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

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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 (α) and terminal (β) 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

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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* (6.2×10^7 to 7.0×10^7 cells/ml) and *N. multiformis* (2.3×10^7 to 2.5×10^7 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* (7.4×10^7 to 1.9×10^8 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* (1.9×10^7 to 3.8×10^7 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 (2.3×10^7 to 2.5×10^7 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* (1.2×10^9 to 4.9×10^9 cells/ml) and *P. aureofaciens* (4.4×10^9 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 (μM)	$\delta^{15}N$–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)	$[N_2O]$ (μM)	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

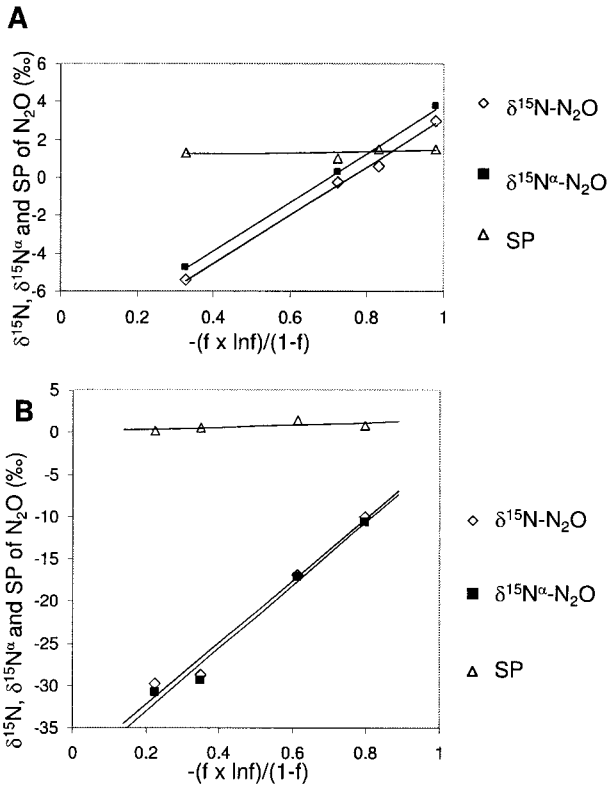

FIG. 1. $\delta^{15}N$, $\delta^{15}N^{\alpha}$, and SPs of N_2O produced in concentrated cell suspensions of *P. chlororaphis* (A) and *P. aureofaciens* (B), with NO_3^- as the substrate. The isotopic enrichment factors for $\delta^{15}N$, $\delta^{15}N^{\alpha}$, and site preference were 12.7, 12.9, 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 μM and 3.4 μM, 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

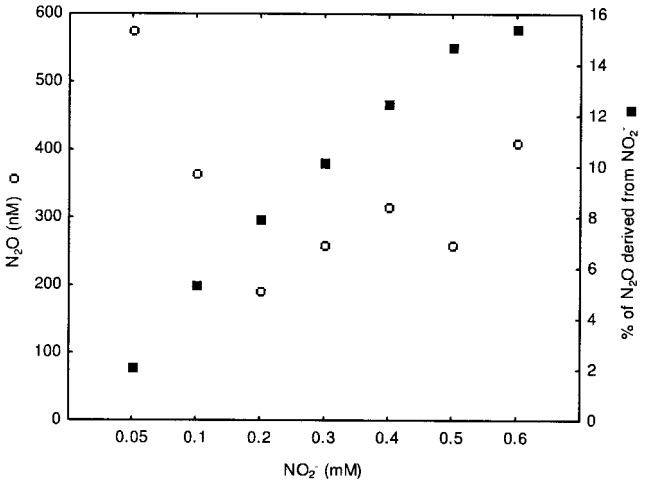

FIG. 2. N_2O concentration (○) and % of N_2O derived from NO_2^- reduction versus NH_2OH oxidation (■) in concentrated cell suspensions of *N. europaea* with various NO_2^- concentrations (0.05 to 0.6 mM of ^{15}N-enriched NO_2^-).

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).

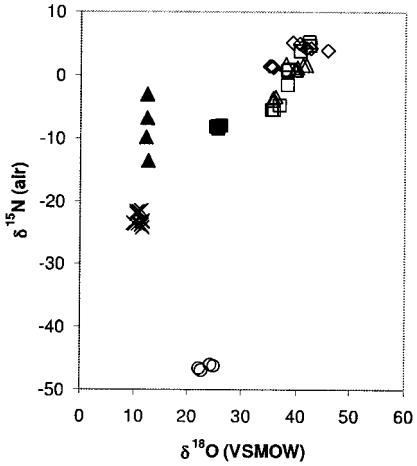

FIG. 3. $\delta^{18}O$ and $\delta^{15}N$ values (reported relative to air and VSMOW standards, respectively) for N_2O produced by *Nitrosomonas europaea* with NH_4^+ as a substrate (○), *Nitrosomonas europaea* with NH_2OH as a substrate (□), *Nitrosospira multiformis* with NH_2OH as a substrate (△),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