^-$ as the substrate. The isotopic enrichment factors for $\delta^{15}N$, $\delta^{15}N^{\alpha}$, and site preference were 12.7, 12.9, and 0.3, respectively, for cell suspensions of *P. chlororaphis* (A) and 36.7, 37.4, and 1.3, respectively, for *P. aureofaciens* (B).

**Relative importance of $NO_2^-$ reduction and $NH_2OH$ oxidation on $N_2O$ production.** Concentrated *N. europaea* cell suspensions in the presence of $NO_2^-$ and $NH_2OH$ with a high liquid S/V ratio produced four times more $N_2O$ than those with a low S/V ratio (15.0 $\mu M$ and 3.4 $\mu M$, respectively). The $N_2O$ in the low-S/V-ratio experiment was more highly enriched in $^{15}N$ than that produced in the high-S/V-ratio experiment (283‰ and 114‰, respectively). Relative to the high-S/V-ratio condition, isotope mass balance indicated that the production of $N_2O$ from $NO_2^-$ reduction was approximately two times greater in the low-S/V-ratio experiment (6.8 and 16.6%, respectively).

The concentration of $N_2O$ in cultures of *N. europaea* incubated with a high S/V ratio initially decreased for $NO_2^-$ concentrations between 0.05 and 0.2 mM and then increased for concentrations between 0.2 and 0.6 mM $NO_2^-$ (Fig. 2). The percentage of $N_2O$ produced from the reduction of $^{15}N$-enriched $NO_2^-$ increased nearly linearly, from 2.0 to 15.3%, over the $NO_2^-$ concentration range from 0.05 to 0.6 mM. Since the S/V ratio controls the diffusion of $O_2$, our results are consistent with those of an earlier study for *N. europaea* that found little

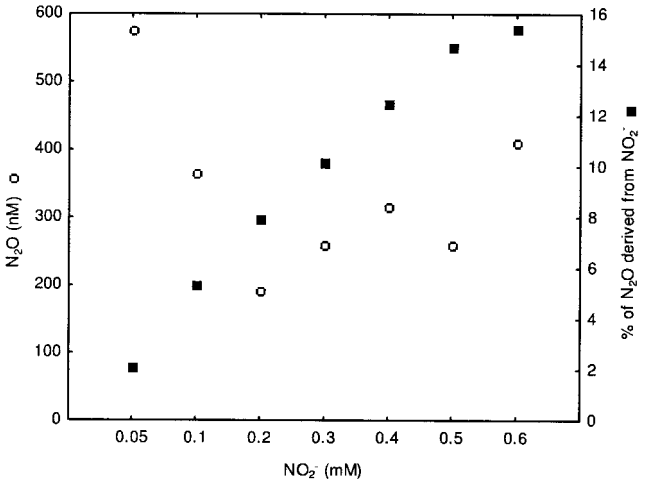


FIG. 2. $N_2O$ concentration (○) and % of $N_2O$ derived from $NO_2^-$ reduction versus $NH_2OH$ oxidation (■) in concentrated cell suspensions of *N. europaea* with various $NO_2^-$ concentrations (0.05 to 0.6 mM of $^{15}N$-enriched $NO_2^-$).

or no effect of $pO_2$ on $N_2O$ production if $NO_2^-$ concentrations were >0.05 mM (1).

**$\delta^{15}N$–$N_2O$ versus $\delta^{18}O$–$N_2O$.** The bulk nitrogen isotopic composition of $N_2O$ was nonetheless distinct for $N_2O$ produced from $NH_2OH$ oxidation and $NO_2^-$ reduction by *N. multiformis* (Fig. 3). $N_2O$ produced during $NH_2OH$ oxidation by *N. europaea*, *N. multiformis*, and *M. trichosporium* could not be distinguished on the basis of $\delta^{18}O$ and $\delta^{15}N$ values (Fig. 3). The oxygen isotope ratios of $N_2O$ produced by $NO_2^-$ reduction in *P. aureofaciens* and *P. chlororaphis* differed by 12‰ (Fig. 3). Nitrite reduction by *N. multiformis* produced $N_2O$ with a $\delta^{15}N$ value that was different from that produced by *P. aureofaciens* and *P. chlororaphis* (Fig. 3). Nitrous oxide produced by *N. europaea* with $NH_4^+$ as a substrate could be distinguished from that produced by denitrification and nitrifier denitrification on the basis of $\delta^{15}N$ values (Fig. 3).

