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Variable carbon isotope fractionation expressed by aerobic CH_4-oxidizing bacteria

Alexis S. Templeton [a,*], Kung-Hui Chu [b,c], Lisa Alvarez-Cohen [b], Mark E. Conrad [d]

[a] Department of Geological Sciences, University of Colorado, 2200 Colorado Ave., Boulder, CO 80309-0399, USA
[b] Department of Civil and Environmental Engineering, University of California, Berkeley, CA 94720-1710, USA
[c] Department of Civil Engineering, Texas A&M University, College Station, TX 77843-3136, USA
[d] Center for Isotope Geochemistry, Mailstop 70A-4418, E. O. Lawrence Berkeley National Laboratory, Berkeley, CA 94720, USA

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Abstract

Carbon isotope fractionation factors reported for aerobic bacterial oxidation of CH_4 ($\alpha_{CH_4-CO_2}$) range from 1.003 to 1.039. In a series of experiments designed to monitor changes in the carbon isotopic fractionation of CH_4 by Type I and Type II methanotrophic bacteria, we found that the magnitude of fractionation was largely due to the first oxidation step catalyzed by methane monooxygenase (MMO). The most important factor that modulates the ($\alpha_{CH_4-CH_3OH}$) is the fraction of the total CH_4 oxidized per unit time, which strongly correlates to the cell density of the growth cultures under constant flow conditions. At cell densities of less than 0.1 g/L, fractionation factors greater than 1.03 were observed, whereas at cell densities greater than 0.5 g/L the fractionation factors decreased to as low as 1.002. At low cell densities, low concentrations of MMO limit the amount of CH_4 oxidized, while at higher cell densities, the overall rates of CH_4 oxidation increase sufficiently that diffusion of CH_4 from the gaseous to dissolved state and into the cells is likely the rate-determining step. Thus, the residual CH_4 is more fractionated at low cell densities, when only a small fraction of the total CH_4 has been oxidized, than at high cell densities, when up to 40% of the influent CH_4 has been utilized. Therefore, since Rayleigh distillation behavior is not observed, $\delta^{13}C$ values of the residual CH_4 cannot be used to infer the amount oxidized in either laboratory or field-studies. The measured ($\alpha_{CH_4-CH_3OH}$) was the same for both Type I and Type II methanotrophs expressing particulate or soluble MMO. However, large differences in the $\delta^{13}C$ values of biomass produced by the two types of methanotrophs were observed. *Methylosinus trichosporium* OB3b (Type II) produced biomass with $\delta^{13}C$ values about 15‰ higher than the dissimilated CO_2, whereas *Methylomonas methanica* (Type I) produced biomass with $\delta^{13}C$ values only about 6‰ higher than the CO_2. These effects were independent of the magnitude of the initial carbon isotope fractionation caused by MMO and were relatively constant despite changing ratios of assimilatory to dissimilatory carbon transformation by the organisms. This suggests that the difference in biomass carbon isotopes is primarily due to differences in the fractionation effect at the formaldehyde branch point in the metabolic pathway, rather than assimilation of CO_2 by Type II methanotrophs.

1. Introduction

Stable isotopic measurements and mass-balance approaches are widely used to evaluate global sources and sinks of CH_4 (e.g., Whiticar and Faber, 1986; Whiticar, 1990) and to quantify the key role that CH_4-oxidizing bacteria play in the consumption of CH_4 in terrestrial environments (e.g., Ciccerone and Oremland, 1988; Happel et al., 1995; Revesz et al., 1995; Chanton et al., 1997; Tyler et al., 1997; Hornibrook et al., 1997; Snover and Quay, 2000). However, it is particularly challenging to reconstruct the flux of carbon in environments where strong seasonal fluctuations in the production and consumption of CH_4 occur (e.g., Bergamaschi, 1997; Conrad et al., 1999; Avery and Martens, 1999; Chanton and Liptay, 2000; Marik et al., 2002), or where a continuous flux of CH_4 is significantly oxidized during transport to the atmosphere (e.g., Tyler et al., 1997; Bergamaschi et al., 1998; Liptay et al.,

* Corresponding author.
E-mail address: alexis.templeton@colorado.edu (A.S. Templeton).

