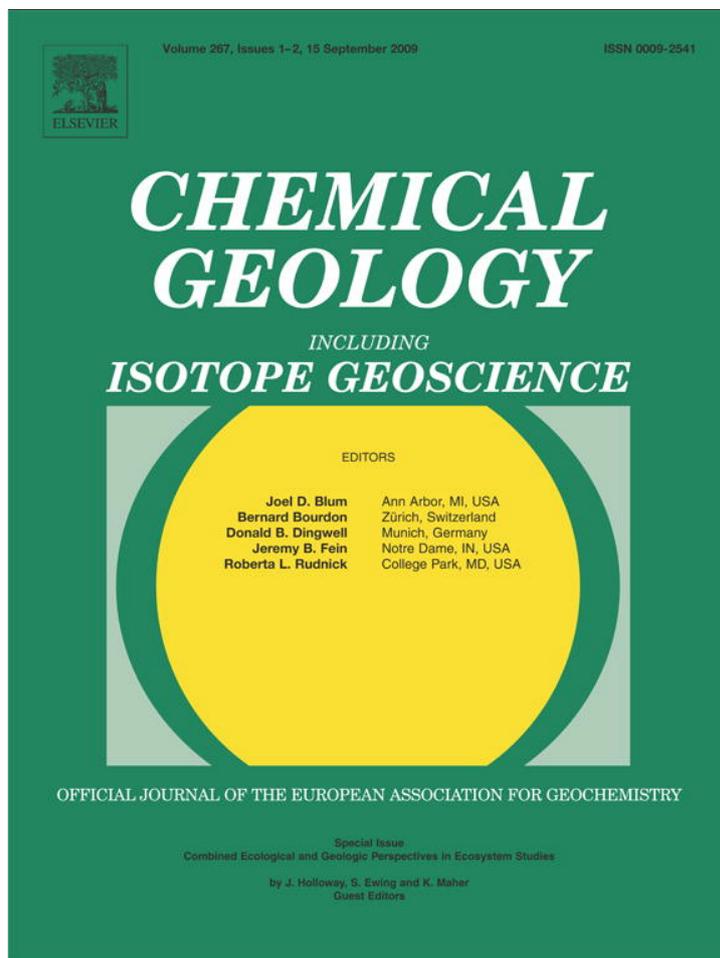


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The $\delta^{15}\text{N}$ and $\delta^{18}\text{O}$ values of N_2O produced during the co-oxidation of ammonia by methanotrophic bacteria

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ABSTRACT

In order to determine if the $\delta^{15}\text{N}$ and $\delta^{18}\text{O}$ values of N_2O produced during co-oxidation of NH_4^+ by methanotrophic (methane oxidizing) bacteria can be isotopically distinguished from N_2O produced either by autotrophic nitrifying or denitrifying bacteria, we conducted laboratory incubation experiments with pure cultures of methanotrophic bacteria that were provided NH_4Cl as an oxidation substrate. The N_2O produced during NH_4^+ oxidation by methanotrophic bacteria showed nitrogen isotope fractionation between NH_4^+ and N_2O ($\epsilon_{\text{N}_2\text{O}-\text{NH}_4^+}$) of -48 and -55% for *Methylomonas methanica* and *Methylosinus trichosporium*, OB3b respectively. These large fractionations are similar to those previously measured for autotrophic nitrifying bacteria and consistent with N_2O formation by multiple rate limiting steps that include NH_4^+ oxidation by the methane monooxygenase enzyme and reduction of NO_2^- to N_2O . Consequently, N_2O formed by NH_4^+ oxidation via methanotrophic or autotrophic nitrifying bacteria might generally be characterized by lower $\delta^{15}\text{N}_{\text{N}_2\text{O}}$ values than that formed by denitrification, although this also depends on the variability of $\delta^{15}\text{N}$ of available nitrogen sources (e.g., NH_4^+ , NO_3^- , NO_2^-). Additional incubations with *M. trichosporium* OB3b at high and low CH_4 conditions in waters of different $\delta^{18}\text{O}$ values revealed that 19–27% of the oxygen in N_2O was derived from O_2 with the remainder from water. The biochemical mechanisms that could explain this amount of O_2 incorporation are discussed. The $\delta^{18}\text{O}$ of N_2O formed under high CH_4 conditions was $\sim +15\%$ more positive than that formed under lower CH_4 conditions. This enrichment resulted in part from the incorporation of O_2 into N_2O that was enriched in ^{18}O due to an isotope fractionation effect of $-16.1 \pm 2.0\%$ and $-17.5 \pm 5.4\%$ associated with O_2 consumption during the high and low methane concentration incubations, respectively. Therefore, N_2O formed by NH_4^+ oxidation via methanotrophic or autotrophic nitrifying bacteria can have very positive $\delta^{18}\text{O}_{\text{N}_2\text{O}}$ values if the O_2 incorporated is previously enriched in ^{18}O from high rates of respiration. Nitrous oxide was collected from various depths in soils overlying a coal-bed methane seep where methanotrophic bacteria are naturally enriched. In one sampling when soil methane concentrations were very high, the $\delta^{18}\text{O}_{\text{VSMOW}}$ values of the N_2O were highly enriched ($+50\%$), consistent with our laboratory experiments. Thus, soils overlying methane seeps could provide an ^{18}O -enriched source of atmospheric N_2O .

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1. Introduction

Nitrous oxide is an active atmospheric trace gas and its concentration is increasing at a rate of 0.25% per year. In the troposphere N_2O acts as a stable greenhouse gas (Lashof and Ahuja, 1990), while in the stratosphere it plays an important part in the catalytic destruction of ozone (Cicerone, 1987). Nitrous oxide is primarily formed by microbial pro-

cesses of nitrification and denitrification in soil, marine and freshwater environments (Yoshinari, 1976; Bremner and Blackmer, 1978; Elkins et al., 1978; Freney et al., 1978; Duxbury et al., 1982; Yoshida et al., 1989; Mosier et al., 1991). Soil and oceanic emissions are currently thought to be the two most important natural sources of atmospheric N_2O .

Based on stable isotopic ($\delta^{15}\text{N}$ and $\delta^{18}\text{O}$) measurements of N_2O , the global N_2O budget is not balanced. The average $\delta^{15}\text{N}_{\text{N}_2\text{O}}$ and $\delta^{18}\text{O}_{\text{N}_2\text{O}}$ values of atmospheric sources from soils and oceans do not agree with the isotopic composition of tropospheric N_2O (Kim and Craig, 1993; Rahn and Wahlen, 2000). Microbial nitrification includes the oxidation of NH_3 by ammonium oxidizing bacteria (AOB), which obtain energy from the reaction, and the co-oxidation of NH_4^+ by methane oxidizing bacteria (methanotrophs). Although it is known that nitrous oxide produced by nitrifiers gains its N from the ammonia substrate

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and its oxygen from both water and O₂, there is still debate about the exact mechanism by which nitrifier N₂O is formed (Stein and Yung, 2003; Schmidt et al., 2004). Studies to date have shown that nitrification by AOB tends to produce N₂O that is generally isotopically depleted in ¹⁸O and ¹⁵N relative to tropospheric N₂O (Wahlen and Yoshinari, 1985; Yoshida, 1988; Sutka et al., 2006), while N₂O originating during denitrification tends to be more enriched in ¹⁵N and ¹⁸O than that produced by nitrifiers (Wahlen and Yoshinari, 1985; Yamazaki et al., 1987; Webster and Hopkins, 1996; Mandernack et al., 2000b; Sutka et al., 2006). Although both of these are major processes of N₂O formation in soils that contribute significantly to atmospheric N₂O (Firestone et al., 1980; Yamulki et al., 2000), isotopic measurements of soil N₂O have thus far been considerably more negative than the average tropospheric values of +7.0(±atm. N₂) and +44.2(±VSMOW) for δ¹⁵N and δ¹⁸O, respectively (Wahlen and Yoshinari, 1985; Kim and Craig, 1993; Pérez et al., 2000; Yamulki et al., 2001; Rock et al., 2007). More work is therefore needed to identify the bacterial controls on the isotopic signatures of different soil N₂O sources. Much of the previous work on the isotopic composition of N₂O produced during microbial nitrification has focused on AOB. This includes recent reports illustrating the utility of nitrogen isotopomer measurements of N₂O to discern its microbial source (e.g. nitrification versus denitrification) (Yamulki et al., 2001; Sutka et al., 2003, 2006).

Despite the ecological ubiquity of methanotrophs, much less is known about the isotopic composition of N₂O produced during their co-oxidation of ammonium. Methanotrophs are present both in high methane environments (geologic methane seeps, landfills, wetlands) as well as low-methane environments (upland soils) where they oxidize atmospheric methane. Because of the similarity between the methane monooxygenase (MMO) enzymes in methanotrophs and ammonia monooxygenase (AMO) enzymes in nitrifiers, each type of organism is capable of oxidizing each other's substrates (Hutton and ZoBell, 1953; Whittenbury et al., 1970; Dalton, 1977; O'Neill and Wilkinson, 1977; Yoshinari, 1985; Bedard and Knowles, 1989; Megraw and Knowles, 1989; Holmes et al., 1995; Schnell and King, 1995). Both MMO and AMO can mediate the oxidation of CH₄ and NH₄⁺ by O₂ to methanol and hydroxylamine, respectively. The co-oxidation of NH₄⁺, and subsequent N₂O formation, by methanotrophic bacteria has previously been reported (Yoshinari, 1985; Megraw and Knowles, 1989; Roy and Knowles, 1994; King and Schnell, 1994; Mandernack et al., 2000a). Methanotrophs cannot produce energy via ammonia oxidation and require CH₄ for sustained N₂O production. However, many native grassland and forest soils both consume atmospheric CH₄ and emit N₂O (Keller et al., 1983, 1986; Mosier et al., 1991; Castro et al., 1993; Neff et al., 1994). Mandernack et al. (2000a) identified landfills as a source of N₂O and provided evidence suggesting it was formed by methanotroph based nitrification in the cover soils. Therefore, the formation of N₂O by methanotrophic bacteria in soils may be a common occurrence.

In this study, we assessed the nitrogen and oxygen isotope systematics associated with the co-oxidation of NH₄⁺ by *Methylobacterium methanica* (a type I methanotroph) and *Methylosinus trichosporium* OB3b (a type II methanotroph) in order to determine if they were substantially different than that previously reported for autotrophic nitrifiers (Yoshida, 1988; Sutka et al., 2006) and to determine if they were affected by ambient methane concentrations. In addition, we measured δ¹⁸O values of N₂O produced by *M. trichosporium* OB3b grown in media with varying δ¹⁸O_{H₂O} values to assess whether the relative percent incorporation of oxygen from H₂O and O₂ into the N₂O product are consistent with mechanisms of N₂O formation previously proposed for AOB. Because our experiments were closed systems and O₂ is isotopically fractionated during microbial respiration and reaction with methane monooxygenase enzymes (Lane and Dole, 1956; Stahl et al., 2001), it was also necessary to measure and account for Rayleigh distillation of ¹⁸O₂ in our experiments.