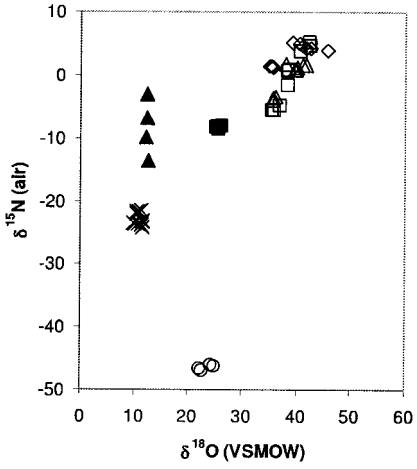


FIG. 3. $\delta^{18}O$ and $\delta^{15}N$ values (reported relative to air and VSMOW standards, respectively) for $N_2O$ produced by *Nitrosomonas europaea* with $NH_4^+$ as a substrate (○), *Nitrosomonas europaea* with $NH_2OH$ as a substrate (□), *Nitrosospira multiformis* with $NH_2OH$ as a substrate (△), *Methylosinus trichosporium* with $NH_2OH$ as a substrate (◇), *Nitrosospira multiformis* with $NO_2^-$ as a substrate (×), *Pseudomonas aureofaciens* with $NO_2^-$ as a substrate (▲), and *Pseudomonas chlororaphis* with $NO_2^-$ as a substrate (■).

## DISCUSSION

**Potential for $NO_2^-$ reduction in *N. europaea* cell suspensions.** By varying the liquid S/V ratio of *N. europaea* cultures, we showed that increased oxygen diffusion decreases the importance of $NO_2^-$ reduction relative to $NH_2OH$ oxidation in $N_2O$ production. The experiments demonstrated that there was an increase in the relative importance of $NO_2^-$ reduction relative to $NH_2OH$ oxidation in $N_2O$ production when there was a low S/V ratio in *N. europaea* cultures. However, even under conditions of high liquid S/V ratios, the production of $N_2O$ from $NO_2^-$ reduction was increased by elevating the concentration of $NO_2^-$ in the sample. Anderson et al. (1) found that $NO_2^-$ concentrations of >0.05 mM can stimulate $NO_2^-$ reduction. More recently, Beaumont et al. (3) found that NirK was expressed aerobically in response to increasing concentrations of $NO_2^-$, demonstrating the potential for aerobic denitrification by nitrifiers. We suggest that the variation in the SPs of $N_2O$ produced during nitrification could have been the result of a contribution of $NO_2^-$ reduction to $N_2O$ production stimulated by low oxygen concentrations or an increase in $NO_2^-$ concentration in the liquid phase of the concentrated cell suspensions, as described by Sutka et al. (27).

**Bulk $\delta^{15}N$ and $\delta^{18}O$ as a basis to differentiate $N_2O$ production during nitrification and denitrification.** Distinctions in the bulk $\delta^{15}N$ and $\delta^{18}O$ values for $N_2O$ from different sources provide a basis for evaluating sources of $N_2O$ in the troposphere (9, 19, 24). Nonetheless, the tendency is for production pathways to produce $N_2O$ with a wide range of isotope values such that source apportionment is difficult. Our results further document a wide range of isotope values for $N_2O$ produced both within replicate cultures carrying out the same process and between microbial production pathways. For example, during the reduction of $NO_2^-$ by *P. aureofaciens*, the $\delta^{15}N$ value for $N_2O$ became more depleted in $^{15}N$ as $N_2O$ was produced (Fig. 3).

The evaluation of $\delta^{18}O$ data for $N_2O$ is particularly challenging because isotope pathways reflect not only the source of atomic O but also the tendency for intermediate compounds of $N_2O$ production to exchange O with water. Ostrom et al. (18) proposed that the observed shifts in the $\delta^{18}O$ value of $N_2O$ with depth in the ocean reflects a predominance of $N_2O$ derived from $NH_4^+$ oxidation, with the preponderance of $N_2O$ from $NO_2^-$ reduction within a comparatively narrow depth interval. Our results are consistent with this dual-source interpretation, as $N_2O$ produced by nitrifier denitrification was markedly depleted in $^{18}O$ relative to that produced by $NH_4^+$ oxidation by *N. europaea* and $NO_2^-$ reduction by *N. multiformis* (Fig. 3). Despite the distinction in $\delta^{18}O$ values between $NH_2OH$ oxidation and nitrifier denitrification, a variation of approximately 12‰ was evident between cultures of two denitrifiers (*P. chlororaphis* and *P. aureofaciens*) carrying out $NO_2^-$ reduction. This indicates that there may not be a uniform oxygen isotope signature for $N_2O$ production by denitrifiers. The substrate, $NO_2^-$ ($\delta^{15}N$ = 1.5‰), was identical in the two

*Pseudomonas* experiments; therefore, isotopic variation is likely due to the exchange of oxygen atoms between intermediates and water, as discussed by Casciotti et al. (7). Schmidt et al. (25) discussed the difficulty of using isotopic values to differentiate between nitrification and denitrification. The current study confirms the challenges of using bulk nitrogen and oxygen isotopes as indications of the biogenic source. However, site-specific isotope characterization can differentiate between nitrification and denitrification.