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1998; Bilek et al., 1999; Chanton et al., 1999; Börjesson et al., 2001; Bastviken et al., 2002; Crossman et al., 2004; Teh et al., 2005). In order to use the $\delta^{13}C$ values of residual CH_4 to determine the fraction oxidized, it is necessary to know the degree of isotopic fractionation caused by microbial oxidation of CH_4. Previous laboratory and field studies have reported a large range of values, from 1.003 to 1.039, for the kinetic isotope effect (k^{12}/k^{13}) caused by microbial CH_4 oxidation (e.g., Barker and Fritz, 1981; Coleman and Risatti, 1981; Happel et al., 1994; Summons et al., 1994; Reeburgh et al., 1997; Zyakun and Zakharchenko, 1998; Jahnke et al., 1999). This variability introduces a high degree of uncertainty into any study using isotope analyses to quantify CH_4 oxidation. For example, it is common to calculate attenuation coefficients for CH_4 oxidation that exceed 100% (e.g., Happel et al., 1994). Therefore, it is critical to understand why and when the fractionation factors for CH_4–biomass and CH_4–CO_2 can be expected to range from values larger than 30‰ to less than 5‰.

Zyakun et al. (1979) suggested that the fractionation factor for biological CH_4 oxidation is partially determined by the rate of dissolution of CH_4 into the media and partially determined by the fractionation caused by bacterial oxidation. However, in field studies, it remains unknown whether physical processes (i.e., diffusion through the soil), the metabolic activity of microorganisms, or a combination of both control the observed kinetic isotope effect (e.g., Tyler et al., 1994). When modeling the carbon isotopic enrichment associated with bacterial oxidation of CH_4, it is typically assumed that the magnitude of the CH_4–CO_2 fractionation factor is constant, despite the possibility for large variations in the rate of CH_4 transfer and oxidation. To effectively predict the carbon isotopic signature of residual CH_4 and CO_2 produced during CH_4 oxidation, it is essential to understand the importance of dynamic variables such as CH_4 concentration and metabolic characteristics of the bacteria, in order to determine whether the fractionation is controlled by diffusion of CH_4 across the air–water interface or by key enzymes active in the oxidation and assimilation of CH_4.

The diverse metabolic characteristics of methanotrophic bacteria could have a significant effect on carbon isotopic fractionation during CH_4 oxidation. There are three different classes of methanotrophic bacteria, Type I, Type II, and Type X (Hanson and Hanson, 1996). Type I and Type X bacteria both primarily use the ribulose monophosphate pathway (RuMP) to assimilate carbon from formaldehyde. Type X methanotrophs are also capable of assimilating low levels of CO_2 using ribulose bisphosphate carboxylase (Taylor et al., 1981). Type II methanotrophs assimilate carbon via two different pathways, with 50–70% of biomass derived from formaldehyde via the serine pathway and the remainder from CO_2 fixation using phosphenolpyruvate (PEP) carboxylase (Hanson and Hanson, 1996) (Fig. 1).

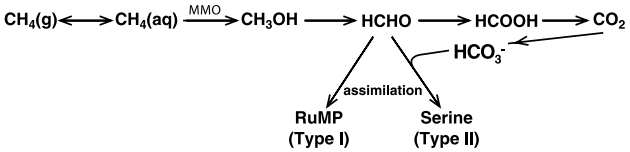

Fig. 1. Schematic diagram of the intermediates formed during CH_4 oxidation, showing the partitioning of carbon at formaldehyde between the assimilatory versus dissimilatory pathways.

In addition to the differences in assimilatory pathways, methanotrophic bacteria express different forms of the key enzyme used for the conversion of dissolved CH_4 to CH_3OH, methane monooxygenase (MMO). All methanotrophs are capable of producing a particulate form of MMO (pMMO) when copper is present (Cook and Shiemke, 1996). Under copper limited conditions, Type II, some Type I and Type X methanotrophs produce a soluble form of MMO (sMMO). Although the overall reaction catalyzed by the two types of enzymes is identical, the catalytic mechanism is not the same due to differences in the subunits that comprise the enzyme and the structure of the active site (Lieberman and Rosenzweig, 2005). Soluble MMO also exhibits a much broader substrate specificity than pMMO (Elliott et al., 1997). Thus, the enzymes may exhibit dissimilar fractionation behavior.