We compare our laboratory results with isotopic measurements of N₂O from native soils overlying a coal bed methane seep where

methanotrophic bacterial populations were naturally enriched. The field measurements were made in order to assess whether methanotrophic production of soil N₂O was apparent from the measured δ¹⁵N_{N₂O} and δ¹⁸O_{N₂O} values, and whether these soils represented a biogenic source of N₂O that was isotopically enriched relative to tropospheric N₂O.

2. Methods

2.1. Methanotrophic bacterial cultures for δ¹⁵N_{N₂O} measurements

The two strains of methanotrophic bacteria, *M. trichosporium*, OB3b, a type II methanotroph, and *M. methanica*, a type I methanotroph, used for experiments 1–3 (Table 1) were obtained in 1997 from Dr. Richard Hanson at the University of Minnesota. The strain of *M. trichosporium* OB3b used for experiments 4–13 (Table 1) was obtained from Mary Lidstrom, University of Washington, Seattle WA. Prior to all experiments both strains were grown as batch cultures in 100 mL of phosphate buffered NMS mineral medium, final pH 6.8, contained in 250 mL serum vials. The NMS media was made as follows: 1 g L⁻¹ MgSO₄·7H₂O, 0.26 g L⁻¹ CaCl₂·2H₂O, 1 g L⁻¹ KNO₃, 2 mL L⁻¹ of Widdel's trace metal solution (Widdel and Pfennig, 1977), and 2 mL L⁻¹ of a 0.24 M (total phosphate) stock solution of equimolar Na₂HPO₄·7H₂O/KH₂PO₄ buffer. Methane and O₂ were periodically added and cultures were used as inoculants when sufficient growth was observed. All equipment and reagents used in the experiments were sterilized by either autoclave or filtration.

Experiments 1–3 were performed in 20-liter glass carboys containing 4 L of NMS medium inoculated with two serum vial cultures. The carboys were fitted with stoppers and sterile 0.2 μm filters to allow the cultures to be purged with sterilized gases. Carbon dioxide (120–180 mL) was added to each carboy at the start of the incubations to facilitate initial growth. The cultures were magnetically stirred and continuously sparged with a mixture of CH₄ and zero air for 3 to 4 days until the cultures became sufficiently turbid. Then 5 M NH₄Cl with a pre-measured δ¹⁵N value of +4.5‰ was added to a final concentration of 20 mM. The carboys were again sparged for 1 h with the CH₄ and zero air, re-sealed, incubated for 24 h without sparging.

The headspace gas of each carboy was sampled immediately after sealing (T₀) and again 24 h later (T₁). Headspace gas concentrations of CH₄, O₂ and CO₂, and N₂O were simultaneously measured on a Shimadzu GC17 gas chromatograph using FID, TCD, and ECD detectors, respectively. Immediately after T₁, cultures were poisoned with 200 mL of 10 M NaOH to assure a final pH of ≥9.5. This also sequestered a majority of the headspace CO₂ as aqueous carbonate prior to cryogenic distillation of the N₂O from the headspace gas.

2.2. Collection of liquid subsamples for cell counts, δ¹⁸O_{H₂O} and δ¹⁵N_{NH₄⁺} analyses

Duplicate aliquots of culture media were collected each for total cell counts, δ¹⁸O of water, and δ¹⁵N analysis of the NH₄Cl at both T₀ and T₁ time points. The δ¹⁵N analyses of the NH₄⁺ were made directly on salts obtained by evaporation of the two aliquots. Prior to evaporation, ultra clean HCl was added to one of the aliquots to achieve a final pH of 2. Approximately 50 μL of 10 M NaOH was added to the other aliquot to achieve a final pH of ≥10. Ammonium and NO₃⁻ were the only sources of nitrogen added to the experiments (with NH₄⁺ in excess) and both species are preserved at low pH (HCl-amended aliquot), resulting in a δ¹⁵N value for total nitrogen. The basified sample was purged for 45–60 min with N₂ to drive off NH₄⁺ as NH_{3(g)} and measured for δ¹⁵N of the remaining nitrate, which was within 1‰ of the δ¹⁵N of the nitrate added to the media, thus confirming that removal of the NH_{3(g)} was complete (data not shown). Using the total N concentration values from the mass spectrometric analysis of the dried salts, the δ¹⁵N_{NH₄⁺} value was calculated by mass balance from the difference in δ¹⁵N values of the

Table 1
Measured and calculated parameters for methanotroph incubation experiments.

Expt. #	Strain	Experiment type	Size	Direct cell count ($\times 10^8 \text{ mL}^{-1}$)		CH ₄ added at T ₀ (% by volume)	Headspace Gas Concentrations at end (by volume)				$\delta^{15}\text{N}_{\text{NH}_4}$ (‰) ^a		$\delta^{15}\text{N}_{\text{N}_2\text{O}}$ at end (‰) ^a	$\delta^{18}\text{O}_{\text{H}_2\text{O}}$ (‰) ^b	$\delta^{18}\text{O}_{\text{N}_2\text{O}}$ at end (‰) ^a	Calc. $\delta^{18}\text{O}_{\text{O}_2}$ at end (‰) ^b
				T ₀	T ₁		CH ₄ (%)	O ₂ (%)	CO ₂ (%)	N ₂ O (ppm)	Initial	End				
1	<i>M. m.</i>	Type I	20 L carboy	2.2	2.4	5.9	4.5	15.4	46		9.0	8.3	−39.4	−14.6	−10.1	
2	<i>M. t.</i>	Type II/low CH ₄	20 L carboy	1.3	1.4	8.2 ^c	6.2	15.3	66		9.8	8.6	−46.0	−17.0	21.2	29.1
3	<i>M. t.</i>	Type II/low CH ₄	20 L carboy	1.5	2.2	7.2 ^c	4.6	16.3	67		9.1	10.8	−44.5	−17.0	25.0	28.0
4	<i>M. t.</i>	Type II/low CH ₄	250 mL flask			0.6	0.7	18.1	1.1	30			−40.7	18.6	44.6	26.1
5	<i>M. t.</i>	Type II/low CH ₄	250 mL flask			0.6	0.0	18.7	1.1	14			−42.2	18.6	52.5	25.5
6	<i>M. t.</i>	Type II/low CH ₄	250 mL flask			0.6	0.8	18.3	0.8	27			−38.9	−2.7	25.6	25.9
7	<i>M. t.</i>	Type II/high CH ₄	250 mL flask			5.4	0.2	9.9	4.9	42			−43.6	18.6	64.8	36.7
8	<i>M. t.</i>	Type II/high CH ₄	250 mL flask			5.4	0.1	10.7	4.4	24			−42.0	−2.7	47.7	34.6
9	<i>M. t.</i>	Type II/high CH ₄	250 mL flask			5.4	0.1	11.7	3.9	19			−41.8	−2.7	46.9	33.1
10	<i>M. t.</i>	Type II/high CH ₄	1 L vial			7.5 ^d	0.1	7.8	6.2	5			−38.9	−15.4	36.6	45.8 ^e
11	<i>M. t.</i>	Type II/high CH ₄	1 L vial			7.5 ^d	0.1	7.7	6.3	5			−39.0	−15.4	37.8	43.8 ^e
12	<i>M. t.</i>	Type II/low CH ₄	1 L vial			1.5 ^d	0.3	17.5	2.1	1				−15.4		30.0 ^e
13	<i>M. t.</i>	Type II/low CH ₄	1 L vial			1.5 ^d	0.3	17.2	2.1	1				−15.4		29.7 ^e

M. m. = *Methylobomonas methanica*; *M. t.* = *Methylosinus trichosporium* OB3b.

^a Relative to Air-N₂.

^b VSMOW.

^c Although these initial CH₄ concentrations were relatively high, these experiments were classified as low-methane because the net consumption of CH₄ and O₂ during the experiment more closely resembled that of the other low-methane experiments.

^d Includes an additional 0.5% CH₄ added with NH₄Cl solution.

^e Directly measured values.

acidified (NH₄⁺ + NO₃[−]) and basified (NO₃[−] only) samples. The $\delta^{15}\text{N}$ values of the salts were determined with an elemental analyzer interfaced with a Micromass Optima isotope ratio mass spectrometer. Standard normalization procedures were applied by analyzing the USGS 25 and USGS 26 ammonium sulfate standards using the $\delta^{15}\text{N}$ values of −30.41 and 53.75‰, respectively, recommended by (Böhlke and Coplen, 1995). Replicate analyses agreed to within $\pm 0.2\%$. There was good reproducibility of the measured $\delta^{15}\text{N}_{\text{NH}_4}$ values from the three different carboys experiments using this approach (Table 1), although they were ~4‰ enriched relative to the NH₄Cl added. Replicate analyses of some of these samples did occasionally show variations as large as 1‰ (data not shown). Consequently, we present our estimates of $\varepsilon^{15}\text{N}_{\text{N}_2\text{O}-\text{NH}_4}$ to the nearest whole value. Given the large isotopic fractionations observed in this study (>40‰), this has no effect on the conclusions drawn.

2.3. Experiments to determine the contribution of O₂ versus H₂O oxygen to methanotrophic N₂O

Experiments 4 through 9 were designed to determine the contribution of O₂ versus H₂O to the oxygen in N₂O formed by oxidation of NH₄⁺ by *M. trichosporium*. Two batches of NMS medium were made from waters with $\delta^{18}\text{O}_{\text{VSMOW}}$ values (−2.7 and +18.6‰) that were distinct from the carboy experiments. Duplicate experiments were set up with each medium (total of 4 experiments). Glass Erlenmeyer flasks (250 mL) with 100 mL of medium and stoppers with 0.2 μm gas filters were inoculated with 1 mL of *M. trichosporium* OB3b culture from the serum vial cultures previously described. The cultures were sparged for 10 days with CH₄ and air and mixed using an orbital shaker (~100 rpm). At 10 days, the cultures were sparged with only air for ~1 h and then the flasks were re-sealed. One half milliliter of 5 M NH₄Cl (final concentration 25 mM) and 10 mL of CH₄ (final headspace concentration 5.4% by volume) were injected by syringe. Concentrations of N₂O in the headspace were monitored for ~1.5 days when they became high enough for isotope analysis, at which time the headspace gas was cryogenically distilled as described below. One of the samples with $\delta^{18}\text{O}_{\text{H}_2\text{O}} = +18.6\%$ was lost during cryogenic distillation. After the headspace gas was removed, each flask was purged with sterile air and re-sealed in preparation for the low headspace CH₄ experiments. For these experiments, an additional 0.5 mL of 5 M NH₄Cl and 1 mL of CH₄ (headspace concentration 1.1%)

were added to each flask and they were incubated again as previously described.