**Site preferences of $N_2O$ produced by ammonia- and methane-oxidizing organisms.** The majority of information on the genetics and biochemical pathways of ammonia-oxidizing bacteria derives from studies of *N. europaea*. However, the genus *Nitrosomonas* is not as dominant in soils and waters as other nitrifiers such as *Nitrosospira* (6, 15, 24). In this study, the average SPs of $N_2O$ produced during $NH_2OH$ oxidation by *N. multiformis* and *N. europaea* were similar (33.5 ± 1.2‰ and 32.5 ± 0.6‰, respectively). In addition, the SPs of $N_2O$ produced in *N. europaea* batch cultures with $NH_4^+$ and in concentrated cell suspensions were similar (31.4 ± 4.2‰ and 33.5 ± 1.2‰, respectively). This similarity is particularly startling given that the substrates were similar in $\delta^{15}N$ yet had large differences in bulk $\delta^{15}N$ values (Tables 1 and 2). This result indicates that bulk N isotope fractionation during nitrification occurs mainly during the conversion of $NH_4^+$ to $NH_2OH$, and furthermore, that the SP is constant even though differences are evident in bulk $\delta^{15}N$ fractionation. Thus, despite variations in substrates and physiological differences between the genera *Nitrosomonas* and *Nitrosospira*, SP values of 32 to 35‰ for $N_2O$ produced by $NH_2OH$ and $NH_4^+$ oxidation can be applied to ammonia-oxidizing organisms as a whole.

Methanotrophs are divided into three groups (types I, II, and X) on the basis of phylogeny and ecology (13). The SP of $N_2O$ produced by $NH_2OH$ oxidation in cultures of *M. trichosporium* (35.6 ± 1.4‰) in this study was similar to that we previously reported for *M. capsulatus* Bath (30.8 ± 5.9‰) (27). In addition, the SPs of $N_2O$ produced during $NH_2OH$ oxidation by *N. multiformis* and *N. europaea* were similar to those of $N_2O$ produced by *M. trichosporium* and *M. capsulatus* Bath. The results indicate that large SPs of 32 to 35‰ are characteristic of nitrification, regardless of whether it is catalyzed by a methane or $NH_4^+$ oxidizer.

**Site preference of $N_2O$ produced during denitrification.** The SP reported here for $N_2O$ produced by denitrification and nitrifier denitrification (~0‰) (Table 3) is similar to the value of −5‰ reported by Toyoda et al. (29), who used cultures of *Pseudomonas denitrificans*. However, Toyoda et al. (29) found that the SP of $N_2O$ produced by *Pseudomonas fluorescens* was approximately 24‰. They suggested that variations in SP resulted from the production of $N_2O$ by abiological reactions within the culture, because the $N_2O$ production rates were low in the *P. fluorescens* experiments and $NO_2^-$ may have accumulated to high concentrations (29). Consequently, the SP value of 24‰ may be anomalous and not characteristic of $N_2O$ production by denitrifying bacteria. Since this study included *P. chlororaphis*, which possesses a cd1-type $NO_2^-$ reductase (31), and *P. aureofaciens*, which has a Cu-containing $NO_2^-$ reductase (8), and there was no difference in the SPs for the $N_2O$ produced, we can conclude that the type of $NO_2^-$ reductase does not influence the SP during denitrification. Consequently, our results and those of Toyoda et al. (29) indicate a consistent SP of approximately 0‰ for $N_2O$ production by denitrifiers, regardless of the enzyme involved.

**Site preference and field studies.** Our study demonstrates that the SP of $N_2O$ produced by denitrification, whether catalyzed by *Pseudomonas* cultures or $NH_4^+$ oxidizers (nitrifier denitrification), is approximately 33‰ lower than that produced by nitrification. Furthermore, our work and that of others (27, 29) demonstrate that in contrast to bulk isotope values, SP is conservative and independent of the substrate isotopic composition, which lays a foundation for the use of isotopomers to evaluate origins in field studies. Yamulki et al. (31) observed a change in SP, from between 5 and 9‰ to approximately −2‰, over a 24-h period in urine-amended grassland soil that was attributed to a shift in the relative importance of production from nitrification to denitrification. Based on isotope mass balance and our SP values of 33‰ and 0.1‰ for nitrification and denitrification, respectively, we estimate that 84% of $N_2O$ production was from denitrification in the first time period, with 100% being from denitrification in the second time period. These results confirm the initial suggestions of Yamulki et al. (31) and provide a quantification of the importance of nitrification and denitrification.

Although $N_2O$ reduction is a process that could influence SP, preliminary evidence from soil mesocosm experiments indicates that $N_2O$ reduction results in a negligible change in SP for the majority of a reaction (21). In addition, Firestone and Tiedje (10) observed that $N_2O$ consumption lagged behind production as a consequence of a delay in the synthesis of reducing enzymes. The impact of $N_2O$ reduction on SP is likely minimal in studies of episodic fluxes that are stimulated by the onset of anoxic conditions, such as the Yamulki study (31).

**Concluding remarks.** The SP of $N_2O$ is a robust and quantitative indicator of the microbial origins of this important greenhouse gas. In contrast to traditional bulk stable isotope analyses, the SP is not affected by isotopic fractionation. This approach may now provide important insights into management activities directed toward curtailing $N_2O$ emissions.

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