To fully develop the theoretical framework for understanding which factors (growth stage, MMO type, culture density, carbon-assimilation pathway, and degree of oxidation) exert the largest control on the carbon isotopic fractionation behavior associated with methanotrophs, it is necessary to simultaneously measure the isotopic composition of the CH_4, CO_2 and biomass in order to quantify the complete carbon balance during cell growth. In that way it is possible to balance the $\delta^{13}C$ values of the combined catabolic (CO_2 production) and anabolic (biomass synthesis) by-products with the isotopic shift in the residual CH_4. The observed isotopic signatures of CO_2 and cell biomass reflect the relative flows of carbon through specific biochemical pathways and the partitioning of carbon isotopes at branch points (Blair et al., 1985; Hayes, 1993). Since the flow of carbon through the assimilatory versus dissimilatory pathways varies widely between Type I and Type II methanotrophs, there is the possibility that variation in the $\delta^{13}C$ values of generated biomass and CO_2 could provide a distinct signal that can be used to monitor the distribution and activity of each type of population.

This study involved a series of experiments conducted with flow-through chemostats inoculated with mixed and pure cultures of methanotrophs. Dynamic variables such as CH_4 concentration, cell-density and metabolic characteristics of the bacteria were manipulated to assess whether the magnitude of isotopic fractionation was controlled by the overall rate of CH_4 oxidation and/or by key enzymes active in the oxidation and assimilation of CH_4. Further, copper concentrations in the culture media were controlled in order to distinguish the isotopic fractionation caused by the expression of sMMO versus pMMO from the effects of

growth stage and carbon assimilation pathway. Experiments were designed to couple continuous-flow conditions to large variations in cell density to measure the carbon isotope fractionation under conditions when only a few percent to as much as 40% of the CH_4 was oxidized, as might occur during the rapid growth and decline of a microbial population of CH_4-oxidizing bacteria. Finally, a predictive model describing how the fractionation factors varied with cell density is used to demonstrate that increases in the cell density of the cultures, which has a direct effect on the amount of CH_4 oxidized per unit time, can be used to quantitatively explain a significant reduction in the overall fractionation of the residual CH_4.

2. Materials and methods

2.1. Microorganisms and culture conditions

All cultures for these experiments were grown on a nitrate minimal salts (NMS) medium that has been successfully used for growing mixed and pure methane-oxidizing bacteria in a number of prior studies (e.g., Chang and Alvarez-Cohen, 1995; Chu and Alvarez-Cohen, 1996; Chu and Alvarez-Cohen, 1998). The details of the preparation of the NMS medium can be found in the cited studies. Briefly, the pH of the medium was maintained around 6.8–7.0 by phosphate buffer and no bicarbonate was added to the medium. However, the medium was prepared in equilibrium with the atmosphere and did contain ~0.2 mM total dissolved inorganic carbon with a $\delta^{13}C$ value of $-10‰$. To induce expression of sMMO in the cultures, copper-free NMS was used. To ensure that no copper was present in the experiments, all the glassware was cleaned by a dish-washing machine following three rises of distilled water. The cleaned glassware was then autoclaved. For pMMO experiments, 2 and 5 μM copper sulfate were added to the cultures for *M. methanica* and *M. trichosporium*, respectively.

A mixed culture of predominantly type II methanotrophs was grown at 22–24 °C in a 10 L chemostat equipped with ports for the introduction and removal of media and headspace gases (Chang and Alvarez-Cohen, 1995). A few days prior to the experiment, the chemostat culture was diluted to produce 3 L of suspended cells with a cell density of 0.1 g/L. The culture was maintained on copper-free NMS flowing through the chemostat at a rate of 25 ml/h (giving an average liquid retention rate of 5 days). The cells were also supplied with a continuous flow of CH_4 mixed with air at a rate of ~275 ml/min. The concentration of CH_4 was 20% by volume on the first day of the experiment and ~12% thereafter (although problems with the flow controller led to intermittent variations between 7.6 and 13.5%). Sampling began at a cell density of 0.73 g/L. Within 9 days the biomass concentration reached 3.8 g/L, and the concentration was maintained at steady-state between 3.8 and 4.0 g/L from that point onward. The influent gas mixture contained near-ambient CO_2 concentrations (~400 ppm) and the effluent CO_2 concentrations ranged from 1.0% to 3.8% over the course of the experiment. Sixty milliliters gas samples for isotopic analyses were extracted from the effluent gas with an airtight syringe and injected into an evacuated vial. Ten milliliters of the effluent media was collected at the same time for isotopic measurement of the biomass.