2.4. Determination of the isotope fractionation effect for O₂ consumption by *M. trichosporium*

M. trichosporium Ob3b cultures were grown in NMS medium ($\delta^{18}\text{O}_{\text{H}_2\text{O}} = -15.4\%$) in 250 mL flasks sparged with CH₄ and air as previously described. After 8 days of growth 200 mL of culture was placed into each of four 1150 mL custom made serum vials. Two vials were over-pressurized with 350 mL compressed air and 100 mL CH₄ to obtain “high” CH₄ headspace concentrations. The other two vials were over-pressurized with 150 mL air and 10 mL CH₄ in order to achieve “low” CH₄ headspace concentrations. The vials were allowed to equilibrate while shaken for 10 min after gasses were added. At T₀ and subsequent time points 0.2 mL samples were taken from the vials via gas tight syringe and immediately injected at atmospheric pressure onto a gas chromatograph as previously described. Duplicate samples measured for each timepoint were typically within 0.7, 0.2, and 0.2 absolute percent by volume for O₂, CH₄, and CO₂, respectively. Samples for $\delta^{18}\text{O}_{\text{O}_2}$ isotope measurements were taken at selected timepoints via a gas-tight glass syringe under the *in situ* pressure within the vial. After collection, the syringe valve was closed, removed from the vial, and the degree of gas expansion in each sample vial was recorded in order to determine the headspace pressure at each time point. The sample was then injected into an evacuated 7 mL Vacutainer, resulting in a slight over-pressure necessary for storage until $\delta^{18}\text{O}_{\text{O}_2}$ analysis. At one time point for each of experiment 10 (T₃) and 11 (T₅), triplicate $\delta^{18}\text{O}_{\text{O}_2}$ samples were taken and standard deviations for their $\delta^{18}\text{O}_{\text{O}_2}$ values were 1.1 and 0.3‰, respectively. In order to also generate N₂O in these experiments 1 mL of 5 M NH₄Cl was added to each of the four vials to a final concentration of 25 mM after about 1.5 days, when the remaining CH₄ in the vial headspace was $\leq 5\%$ of the initial amount. An additional 2 and 5 mL of CH₄ were added to each of the high and low methane vials in order to provide substrate for the co-oxidation of ammonia. Approximately 5 days after T₀, the headspace CH₄ concentrations were <0.1% by volume in experiments 10 and 11 and <0.5% by volume in experiments 12 and 13. The experiment was stopped and final samples were taken for headspace gas concentration and $\delta^{18}\text{O}_{\text{O}_2}$ measurements. Nitrous oxide was then cryogenically distilled from the remaining headspace gas in each of the two high methane vials.

2.5. Stable isotope measurements of H₂O and O₂

The $\delta^{18}\text{O}$ value of water was determined by analyzing CO₂ gas that had equilibrated with 200 μl aliquots at 40 °C in septum-capped vials. Raw data were corrected for the H₂O–CO₂ isotopic fractionation, and then adjusted for small instrumental effects using results obtained for water standards that had been previously calibrated against VSMOW and SLAP. Duplicate analyses normally agreed to within $\pm 0.1\%$.

For isotopic analysis of O₂, aliquots of head space gas were passed through a trap immersed in liquid nitrogen to remove H₂O, CO₂, N₂O, and other condensable species, and then admitted to the bellows of a Finnigan MAT 252 mass spectrometer. The $\delta^{18}\text{O}$ of O₂ was determined by comparison to a pure O₂ working standard after balancing the major ion beams (ion mass/ion charge = 32). The head space gases also contained N₂ and Ar; these were admitted to the mass spectrometer along with the O₂ because they do not condense in a liquid nitrogen trap. As a consequence, the total gas pressure in the mass spectrometer ion source was higher during measurement of unknowns than during measurement of the working standard. Analyses of O₂ in laboratory air ($\delta^{18}\text{O} = 23.5\%$) (Kroopnick and Craig, 1972), carried out using the same liquid nitrogen purification step, revealed that the presence of N₂ and Ar resulted in $\delta^{18}\text{O}$ values that were low by 2.0‰. Tests carried out by Emerson et al. (1999) using a Finnigan MAT 251, which has an ion source virtually identical to the Finnigan MAT 252 source, suggest that the magnitude of the $\delta^{18}\text{O}$ error increases with dilution of the O₂. The head space gases analyzed in this study had O₂/N₂ + Ar ratios ranging from air-like (0.265) to as low as 0.035 (Tables 1 and 2; N₂ + Ar calculated by difference from 100%). The increase in the $\delta^{18}\text{O}$ error over this composition range is only 0.1‰ (Fig. 4 of (Emerson et al., 1999) in this range O₂/N₂ is a very close approximation of O₂/(N₂ + Ar). This is insignificant for the purposes of the present study. Thus, a uniform correction of 2.0‰ was applied to the O₂ isotopic results for all head space gases. Replicate measurements agreed to within $\pm 0.8\%$ or better.

2.6. Field study of N₂O, NH₄⁺ and NO₃⁻ from methane-consuming soils

To compare the laboratory studies with a natural environment where N₂O is produced by methanotrophic bacteria, we measured the $\delta^{15}\text{N}_{\text{N}_2\text{O}}$ and $\delta^{18}\text{O}_{\text{N}_2\text{O}}$ values from cover soils overlying a coal bed methane seep. Soil gas samples were collected from a 1 meter depth soil gas probe installed by the Bureau of Land Management at a coal-bed methane seep approximately 6.5 km north of Bayfield, Colorado, USA. Additional samples were collected from other depths using 1/2 inch-diameter stainless steel soil gas probes. Collections were made under contrasting soil moisture conditions on 8-3-03 (dryer) and 5-11-04 (wetter). For the latter collection soil samples were also collected. Field capacity and concentrations of NH₄⁺ and NO₃⁻ (standard KCl extraction method; Keeney and Nelson, 1982) were determined on these samples by the Colorado State University Soil Testing Laboratory in Fort Collins, CO. Percent soil moisture was determined comparing wet and dry (overnight at 105 °C) soil weights and was then converted to percent field capacity.

Prior to soil gas collection, approximately three static volumes of gas were purged from the probe or well. Samples for concentration measurements were collected into either 10 mL glass syringes that were sealed by inserting the needle into a rubber stopper, or in 7 mL Vacutainer tubes containing a small amount (~1 mL) of acidified (~0.1 M), He-purged water. The Vacutainers were inverted after collection to maintain a gas tight seal. The samples were analyzed for CH₄, CO₂, O₂, and N₂O concentrations within 24 to 36 h of collection by gas chromatography as described in Section 2.1. Samples for isotopic analysis of N₂O were collected in either evacuated serum vials or 15 mL Vacutainers with acidified water as previously described. A duplicate sample from the 100 cm depth (5-11-04) was collected in a 150 mL stainless steel gas sampling bottle with gas tight valves.

Table 2

Data from experiments 10–13 used for determining the O₂ fractionation effects during the oxidation of CH₄ by *M. trichosporium* OB3b.

Time (days)	Total mmoles in headspace gas + dissolved in medium			¹⁸ O/ ¹⁶ O of O ₂ ($\times 10^{-3}$)	$\delta^{18}\text{O}$ of O ₂ (‰ VSMOW)
	CH ₄	CO ₂	O ₂		
<i>Experiment 10</i>					
0.00	3.5	0.0	10.5	2.05814	26.4
0.08	3.1	0.3	9.8	2.05734	26.0
0.22	2.4	0.9	8.8	2.06255	28.6
0.38	1.7	1.4	7.4	2.06803	31.3
0.61	1.0	2.0	6.2	2.06876	31.7
0.88	0.6	2.3	5.2	2.07398	34.3
1.13	0.4	2.7	4.9		
1.24	0.3	3.1	4.8	2.07959	37.1
1.60	0.2	3.3	4.5	2.08862	41.6
1.63	0.3	3.5	4.5		
3.11	0.2	4.0	3.9		
4.22	0.1	4.1	3.6		
5.14	0.1	4.0	3.4	2.09704	45.8
<i>Experiment 11</i>					
0.00	3.4	0.0	10.0	2.05413	24.4
0.08	3.2	0.4	9.9	2.05934	27.0
0.22	2.2	1.0	8.2	2.06295	28.8
0.40	1.6	1.6	7.2	2.06455	29.6
0.62	1.0	2.1	6.1	2.06937	32.0
0.88	0.6	2.5	5.3	2.07404	34.3
1.13	0.4	2.8	4.8		
1.24	0.3	2.8	4.6	2.08440	39.5
1.60	0.2	3.3	4.4	2.08180	38.2
1.62	0.3	3.4	4.4		
3.11	0.1	3.9	3.9		
4.21	0.1	4.1	3.6		
5.25	0.1	3.9	3.3	2.09303	43.8
<i>Experiment 12</i>					
0.00	0.4	0.0	8.5	2.05693	25.8
0.40	0.2	0.4	8.2	2.05774	26.2
0.87	0.1	0.6	7.3	2.05894	26.8
1.51	0.0	0.9	7.0	2.06155	28.1
1.55	0.2	1.0	7.4		
3.09	0.1	1.4	6.9		
4.20	0.1	1.4	6.7		
5.32	0.1	1.6	6.6	2.06536	30.0
<i>Experiment 13</i>					
0.00	0.4	0.0	8.7	2.05352	24.1
0.40	0.1	0.4	7.9	2.05914	26.9
0.87	0.1	0.6	7.2	2.06135	28.0
1.51	0.0	0.8	7.2	2.06395	29.3
1.54	0.2	0.9	7.3		
3.09	0.1	1.2	6.7		
3.54	0.1	1.4	6.7		
5.35	0.1	1.5	6.6	2.06475	29.7

2.7. Cryogenic purification of headspace N₂O for $\delta^{15}\text{N}$ and $\delta^{18}\text{O}$ analysis

The headspace gas of each experiment was cryogenically distilled to isolate purified N₂O for isotopic analysis. The gas was introduced into a vacuum line by attaching vacuum tubing directly to the incubation vessel (experiments 1–3 and 10–13) or by transferring the headspace gas via a 100 mL gas tight syringe (experiments 4–9). The samples flowed through a series of traps: (1) ascarite, (2) dry ice/ethanol, (3–5) liquid N₂, (6) ascarite, (7) liquid N₂. The ascarite traps removed CO₂ which freezes out with N₂O in liquid N₂; the dry/ice ethanol trap removed water vapor. The purified N₂O samples were collected and stored in flame sealed tubes.