Pure cultures of a Type I methanotroph *M. methanica* and a Type II methanotroph *M. trichosporium* OB3b (ATCC #35070 and ATCC #35067) were cultured in a 500 ml KIMAX flask (24/40 joint) sealed with a specially made glass adapter with three outlets for the continuous introduction and removal of headspace gases and the periodic removal of medium containing cells. Starter cultures were grown on NMS and the expression of sMMO or pMMO controlled by the omission or addition of copper sulfate. These experiments were conducted at 22–24 °C in duplicate flasks containing 200 ml of growth media with an initial cell density of approximately 0.03 g/L. Flasks were mixed by magnetic stir bars and fed a gas mixture of air containing ~14% CH_4 and 20% O_2 at a rate of 5 ml/min through a 23-gauge long needle to produce tiny bubbles to enhance solubility. No CO_2 was added to the gas mixture (beyond ~400 ppm contained in the air), but no lag was observed for *M. trichosporium* relative to *M. methanica*, nor was there a significant difference during early growth in the effluent CO_2 concentrations. The effluent gases were continuously flushed through a 60 ml serum bottle, which was sealed and replaced when a sample for isotopic analysis was collected. Cell suspensions (15 ml) for isotopic measurement of the biomass and for the colorimetric sMMO enzyme activity assay were withdrawn by syringe at a fraction of the gas sampling time points. Cell growth was monitored by optical density measured at 600 nm using a spectrophotometer (Spectornic 20D+, Milton Roy Company). Optical density was correlated with dry cell mass measured from the difference of cell weights after drying at 105 °C overnight and after combustion at 550 °C for 2 h. The optical density (OD) and cell density were linearly correlated below $OD = 1$ (cell density $(g/L) = 0.64 \times OD$ for *M. methanica*, or $0.62 \times OD$ for *M. trichosporium*).

A naphthalene oxidation assay modified from Brusseau et al. (1991) was used to detect the activity of sMMO in the cultures. One milliliter cell suspensions were combined with 1 ml naphthalene stock solution (234 mM at 25 °C) and 20 mM formate in a 3 ml vial sealed with a 13 mm Teflon-lined rubber septa (Alltech Co.). The vial was then incubated at 35 °C on a shaker at 150 rpm for 60 min before the addition of 100 ml of freshly made 0.2% (w/v) tetrazotized *o*-dianisidine. The production of purple-colored naphthol diazo-dye measured by absorbance at 530 nm indicated positive sMMO activity. All samples were measured in duplicate. Reaction mixtures containing only cells and formate (no naphthalene) were used as blank controls.

2.2. Isotopic analyses

The concentrations of CH_4, O_2, CO_2, and N_2(+Ar) in influent and effluent gas samples were measured by gas chromatograph headspace analysis. Each gas sample (0.25 ml) was injected through a 0.5 ml gas-tight pressure-lok Dynatech-Precision syringe (Alltech Co., IL) into a Hewlett Packard HP 5890 series II gas chromatograph equipped with a thermal conductivity detector and a CTR1 column (Alltech Co., IL). Scotty certified multi-component gas mixtures (Alltech Co., IL) were used for calibration and the measured gas concentrations were normalized to 100%.

For isotopic analysis of CH_4 and CO_2, gas samples were attached to a vacuum line and the CO_2 was cryogenically separated from the non-condensable gases and water vapor. The CH_4 was then passed through a CuO furnace at 900 °C, converted to CO_2 and H_2O, and the resulting CO_2 collected in a sealed Pyrex tube for $\delta^{13}C$ analysis. Biomass samples for carbon isotope measurements were obtained by centrifuging 10 ml of the culture media, decanting the supernatant. The pellet was re-suspended in de-ionized water, centrifuged and decanted again, and then freeze-dried. Approximately 0.5 mg of freeze-dried cells were reacted with 1 g CuO and 500 mg Cu granules in an evacuated Vycor tube at 900 °C for 2 h to produce CO_2. The CO_2 was cryogenically purified, measured, and sealed in a Pyrex tube. In addition, the carbon isotope composition of dissolved inorganic carbon compounds (DIC) in a limited number of the culture samples were analyzed by acidifying the samples with phosphoric acid and collecting the resulting CO_2 for isotopic analysis.