2.8. Measurement of $\delta^{18}\text{O}_{\text{N}_2\text{O}}$ and $\delta^{15}\text{N}_{\text{N}_2\text{O}}$ values by isotope ratio mass spectrometry

Isotopic measurements of purified N₂O from experiments 1 to 3 were performed by dual inlet-isotope ratio mass spectrometry (DI-

IRMS). Purified N₂O was cryogenically separated from the air samples after removal of CO₂ with a column of ascarite. Any remaining contaminants were removed by processing through a gas chromatograph fitted with a thermal conductivity detector (we used a 3/8 inch, stainless steel column of 2-m length packed with Porapak Q and operated at a temperature of 18 °C with ultra high purity helium as carrier gas). The recovered N₂O was then measured directly by stable isotope mass spectrometry on a dual-inlet VG Prism II mass spectrometer. The extraction techniques were tested with artificial air standards made with N₂O working gas of known isotopic composition. Due to the off-line nature of the dual inlet method, there is potential for introduction of trace levels of CO₂ during sample handling prior to and during introduction to the mass spectrometer. To test for this potential contamination and to make corrections if necessary, we monitored two indicators of CO₂ contamination—the beam intensities of $M/z = 12$ (elemental carbon) and $M/z = 22$ (doubly ionized CO₂) (the second ionization efficiency for N₂O is negligible compared to that for CO₂). Corrections were derived from empirical relationships developed by analyses of N₂O standards spiked with known quantities of CO₂. The sensitivity of N₂O isotope analysis to CO₂ contamination in our mass spectrometer is 6.8‰/‰ CO₂ for δ¹⁵N and 12.4‰/‰ CO₂ for δ¹⁸O. Typical corrections, if necessary at all, are less than a few tenths of a per mil (Rahn and Wahlen, 1997).

Isotopic measurements of purified N₂O from experiments 4 to 11 and of unpurified N₂O from the coal-bed methane seep were made with a Trace Gas Analyzer (TGA) (GV Instruments) interfaced to an Isoprime IRMS (GV Instruments). The TGA was fitted with the direct injection attachment set up for N₂O analysis with preparatory and gas chromatograph backpressures set at 5 and 10 psi, respectively. It is not necessary to monitor for CO₂ contamination with the mass spectrometer because the gas chromatograph onboard the TGA separates any contaminant CO₂ from N₂O before it is introduced into the IRMS. A calibrated N₂O isotope standard obtained from Dr. N. Yoshida (Tokyo Institute of Technology) was used to calibrate high purity N₂O as a laboratory reference against which all samples were measured. Samples were introduced into the TGA by syringe and duplicate or triplicate analyses were performed on each sample. Standard deviations (triplicate analyses) and absolute differences (duplicate analyses) were typically <1‰ and always <2‰ for both δ¹⁸O and δ¹⁵N. The deconvolution algorithm described by (Kaiser, 2002) was used to correct for isotopologue contributions to masses 45 and 46. Diluted aliquots of the N₂O standard were analyzed alternately with samples to account for system fractionation effects. These effects ranged from approximately 0 to +2.5‰ for δ¹⁵N and 0 to +1‰ for δ¹⁸O. Sample values were corrected by subtracting the average daily fractionation effects from the measured values. The 8-3-03 field samples were initially measured against a lower purity N₂O standard which was later calibrated against the high purity N₂O standard.

All δ¹⁸O values are reported with reference to the Vienna Standard Mean Ocean Water (VSMOW) and δ¹⁵N values are referenced to N₂ in air.

3. Results

3.1. Methanotrophic cultures

Table 1 summarizes the parameters of the incubation experiments with *M. methanica* and *M. trichosporium*. Based on the high cell counts of $\sim 2 \times 10^8$ cells mL⁻¹ for experiments 1–3, and the lack of significant growth during the 1 day incubation, it appears the bacteria were near the stationary phase of growth. Substantial concentrations of N₂O were produced in the headspace of all experiments except 12 and 13. Concentrations of N₂O in these experiments were too small for accurate isotopic measurements. Molecular oxygen consumption and CO₂ production occurred during all of the incubations indicating that the methanotroph cultures were active. For most experiments, CH₄

consumption was the dominant metabolic process. However, for some experiments (e.g., 4 and 12) the amounts of oxygen consumed and CO₂ produced cannot be explained by methane oxidation alone. An aliquot of the methanotrophic media from these experiments was streaked onto plates of standard nutrient agar. Lack of bacterial growth on these plates confirmed that contamination by heterotrophic bacteria was not the cause of CO₂ production (data not shown). It is possible that intermediates of methane oxidation, or other intracellular energy stores, were also being metabolized during these low methane experiments. We have observed on several occasions the continued production of CO₂ for several hours by cultures of *M. trichosporium* after initial growth on methane and after the methane headspace gas has been completely purged (data not shown).

3.2. Methanotrophic cultures, N₂O–NH₄

The N₂O produced by *M. methanica* in experiment 1 and *M. trichosporium* in experiments 2 and 3 was depleted in ¹⁵N relative to the NH₄⁺ substrate. The change in δ¹⁵N of the NH₄⁺ throughout the 1 day incubation was small or negligible, permitting a straightforward calculation of the nitrogen isotope enrichment factor based on the measured differences between δ¹⁵N_{NH₄⁺ and δ¹⁵N_{N₂O}. An approximation of ε¹⁵N_{N₂O–NH₄⁺, calculated in this paper as δ¹⁵N_{N₂O}–δ¹⁵N_{NH₄⁺, from the three carboy experiments ranged from –48 to –56‰. Although the δ¹⁵N_{NH₄⁺ values were not measured for experiments 4–11, the same nitrogen salts were used for all experiments and measured δ¹⁵N_{N₂O} values were similar to those for experiments 1–3 (Table 1).}}}}

3.3. Methanotrophic cultures, N₂O–H₂O/O₂

Similar to δ¹⁵N_{N₂O} values, the isotopic composition of N₂O oxygen is controlled by both the isotopic composition of the source oxygen and any net fractionation associated with biochemical reactions. However, unlike N₂O–nitrogen, δ¹⁸O values of N₂O were more scattered, which likely results from having two potential oxygen sources: H₂O and O₂. The δ¹⁸O value of N₂O produced during co-oxidation by methanotrophs can be written as a mass balance of the δ¹⁸O values of the source H₂O and O₂ and their associated fractionations (Sessions and Hayes, 2005).

$${}^{18}R_{N_2O} = F_{H_2O} \alpha_{N_2O-H_2O} {}^{18}R_{H_2O} + (1-F_{H_2O}) \alpha_{N_2O-O_2} {}^{18}R_{O_2} \quad (1)$$

Where ¹⁸R_{N₂O} is the ¹⁸O/¹⁶O ratio in N₂O, F_{H₂O} is the fraction of N₂O oxygen that is derived from water and α is the isotopic fractionation factor.

$$\alpha_{N_2O-O_2} = \frac{{}^{18}R_{N_2O}}{{}^{18}R_{O_2}} \quad (2)$$

Eq (1) can be rewritten as,

$$\delta^{18}O_{N_2O} = F_{H_2O} (\alpha_{N_2O-H_2O} \delta^{18}O_{H_2O} + \epsilon_{N_2O-H_2O}) + (1-F_{H_2O}) (\alpha_{N_2O-O_2} \delta^{18}O_{O_2} + \epsilon_{N_2O-O_2}) \quad (3)$$

where

$$\delta^{18}O_X = \frac{{}^{18}R_X}{{}^{18}R_{std}} - 1 \quad (4)$$

and

$$\epsilon \equiv \alpha - 1 \quad (5)$$

Note that factors of 1000 are not used for simplification (Sessions and Hayes, 2005).

In the case where the only experimental variable is the $\delta^{18}\text{O}$ value of water, the slope of a linear regression of $\delta^{18}\text{O}_{\text{N}_2\text{O}}$ on $\delta^{18}\text{O}_{\text{H}_2\text{O}}$ will approximate $F_{\text{H}_2\text{O}}$ as long as $\alpha_{\text{N}_2\text{O}-\text{H}_2\text{O}}$ is close to unity (Fig. 1).

$$\delta^{18}\text{O}_{\text{N}_2\text{O}} = F_{\text{H}_2\text{O}}\alpha_{\text{N}_2\text{O}-\text{H}_2\text{O}}\delta^{18}\text{O}_{\text{H}_2\text{O}} + (1-F_{\text{H}_2\text{O}})(\alpha_{\text{N}_2\text{O}-\text{O}_2}\delta^{18}\text{O}_{\text{O}_2} + \epsilon_{\text{N}_2\text{O}-\text{O}_2}) + F_{\text{H}_2\text{O}}\epsilon_{\text{N}_2\text{O}-\text{H}_2\text{O}} \quad (6)$$

The slope of the regression for all data points is 0.69 ± 0.20 ($p < 0.001$) and the correlation is not very strong ($R^2 = 0.59$; regression not shown in Fig. 1). The scatter in the data can be explained by an additional experimental variable, namely, varying $\delta^{18}\text{O}_{\text{O}_2}$ values in the experiments due to isotopic fractionation effects associated with the consumption of O_2 by methanotrophs. In order to assess the dynamics of $^{18}\text{R}_{\text{O}_2}$ in our closed system incubations, we measured the ^{18}R of headspace O_2 at several time points during experiments with high and low methane concentrations (Table 2, Fig. 2). The observed ^{18}O enrichment in $^{18}\text{R}_{\text{O}_2}$ is described by the Rayleigh distillation equation (Scott et al., 2004).

$$\frac{^{18}\text{R}_t}{^{18}\text{R}_0} = \left(\frac{N_t}{N_0}\right)^\epsilon \quad (7)$$

Where $^{18}\text{R}_0$ and $^{18}\text{R}_t$ are the $^{18}\text{O}:^{16}\text{O}$ ratios of O_2 at time zero and time t , respectively; N_0 and N_t are the initial and residual number of moles of O_2 , respectively, and ϵ is the isotope fractionation effect for O_2 consumption by methane oxidation. $\epsilon = (\alpha - 1)$ where

$$\alpha = \frac{^{18}\text{R}_p}{^{18}\text{R}_r} \quad (8)$$

An estimate of ϵ was obtained by a linear regression of the natural log transformed data (Fig. 3) (Scott et al., 2004).

$$\ln(^{18}\text{R}_t) = \epsilon \ln(N_t) + \ln\left(\frac{^{18}\text{R}_0}{N_0^\epsilon}\right) \quad (9)$$

The slope of the line provides direct estimates of ϵ for total O_2 consumption by the methanotrophic cultures, yielding values of