The CO_2 stable isotope ratios were analyzed using a Prism Series II isotope ratio mass spectrometer at the Center for Isotope Geochemistry (CIG) at the Lawrence Berkeley National Laboratory. The carbon isotope ratios are reported relative to V-PDB and are reproducible within $\pm 0.1‰$. The $\delta^{13}C$ values of CH_4 and CO_2 from a series of experiments designed to test the reproducibility of the pure culture experiments were analyzed using a trace gas pre-concentration system linked to the Micromass JA series Isoprime mass spectrometer at CIG. For CH_4, 10 μl of sample was injected into the trace gas system, where the CH_4 was combusted to form CO_2 that was cryo-focused and admitted into the Isoprime in continuous-flow mode for isotope analysis. The reproducibility of the $\delta^{13}C$ values obtained using this system is $\pm 0.5‰$ and the values for these samples were indistinguishable from those obtained using the off-line combustion system. For CO_2 analyses, 0.3–10 ml of gas was injected into the trace gas system, where the CO_2 was cryo-focused and then analyzed with the Isoprime mass spectrometer. The reproducibility of these analyses is $\pm 0.2‰$. The data trends for these experiments were similar to those obtained with the off-line system, and allowed for analyses at much lower CO_2 concentrations.

3. Results

3.1. Chemostat experiment with mixed Type II culture

The data collected for samples from the chemostat experiment with the mixed culture of Type II methanotrophs are given in Table 1. The concentration of CH_4 in the influent gas was initially set at 20% (v/v) during initial growth, and was decreased on day 2 to 12% as the cells in the chemostat approached steady-state. The influent CH_4 concentration intermittently fluctuated between 7.6% and 13.5% due to a malfunctioning regulator. The fraction of influent CH_4 oxidized by the culture increased from less than one-tenth at the start of the experiment to more than one-fourth at the end of the second week. Between 5% and 20% of the oxidized carbon was incorporated into biomass (generally increasing as the culture approached steady-state), with the remainder going to CO_2.

Table 1
Data collected from the mixed culture, large chemostat experiment

Day	% CH_4 in	% CH_4 out	$\delta^{13}C$ CH_4 out (‰)	% CO_2 out	$\delta^{13}C$ CO_2 out (‰)	Cell density (g/L)	$\delta^{13}C$ biomass (‰)
1	20.2	19.0	−17.8	2.0	−43.6	0.88	−22.2
2	9.7	9.3	−19.3	1.0	−32.7	1.1	−18.9
3	9.6	8.1	−19.5	1.8	−32.2	1.4	−17.2
4	9.3	7.7	−19.9	2.7	−29.8	1.6	−16.0
6	12.5	9.6	−20.3	2.9	−27.2	3.0	−12.4
7	7.6	5.5	−21.0	2.9	−25.0	3.3	−12.3
8	12.5	9.5	−20.4	3.3	−26.5		−11.8
9	12.2	9.6	−19.9	2.7	−26.6	3.8	−10.9
10	11.4	9.5	−20.2	2.5	−26.6		−10.0
11						3.8	−9.8
12			−21.2		−23.8		−9.7
13	12.5	9.3		2.5			−9.4
14							−9.6
15	13.0	9.6	−21.1	2.5	−25.6	3.9	−10.0
16	13.5	10.5	−20.1	2.3	−26.6		
17	13.0	10.2	−19.9	3.8	−26.0		
18			−19.7		−26.0		

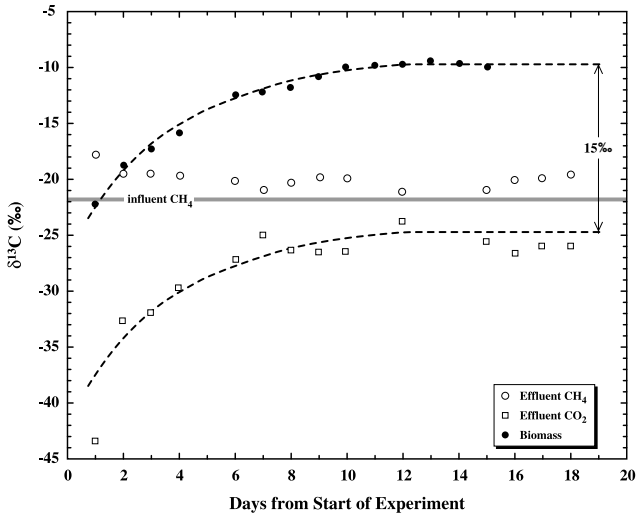

Fig. 2. $\delta^{13}C$ CH_4, CO_2, and biomass versus time (days) for a mixed culture of type II methanotrophs grown in a full chemostat with a liquid retention time of 5 days. Initial cell density of 0.3 g/L increased to a steady-state cell density of 3.8 g/L within 10 days. The filled squares represent isotope data for effluent CH_4, the filled circles represent CO_2 data and the empty circles represent biomass data. The upper dashed curve drawn through the biomass data is a hand-drawn, best-fit line. The lower dashed curve is the same as the upper curve shifted down by 15‰.

The carbon isotope compositions of CH_4, CO_2, and biomass are plotted versus time on Fig. 2. The $\delta^{13}C$ value of the influent CH_4 was −21.8‰. The largest shift in the $\delta^{13}C$ value of the effluent CH_4 (4‰) was measured in the initial sample taken after one day. As the experiment progressed, the shift in the $\delta^{13}C$ value of CH_4 decreased to about 1‰, despite increasing degrees of oxidation. This is the opposite of what would be expected if the fractionation effect was constant throughout the experiment.

The concentration of CO_2 in the effluent gas was initially high (2%) when the amount of influent CH_4 was 20%, but dropped to 1% on day 2 after the influent CH_4 concentration was decreased and then increased to almost 4% later in the experiment. The $\delta^{13}C$ values of the CO_2 in the gaseous effluent increased from −43.6‰ at the start of the experiment to as high as −23.8‰. The difference between the $\delta^{13}C$ values of the effluent CH_4 and CO_2 ($\Delta^{13}C_{CH_4-CO_2}$) decreased from 26‰ at the beginning of the experiment to as low as 3‰.

The cell density in the culture increased from an initial concentration of 0.3–3.8 g/L on day 9 and remained near that concentration for the remainder of the experiment. As with CO_2, the $\delta^{13}C$ values of the biomass increased with time, rising from −22.2‰ to as high as −9.4‰ (Fig. 2). The differences between $\delta^{13}C$ values of the biomass and the CO_2 samples collected on the same day were relatively constant at 15‰ ± 2‰ (1σ). The consistent, large fractionation between the CO_2 and biomass indicates that there is a major discrimination against ^{12}C in the assimilatory pathway of Type II methanotrophs.

3.2. Pure culture isotopic fractionation experiments

The measured concentrations and $\delta^{13}C$ values of CH_4, CO_2, and biomass for the pure culture experiments are given in Tables 2 and 3. The CO_2 concentrations in the influent gas were on the order of 0.04% with a $\delta^{13}C$ value of about −18‰. The measured data in the tables have been corrected for this, but the effect was small for all but the lowest concentration samples. In addition, the DIC concentrations and isotopic compositions in a limited number of samples were measured to insure that it did not represent

Table 2
Results of isotopic fractionation experiments with *M. OB3b* pure cultures