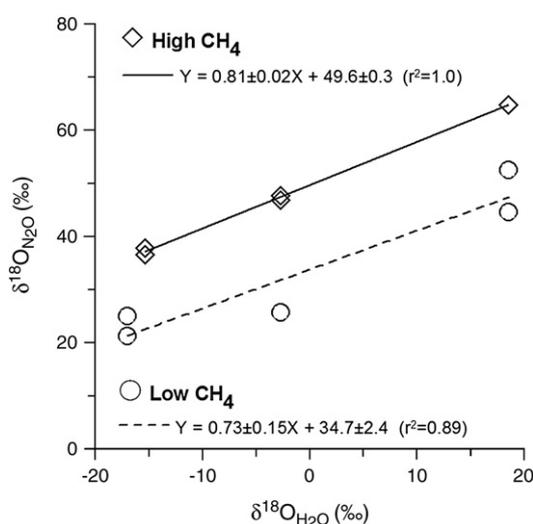


Fig. 1. $\delta^{18}\text{O}_{\text{N}_2\text{O}}$ from various incubations versus the $\delta^{18}\text{O}$ values of the incubation water. When the data are assigned to two groups for high (diamonds) and low (circles) methane experiments the resulting linear regressions with the associated standard errors are $Y = 0.81 \pm 0.02X + 49.6 \pm 0.3$; $r^2 = 1.00$ and $Y = 0.73 \pm 0.15X + 33.7 \pm 2.4$; $r^2 = 0.89$, respectively. By comparison, the regression for the combined data from both groups of experiments is $Y = 0.69X + 41.5$; $r^2 = 0.59$.

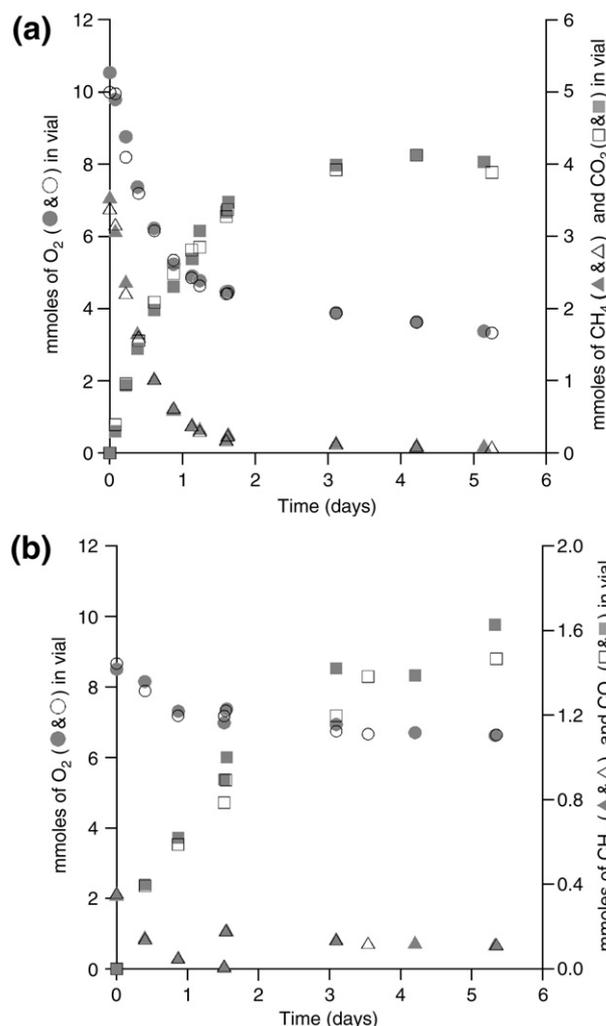


Fig. 2. Change in O_2 (circles), CH_4 (triangles), and CO_2 (squares) in vials incubated over time at high (a, experiments 10 and 11) and low (b, experiments 12 and 13) methane concentrations. Duplicate experiments are represented by open and closed symbols. Note a small amount of additional methane was added at ~ 1.5 days. Values were calculated from measured changes in concentrations of gasses in headspace, headspace pressure, Henry's constants, equilibrium constants for $\text{H}_2\text{CO}_3/\text{HCO}_3^-$ and $\text{HCO}_3^-/\text{CO}_3^{2-}$ (Stumm and Morgan, 1996) and the pH of the culture in each vial (6.8 to 7.1).

$-16.1 \pm 2.0\%$ and $-17.5 \pm 5.4\%$ (95% CI) for the high and low methane consumption experiments, respectively (Scott et al., 2004). These ϵ values, and the measured concentrations of O_2 at the end of each incubation were used to estimate the $\delta^{18}\text{O}$ values of the residual O_2 at the end of each experiment for those experiments in which $\delta^{18}\text{O}_{\text{O}_2}$ values were not directly measured (Table 1). Because both $\delta^{18}\text{O}_{\text{H}_2\text{O}}$ and $\delta^{18}\text{O}_{\text{O}_2}$ values varied across experiments, a dual regression based on Eq. (3) is more appropriate. However, N_2O was produced during co-oxidation of NH_4^+ with CH_4 and the $\delta^{18}\text{O}_{\text{O}_2}$ values at the end of the experiment represent a maximum value for the isotopic composition of O_2 from which N_2O oxygen may have been derived. Therefore, the calculated $\delta^{18}\text{O}_{\text{O}_2}$ values cannot be considered exact. Instead the $\delta^{18}\text{O}_{\text{O}_2}$ values were used to place the data into two groups, generally corresponding to the high and low methane experiments, with more similar isotopic compositions of O_2 during N_2O production (Table 1). The linear regressions of $\delta^{18}\text{O}_{\text{N}_2\text{O}}$ on $\delta^{18}\text{O}_{\text{H}_2\text{O}}$ for the separate data groups are shown in Fig. 1. The slope of the regressions for the two data groups are 0.81 ± 0.02 ($p < 0.001$, $r^2 = 1.00$) and 0.73 ± 0.15 ($p < 0.02$, $r^2 = 0.89$). Both correlations are much better than for the combined data indicating that grouping the data removed much of the scatter due to variable $\delta^{18}\text{O}_{\text{O}_2}$ values (Fig. 1).

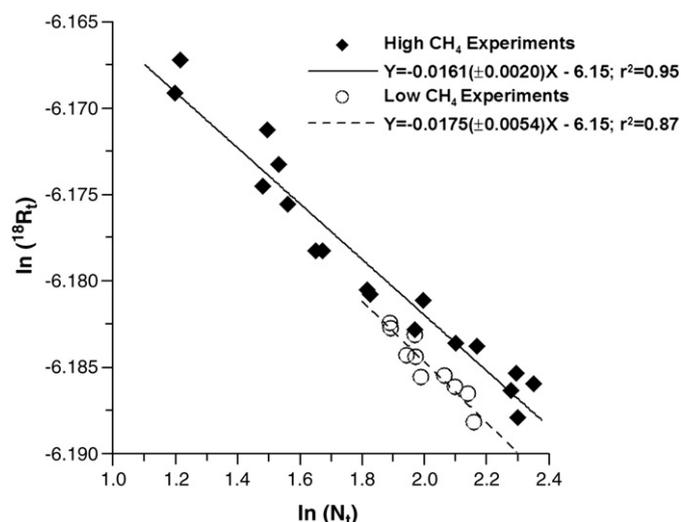


Fig. 3. Rayleigh plots for O_2 consumption showing change in the natural log of $^{18}R_{O_2}$ relative to the number of moles of O_2 remaining (N_i) in the high (experiments 10 and 11) and low (experiments 12 and 13) methane incubation experiments shown in Fig. 2. The slope ($\pm 95\%$ confidence interval) of the linear regressions provides an estimate of the kinetic isotope fractionation effect for O_2 consumption during methane oxidation (Eq. (9)).

For a given $\delta^{18}O_{H_2O}$, the $\delta^{18}O_{N_2O}$ values from the high methane experiments are $\sim 15\%$ more positive than those measured from the low methane experiments (Fig. 1). These more positive values likely reflect, in part, contributions from O_2 that has been isotopically enriched in ^{18}O due to fractionation effects associated with greater O_2 consumption (Fig. 3). The average estimated $\delta^{18}O_{O_2}$ values at the end of the high- CH_4 experiments ($39 \pm 6\%$) was $12(\pm 8)\%$ more positive than the average estimated value at the end of the low- CH_4 experiments ($27 \pm 2\%$). Given this 12% difference, the Rayleigh fractionation effect associated with O_2 consumption does not appear to fully explain the full range of $\delta^{18}O_{N_2O}$ values between the high and low methane experiments. Other factors that could have contributed to the higher $\delta^{18}O_{N_2O}$ in the high CH_4 experiments are 1) the $\delta^{18}O_{O_2}$ values between high and low CH_4 experiments was actually much larger in the vicinity of the cells than was estimated for the overall system, and/or 2) the metabolic state of the methanotrophs, perhaps due to different O_2 concentrations or relative oxidation rates, plays a role in oxygen isotope fractionation effects associated with N_2O production.

The single $\delta^{18}O_{N_2O}$ measurement for *M. methanica* (experiment 1) does not agree with the $\delta^{18}O_{N_2O}$ produced by *M. trichosporium* under similar conditions (experiments 2 and 3). This suggests that the proportions of its oxygen sources and/or oxygen isotopic fractionation effects associated with biochemical pathways are substantially different than those of *M. trichosporium*.

3.4. Field study of methane-consuming soils

The concentration profiles of soil gas N_2O sampled in Aug. 2003 and May 2004 are shown in Fig. 4. In Aug. 2003, the depth (80 cm) of maximum N_2O concentration corresponded with the apparent depth of methane oxidation as evidenced by soil gas profiles of O_2 , CH_4 , and CO_2 (Fig. 4). This suggests that the N_2O observed in Aug. 2003 was produced by co-oxidation of NH_4^+ by methanotrophic bacteria. Additionally, substantial concentrations of diagnostic phospholipid fatty acid (PLFA) biomarkers diagnostic of type II (18:1 ω 8) and type I (16:1 ω 8) methanotrophs were detected throughout the top ~ 1.8 m of these soils at three different times between April 2002 and May 2004 (Mills, 2007). The maximum concentration of N_2O in May 2004 was detected at 100 cm depth. Although the wetter conditions in May 2004 might

suggest that N_2O at this time is derived from denitrification, the expected concomitant decrease in NO_3^- concentrations with depth was not observed (Fig. 4). For the 102 to 122 cm soil interval ~ 5 and 20% of total PLFAs could be attributed to type II and type I methanotrophs, respectively. Therefore, the N_2O maximum observed during the May 2004 collection could have been produced by methanotrophs.

The $\delta^{18}O_{N_2O}$ values for the samples collected in May 2004, when soil gas CH_4 concentrations were much higher, are approximately 20% more positive than the values for the two samples collected in Aug. 2003 (Table 3). If the N_2O was produced predominantly by co-oxidation of NH_4^+ by methanotrophs, these field results parallel those from the laboratory incubations, with more positive $\delta^{18}O_{N_2O}$ values observed at higher concentrations of soil gas CH_4 . Although there was variation in the $\delta^{15}N_{N_2O}$ values for all samples (Table 3), these did not correlate with sampling date or CH_4 concentration.

4. Discussion

4.1. Nitrogen isotope fractionation during methanotrophic oxidation of NH_4^+ to N_2O

Because methanotrophs have previously been shown to fractionate carbon isotopes during co-oxidation of chlorinated solvents (Brungard et al., 2003), the negative nitrogen isotope fractionation effects ($\epsilon_{N_2O-NH_4^+}$) of ~ -48 to -56% that we observed for the co-oxidation of NH_4^+ to N_2O by methanotrophs was somewhat expected (Table 1). However, these large effects are similar to values of ~ -47 to -68% previously measured for pure cultures of *Nitrosomonas europaea*, an autotrophic nitrifying bacteria (Yoshida, 1988; Sutka et al., 2006). This similarity suggests that $\delta^{15}N_{N_2O}$ cannot be used as a tool to discriminate the relative activities of each bacterial group for mediating nitrification and N_2O formation in environmental systems, as proposed by Mandernack et al. (2000b). Although Sutka et al. (2006) used NH_2OH rather than NH_4^+ as a substrate for investigating isotopomeric site preferences during nitrification by methanotrophs, their results are consistent with ours and suggest that there is no

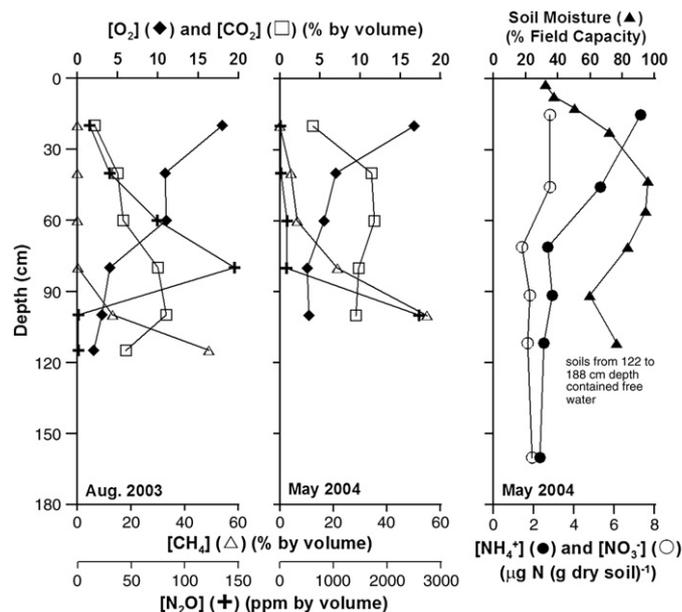


Fig. 4. Depth profiles of soil moisture and concentrations of soil gases (N_2O , CH_4 , O_2 , CO_2), NH_4^+ and NO_3^- measured above a thermogenic methane seep in Southwest Colorado during times which N_2O was collected for isotopic analyses. Although soil moistures were not measured in Aug. 2003, field observations indicated that soils were drier than in May 2004. Soil gas samples from the 115 cm gas probe could not be obtained in May 2004 due to reduced gas permeability in the wetter soil at this depth.

Table 3

Soil gas $\delta^{15}\text{N}_{\text{N}_2\text{O}}$ and $\delta^{18}\text{O}_{\text{N}_2\text{O}}$ values and concentrations of N_2O , CH_4 , O_2 , and CO_2 above a coal-bed methane seep in Southwest Colorado.

Sample date	Depth (cm)	$\delta^{15}\text{N}_{\text{N}_2\text{O}}$ (‰ Air-N ₂)	$\delta^{18}\text{O}_{\text{N}_2\text{O}}$ (‰ VSMOW)	Soil gas concentrations (by volume)			
				N ₂ O (ppm)	CH ₄ (%)	O ₂ (%)	CO ₂ (%)
8-03	60	-8.7	28.5	74	0.0004	14.1	5.7
	80	-7.8	26.3	181	0.022	8.7	9.7
5-04	60	-0.6	46.9	134	6.6	5.4	11.8
	80	-13.1	49.9	128	21.5	3.3	9.8
	100	3.0 (2.2) ^a	47.5 (46.1) ¹	2630	55.3	3.5	8.9

^a Values are for a duplicate sample collected in a 150 mL stainless steel gas sampling bottle.

difference in site preference for nitrification by methanotrophs and autotrophic nitrifiers.

4.2. N₂O formation – oxidation of hydroxylamine versus reduction of nitrite

The kinetic N isotope fractionation for the production of N₂O during the co-oxidation of ammonia by methanotrophs does provide information on the formation pathway. Nitrifiers can produce N₂O by two major pathways: the direct oxidation of NH₂OH and the reduction of NO₂⁻ (Poth and Focht, 1985; Hooper et al., 1990). The latter process, which has been termed “nitrifier denitrification” (Goreau et al., 1980; Poth and Focht, 1985; Webster and Hopkins, 1996; Wrage et al., 2001), is favored under lower O₂ concentrations (Goreau et al., 1980; Yoshinari, 1985; Yoshida, 1988), and is likely responsible for the majority of N₂O formed by nitrification (Poth and Focht, 1985; Hooper et al., 1990). As originally

proposed by Yoshida (1988), the very negative $\delta^{15}\text{N}$ values of N₂O produced during nitrification probably result from cumulative N isotope fractionation effects imparted by AMO (or MMO) and other enzymes that participate later in the N₂O formation pathway. Recent data showing smaller N isotope fractionation effects during the conversion of NH₄⁺ to NO₂⁻ ($\epsilon_{\text{NH}_4^+ \rightarrow \text{NO}_2^-}$) than to N₂O ($\epsilon_{\text{NH}_4^+ \rightarrow \text{N}_2\text{O}}$) (Casciotti et al., 2003) provides evidence that enzymes downstream from monooxygenases also are important in the net N isotope fractionation observed for N₂O formation. Sutka et al. (2003, 2006) directly measured negative fractionations ($\Delta^{15}\text{N}_{\text{N}_2\text{O}-\text{NO}_2^-} = -36\text{‰}$ and -24‰) for the process mediated by these downstream enzymes during nitrifier denitrification by *N. europaea* and *N. multiformis*, respectively. The very negative $\epsilon_{\text{N}_2\text{O}-\text{NH}_4^+}$ values that apparently result from isotope fractionation effects expressed by multiple enzymes are consistent with similarly negative net fractionation observed for the oxidation of CH₄ by methanotrophs. Jahnke et al. (1999) determined that these negative fractionation effects result from both MMO and methanol dehydrogenase enzymes.

In contrast to the very negative fractionation associated with the conversion of NO₂⁻ to N₂O, a small positive fractionation appears to be associated with the oxidation of hydroxylamine to N₂O ($\epsilon_{\text{N}_2\text{O}-\text{NH}_2\text{OH}}$). Sutka et al. (2003, 2006) reported $\epsilon_{\text{N}_2\text{O}-\text{NH}_2\text{OH}}$ values of $\sim +2.3\text{‰}$ and $+5.7\text{‰}$ for *M. capsulatus* and *M. trichosporium*, respectively. A similar $\epsilon_{\text{N}_2\text{O}-\text{NH}_2\text{OH}}$ value ($+2.0\text{‰}$) was reported for *N. europaea* and *N. multiformis* (Sutka et al., 2006), although a value of -26.0‰ has also been reported for *N. europaea* by the same authors (Sutka et al., 2003). Thus the very negative $\delta^{15}\text{N}_{\text{N}_2\text{O}}$ values that we measured in this study suggest that N₂O formation by methanotrophs occurred by the reduction of NO₂⁻. Future investigations using NO₂⁻ as a starting substrate for N₂O formation by methanotrophic pure cultures might help to further test

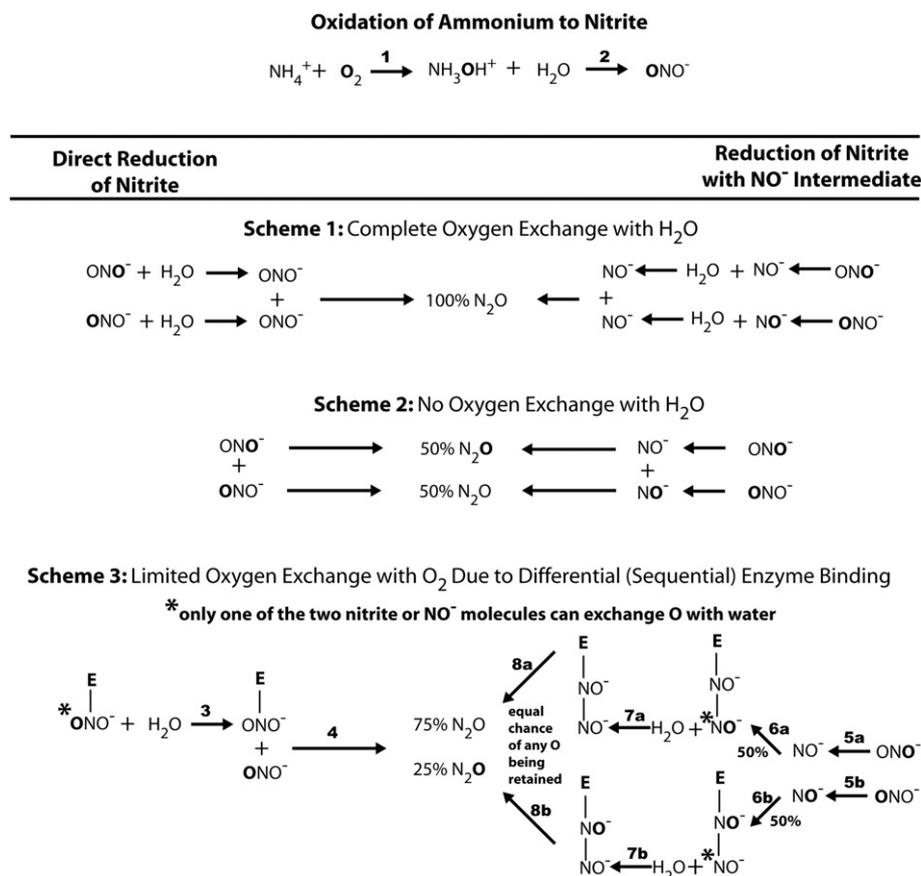


Fig. 5. Previously-proposed reaction schemes for the formation of N₂O during nitrifier denitrification (Anderson et al., 1982; Poth and Focht, 1985; Hooper et al., 1990; Stein and Yung, 2003; Schmidt et al., 2004). Some steps shown here may be in common with N₂O formation by denitrifying bacteria (Garber and Hollocher, 1982; Weeg-Aerssens et al., 1988; Ye et al., 1991). Scheme 3 provides a possible pathway that could explain 25% O₂ incorporation into N₂O during its formation by bacterial NH₄⁺ oxidation either by direct reduction of nitrite or through an NO intermediate during differential (sequential) enzyme binding of NO₂⁻ (see text).

this, although nitrite can impose toxicity effects on methanotrophs (King and Schnell, 1994; Schnell and King, 1994).

The observed correlation between measured $\delta^{18}\text{O}_{\text{N}_2\text{O}}$ values and $\delta^{18}\text{O}_{\text{H}_2\text{O}}$ of the medium water in our incubations are also consistent with N_2O formation by reduction of NO_2^- (Fig. 1). The slope of the regression of $\delta^{18}\text{O}_{\text{N}_2\text{O}}$ on $\delta^{18}\text{O}_{\text{H}_2\text{O}}$ is an estimate of $\alpha_{\text{H}_2\text{O}} \times F_{\text{H}_2\text{O}}$. Although $\alpha_{\text{H}_2\text{O}}$ is not known, our experiments indicate that it is substantially greater than unity. Assuming that $1.00 < \alpha_{\text{H}_2\text{O}} < 1.060$ and incorporating the standard error of the slopes (Fig. 1), estimates of $F_{\text{H}_2\text{O}}$ can be constrained from 0.75 to 0.83 and 0.55 to 0.88 for the high and low CH_4 experiments, respectively. The wider range for the latter is due to larger standard errors in the slope that likely resulted from greater experimental variability, such as the $\delta^{18}\text{O}_{\text{O}_2}$ values at the time of N_2O production. Consequently, the slopes of the regressions in Fig. 1 suggest a stoichiometric ratio for the incorporation of O_2 versus water oxygen of approximately 1:3.

Anderson et al. (1982) showed that one of the oxygen atoms in NO_2^- produced by *Nitrosomonas* is derived from molecular O_2 and is acquired during the initial oxidation of NH_4^+ to NH_2OH . If N_2O in our experiments were formed by the direct oxidation of NH_2OH , we would expect a stoichiometric ratio of 1:1. However, our results are more consistent with $^{15}\text{N}_{\text{NH}_4}$ and $^{18}\text{O}_{\text{H}_2\text{O}}$ labeling studies of N_2O formation by *N. europaea* which showed that only 22 to 31% of the N_2O was derived from O_2 (Hooper et al., 1990). The results of Hooper et al. (1990) indicated that this N_2O was formed by reduction of NO_2^- rather than by oxidation of NH_2OH . Regardless of the exact mechanism for the incorporation of oxygen from O_2 and water, our $\delta^{18}\text{O}_{\text{N}_2\text{O}}$ values are in agreement with N_2O formation by NO_2^- reduction in *M. trichosporium* OB3b. In particular, the high methane experiments, which were associated with higher O_2 consumption and lower O_2 concentrations (Fig. 2), might be expected to favor N_2O production by reduction of nitrite as previous studies have reported (Goreau et al., 1980; Yoshinari, 1985; Yoshida, 1988).

4.3. Comparison of methanotrophic nitrification with previous proposed mechanisms of N_2O formation

The exact mechanism by which N_2O is produced from the reduction of nitrite during nitrification is not known. There are several possible mechanisms that could result in the non-stoichiometric incorporation of H_2O -oxygen into N_2O , such as by isotopic exchange between H_2O and NH_2OH and/or NO_2^- intermediates (Anderson et al., 1982; Ye et al., 1991; Casciotti et al., 2007) or O_2 -oxygen through the production of metabolic water (Kreuzer-Martin et al., 2005). Although we cannot exclude any of these possibilities, the 1:3 O_2 : H_2O oxygen stoichiometric ratio suggested by our results and those of Hooper et al. (1990) provide evidence for another possible alternative, the sequential reduction of two nitrite molecules as discussed below.

Similar to the AMO of nitrifiers (Anderson et al., 1982), after the initial formation of NO_2^- , it is expected that 50% of the oxygen in each nitrite molecule is derived from O_2 due to MMO (steps 1 and 2, Fig. 5). The fate of this O_2 -derived oxygen during the reduction of two nitrite molecules to N_2O likely depends on the reaction pathway. While scheme 3 presented in Fig. 5 is consistent with our observed 1:3 ratio, schemes 1 and 2 are not. This is the case whether the reduction of two nitrite molecules occurs directly as has been shown to occur in some strains of denitrifying bacteria (Ye et al., 1991) or through an NO^- intermediate as previously suggested (Stein and Yung, 2003; Schmidt et al., 2004). While Kool et al. (2007, 2009) have suggested that in soil environments N_2O generally reflects complete oxygen isotopic exchange with water (scheme 1), irrespective of its formation by either nitrification or denitrification, this is clearly not the case for our experiments.

Schemes 2 and 3 illustrate two previously proposed pathways (parallel and sequential, respectively) for the reduction of NO_2^- to N_2O via NO intermediates (Garber and Hollocher, 1982; Shearer and Kohl, 1988; Weeg-Aerssens et al., 1988). In each mechanism nitrite is initially

converted to a nitrosyl (NO^+) intermediate. However, the parallel mechanism, also referred as the “simultaneous” pathway (Stein and Yung, 2003), favors the formation of two independent free or enzymatically bound nitroxyl (E-NO^-) intermediates which can then combine to an N_2O molecule (Garber and Hollocher, 1982). The parallel mechanism appears to result in the binding and reduction of each nitrite molecule in an identical manner. Assuming there was no oxygen isotope exchange with water, this mechanism, as simplified in scheme 2 of Fig. 5, would seemingly favor the incorporation of 50% of the oxygen in N_2O from O_2 .

In contrast, the sequential mechanism (scheme 3, Fig. 5) proposes that, after the first nitrite is reduced to the nitroxyl intermediate, a second nitrite reacts differently by nucleophilic attack on the E-NO^- intermediate to form an $\text{E-N}_2\text{O}_3$ intermediate prior to N_2O formation (Shearer and Kohl, 1988; Weeg-Aerssens et al., 1988). Because the nitrite molecules have different fates during the sequential mechanism, it is feasible that one nitrite experiences complete oxygen isotopic exchange with water and thus carries no O_2 signal, while the other nitrite molecule retains most of its isotopic imprint from O_2 . Therefore, the sequential mechanism could theoretically favor a dissolved oxygen signal in the N_2O product that is close to 25%, or less if isotopic exchange with water also occurs. This might explain, in part, the range of 18 to 27% of O_2 -derived oxygen observed in our experiments and those of Hooper et al. (1990). This mechanism is also supported by the results of Sutka et al. (2006), that showed a clear site preference of $\sim +30\%$ for N_2O formed by autotrophic nitrifiers and methanotrophs as predicted by Stein and Yung (2003) for the sequential pathway. Additional biochemical studies are needed to accurately determine the exact pathways associated with oxygen incorporation into methanotrophically-produced N_2O . Such studies might also include ^{18}O -labeled water to determine the extent of isotopic exchange with water as recently proposed (Kool et al., 2007).

4.4. Oxygen isotope fractionation of H_2O and O_2 during N_2O formation

The Rayleigh plots in Fig. 3 show that molecular oxygen is isotopically fractionated during its consumption by methanotrophs. The fractionation effects of -16.1 and -17.5% that we observed are a result of the combined effects of O_2 consumption by MMO and terminal oxidase enzymes. However, the similarity between our results and fractionation effects previously reported for both MMO activity (-15.2 to -16.7% , Stahl et al., 2001) and dark respiration by unicellular prokaryotes and eukaryotes (-14 to -26% ; Guy et al., 1992, 1993; Stahl et al., 2001) suggests that the effects for both of these enzymatic processes are similar.

Although we cannot exactly determine $\alpha_{\text{N}_2\text{O-H}_2\text{O}}$ and $\alpha_{\text{N}_2\text{O-O}_2}$ from our data, it can be used to constrain relationships between the two fractionation effects (Sessions and Hayes, 2005). To this end, Eq. (1) was solved for $\alpha_{\text{H}_2\text{O}}$ and the data from each experiment were used for the parameters $^{18}R_{\text{N}_2\text{O}}$, $^{18}R_{\text{H}_2\text{O}}$, and $^{18}R_{\text{O}_2}$. A range of hypothetical values were used for $F_{\text{H}_2\text{O}}$ and $\alpha_{\text{N}_2\text{O-O}_2}$. Table 4 shows example results of these calculations with $F_{\text{H}_2\text{O}} = 0.75$ and $\alpha_{\text{N}_2\text{O-O}_2} = 0.983$. The calculated $\alpha_{\text{N}_2\text{O-H}_2\text{O}}$ values produced from each set of data are similar when compared within high and low methane groupings. When O_2 oxygen is incorporated into hydroxylamine by MMO it should undergo an initial fractionation effect of approximately -17% ($\alpha_{\text{N}_2\text{O-O}_2} = 0.983$; Fig. 3). For a $F_{\text{H}_2\text{O}}$ value of 0.75, this corresponds to very large $\alpha_{\text{N}_2\text{O-H}_2\text{O}}$ values of 1.059 and 1.042 for the high and low methane data sets, respectively. Fig. 6a and b show average $\alpha_{\text{N}_2\text{O-H}_2\text{O}}$ values calculated for each data set for a variety of hypothetical $F_{\text{H}_2\text{O}}$ values (x axis) and $\alpha_{\text{N}_2\text{O-O}_2}$ values (isobars).

Once O_2 oxygen is incorporated into nitrite it is subject to the same fractionation effects as nitrite oxygen that is derived from H_2O . Casciotti et al. (2007) have determined a positive oxygen isotope fractionation for the conversion of nitrite to N_2O with ^{18}O preferentially transferred to N_2O . Both H_2O and O_2 derived oxygen should undergo this same effect,

Table 4
Example of $\alpha_{N_2O-H_2O}$ values calculated for $F_{H_2O} = 0.75$ and $\alpha_{N_2O-O_2} = 0.983$.

Group 1 (high CH ₄)							
Exp #	7	8	9	10	11	Average	Std. dev.
$\alpha_{N_2O-H_2O}$	1.060	1.061	1.060	1.056	1.058	1.059	0.002
Group 2 (low CH ₄)							
Exp #	2	3	4	5	6	Average	Std. dev.
$\alpha_{N_2O-H_2O}$	1.042	1.048	1.037	1.048	1.034	1.042	0.006

but the net fractionation for O₂ derived oxygen should be less due to the negative fractionation effect associated with MMO. Thus both $\alpha_{N_2O-H_2O}$ and $\alpha_{N_2O-O_2}$ could be greater than 1, with the former larger than the latter. A very simplistic model can be used to solve for $\alpha_{N_2O-NO_2^-}$ values that satisfy both this scenario and our data. The model does not take into account fractionation effects associated with steps other than those mediated by MMO and nitrite reductase.

The constraints of the model are:

$$\alpha_{N_2O-H_2O} = \alpha_{N_2O-NO_2^-}$$

and

$$\alpha_{N_2O-O_2} = (\alpha_{N_2O-NO_2^-})(\alpha_{NH_2OH-O_2})$$

For a F_{H_2O} value of 0.75 the solutions are $\alpha_{N_2O-O_2} = 1.026$ and $\alpha_{N_2O-H_2O} = 1.044$ for the high methane data (Fig. 6a) and $\alpha_{N_2O-O_2} = 1.013$ and $\alpha_{N_2O-H_2O} = 1.031$ for the low methane data (Fig. 6b). Although the model is simplistic, the solutions show a very large fractionation effect at some step(s) in the production of N₂O is necessary to explain our data, and the reduction of nitrite to N₂O is one likely step. It also shows that if F_{H_2O} is the same for both group 1 and 2 data sets, the net oxygen isotope fractionation was substantially smaller in the low methane experiments. Therefore, the rate of methane oxidation could impact the $\delta^{18}O$ of N₂O produced by methanotroph-mediated nitrification in two ways: increasing the $\delta^{18}O$ value of the O₂ source through Rayleigh distillation and affecting the internal kinetic isotope fractionation associated with N₂O formation. Both of these influences need to be more carefully considered.

Very positive $\delta^{18}O_{N_2O}$ values previously measured from soils were presumed to reflect a relatively large contribution from atmospheric O₂ having a $\delta^{18}O$ value of +23.5‰, and thus to be derived from nitrification (Pérez et al., 2001). However, because denitrification involves the same enzymatic steps as nitrification during the conversion of nitrite to N₂O, internal kinetic isotope fractionation might similarly produce N₂O with high $\delta^{18}O$ values. For example, $\delta^{18}O_{N_2O}$ values measured from two cultures of denitrifying bacteria were enriched up to ~65‰ relative to the water, and ~40‰ relative to the nitrate oxygen (Toyoda et al., 2005). Furthermore, nitrate amended soils produced enriched N₂O with $\delta^{18}O$ values exceeding +50‰, while NH₄⁺-amended soils resulted in $\delta^{18}O$ values as high as +80‰ (Wrage et al., 2004). Although nitrification could result in the most highly enriched $\delta^{18}O_{N_2O}$ values due to incorporation of O₂ and internal isotope fractionation, there is likely to be overlap of $\delta^{18}O_{N_2O}$ values produced from nitrification and denitrification from the same ecosystem.

4.5. $\delta^{18}O_{N_2O}$ and $\delta^{15}N_{N_2O}$ values for soils overlying a thermogenic CH₄ seep

The results of our laboratory experiments suggest that, in environments where methanotrophs mediate N₂O formation by co-oxidation of ammonia, consideration of CH₄ dynamics is important for interpreting the $\delta^{18}O_{N_2O}$ values. It is feasible that high $\delta^{18}O_{N_2O}$ values, which were sometimes more positive than tropospheric N₂O, previously reported by Mandernack et al. (2000b) for landfill soil N₂O emissions may have formed under similar conditions as the high methane experiments

(Fig. 1). The $\delta^{18}O_{N_2O}$ values that we measured for gases collected from soils overlying a coal bed methane seep (Table 3) are consistent with our laboratory results. The less positive $\delta^{18}O_{N_2O}$ values for the August 2003 samples correspond to relatively low CH₄ and intermediate oxygen concentrations in soil gas while the more positive $\delta^{18}O_{N_2O}$ values for the May 2004 samples correspond to high CH₄ and low oxygen concentrations (Table 3).

Although soil gas profiles and wetter soil conditions observed in May 2004 (Fig. 4) suggest that the ¹⁸O-enriched N₂O in these soils could also have resulted from denitrification, soil nitrate concentrations did not decrease in the deeper soils (~90–100 cm) where a drop in soil moisture was also observed. Methanotrophs are often found in microaerophilic environments and have extremely high affinities for O₂ (Ren et al., 1997), thus it is reasonable to attribute the methanotroph biomarker PLFAs detected at the depth of the N₂O maximum in May 2004 (Mills, 2007) to metabolically active methanotrophs. Given that methanotrophs and autotrophic nitrifiers have both been observed to produce elevated amounts of N₂O under low O₂ conditions (Goreau et al., 1980; Yoshinari, 1985; Yoshida, 1988), it is feasible that methanotrophs produced even higher amounts of N₂O under the reduced oxygen conditions at 100 cm during May 2004, resulting in the particularly high concentrations of ¹⁸O-enriched N₂O. Irrespective of the microbial pathway of N₂O production at this time, $\delta^{15}N_{N_2O}$ values that change with soil depth

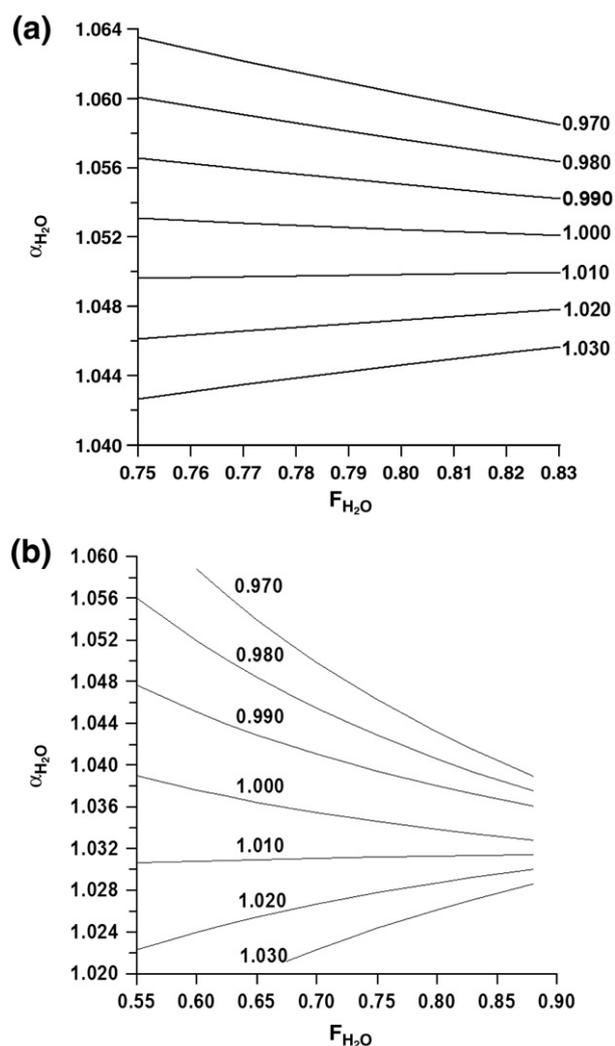


Fig. 6. Average calculated α_{H_2O} values as a function of F_{H_2O} and α_{O_2} values for high (a) and low (b) methane data sets. Various hypothetical α_{O_2} values are represented by isobars.

while $\delta^{18}\text{O}_{\text{N}_2\text{O}}$ remained relatively constant, suggests that microbial processes that affect the isotopic values of N_2O are active throughout the soil column.

From the $\delta^{18}\text{O}_{\text{N}_2\text{O}}$ values we measured at the Pine River methane seep it is possible that other processes such as nitrification by nitrifiers, denitrification, or N_2O reduction may have also contributed to N_2O formation (Mandernack et al., 2000b; Ostrom et al., 2007). However, it does pose intriguing questions about the control of methane dynamics on the $\delta^{18}\text{O}$ of N_2O produced in thermogenic methane seeps and other high methane environments. Considering that soil seepage of thermogenic sources of methane may be much more geographically widespread than previously thought (Klusman and Jakel, 1998; Klusman, 2006; Etiope and Klusman, 2008) additional investigations of nitrogen cycling and N_2O production in these environments are warranted.

5. Conclusions

Large nitrogen isotope fractionations of -48 and -55% were measured during N_2O formation by the co-oxidation of NH_4 from type I and type II methanotrophic bacteria, respectively. These large kinetic isotope fractionations are similar to those previously reported for autotrophic nitrifying bacteria, and consistent with a pathway of N_2O formation that includes reduction of nitrite as an intermediate. Thus, $\delta^{15}\text{N}_{\text{N}_2\text{O}}$ values are not likely to be useful for discriminating N_2O formation by methanotrophic and autotrophic nitrifying bacteria. For the type II methanotroph, *M. trichosporium* OB3b, we observed the incorporation of 19% and 27% O_2 -derived oxygen into N_2O , with the remaining % oxygen derived from H_2O , under high and low methane conditions, respectively. Because the observed O_2 incorporation approximated 25% under both experimental conditions, we speculate that a 25% incorporation of O_2 would be consistent with the sequential and differential binding of two unique NO_2^- or NO molecules, and their subsequent reduction to N_2O . However, the possibility that N_2O forms by the parallel reduction of two nitrite molecules which each undergo a greater degree of oxygen isotopic exchange with ambient water cannot be excluded. Because of greater isotopic enrichment of residual O_2 resulting from enhanced methanotrophic respiration under high methane conditions, the $\delta^{18}\text{O}_{\text{N}_2\text{O}}$ was accordingly enriched by $\sim 15\%$ relative to the low methane conditions. Therefore, in order to discern the biochemical pathways of N_2O formation from measured $\delta^{18}\text{O}_{\text{N}_2\text{O}}$ values, it is also essential to consider the effects of soil respiration on $\delta^{18}\text{O}_{\text{O}_2}$ values and its attendant effects on soil $\delta^{18}\text{O}_{\text{N}_2\text{O}}$ values. Soils with high CH_4 gas concentration overlying coal bed deposits had $\delta^{18}\text{O}_{\text{N}_2\text{O}}$ values that were consistent with formation by methanotrophic bacteria, although denitrification may also have contributed to N_2O formation under the wetter conditions. Nonetheless, methanotrophs may be an important source of ^{18}O -enriched N_2O to the atmosphere in such high methane environments, and might help explain, in part, the current gap between ^{18}O -enriched tropospheric N_2O and previously identified sources of N_2O from soils and oceans.

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