Cell density[a]	% CO_2	$\delta^{13}C_{CO_2}$	% CH_4	$\delta^{13}C_{CH_4}$	$\delta^{13}C_{cells}$
Culture A					
Without copper					
0.06	0.2	−48.6	13.2	−24.4	
0.11	0.6	−53.1	14.2	−22.3	−31.6
0.17	1.4	−46.3	11.7	−18.4	−31.7
0.22	1.5	−37.0	14.5		
0.34	1.9	−35.7	16.2	−24.0	−26.2
0.45	2.6	−38.5	9.9	−17.8	−25.7
0.52	2.4	−36.0	11.2	−19.9	
0.57	3.0	−29.4	3.9	−17.6	−22.6
0.64	3.5	−32.6	11.5	−19.8	−21.8
With copper					
0.04	0.1	−60.1	13.2	−22.3	
0.06	0.2	−58.1	13.2	−20.2	−30.8
0.08	0.4	−55.3	13.0	−21.6	
0.14	0.7	−48.2	12.2	−19.0	−31.8
0.29	1.2	−32.8	11.4	−21.7	−24.0
0.40	1.4	−30.4	11.1	−21.9	−22.4
0.47	1.6	−30.0	11.0	−22.0	−21.0
0.55	2.3	−29.9	11.0	−21.6	−19.8
0.70	2.4	−30.3	11.0	−21.2	−18.7
Culture B					
Without copper					
0.07	0.2	−47.5	13.0	−23.5	
0.14	1.2	−51.6	13.4	−19.6	
0.25	2.2		14.1	−17.8	−23.9
0.29	2.1	−36.4	13.2	−22.2	
0.42	4.6	−34.0	12.8	−19.3	−26.2
0.51	4.3	−32.1	9.4	−20.7	−25.8
0.56	4.1	−31.6	11.2	−20.4	
0.63	3.9	−27.7	3.9	−18.2	
0.65	4.6	−29.4	11.1	−20.1	−20.5
0.67	5.1		11.4		−19.5
With copper					
0.03	0.1	−67.6	12.7	−21.5	
0.05	0.2	−56.5	12.8	−21.5	
0.07	0.4	−56.5	12.5	−20.8	
0.12	0.6	−51.9	12.0	−19.6	−33.5
0.26	1.3	−40.5	10.5	−19.0	−29.2
0.36	1.9	−32.3	9.9	−20.3	−25.3
0.46	2.3	−30.3	9.9	−21.8	−23.1
0.57	2.5	−29.0	9.7	−21.3	−21.2
0.67	1.5	−28.0	11.6	−21.7	−19.5

[a] Cell densities are given in g/L calculated from the measured optical densities of the cultures during the experiments (cell density = 0.643 × optical density).

Table 3
Results of isotopic fractionation experiments with *M. methanica* pure cultures

Cell density[a]	% CO_2	$\delta^{13}C_{CO_2}$	% CH_4	$\delta^{13}C_{CH_4}$	$\delta^{13}C_{cells}$
Without copper					
0.06	0.2		14.9		
0.07	0.2		14.7	−24.3	
0.10	0.3	−38.2	13.2	−24.2	
0.10	0.3	−41.9	14.1	−24.3	
0.12	0.4	−45.1	11.6	−21.4	−37.0
0.15	0.4	−44.2	14.5	−24.0	−38.5
0.16	0.4	−42.5	12.3	−19.8	
0.17	0.4	−43.4	12.6		
0.19	0.4		13.0	−24.0	
0.20	0.1	−45.0	13.2		
0.25	0.3		13.5	−22.9	
0.27	0.3	−42.7	13.1		
0.28	0.4		12.3	−21.7	
0.29	0.3	−40.2	12.8		
0.30	0.3		13.4		
0.30	0.3		15.1		−43.1
0.32	0.3		13.2	−24.4	−41.7
0.32	0.3	−42.3	13.2	−23.8	−41.3
0.34	0.2	−45.0	17.5	−19.2	−43.3
0.35	0.4	−38.5	13.8	−22.4	
0.35	0.2		15.3		
0.36	0.2		15.4		−44.1
0.37	0.3		13.0		
With copper					
0.02	0.0		13.4	−20.2	
0.03	0.1		13.5	−20.3	
0.02	0.1	−48.1	13.6	−19.5	
0.03	0.1	−48.4	13.4	−20.0	
0.05	0.2	−49.0	13.3	−19.7	
0.06	0.2	−50.7	13.1	−19.8	
0.07	0.2		12.8		
0.12	0.3	−46.9	12.8	−18.6	−47.1
0.23	0.5	−44.1	12.6	−18.1	−40.3
0.32	0.8	−39.3	12.0	−18.0	−38.5
0.49	1.1	−30.8	11.0	−19.3	
0.64	1.8	−31.8	10.0	−16.2	−31.7
0.90	1.6	−25.2	10.8	−20.2	−28.0

[a] Cell densities are given in g/L calculated from the measured optical densities of the cultures during the experiments (cell density = 0.612 × optical density).

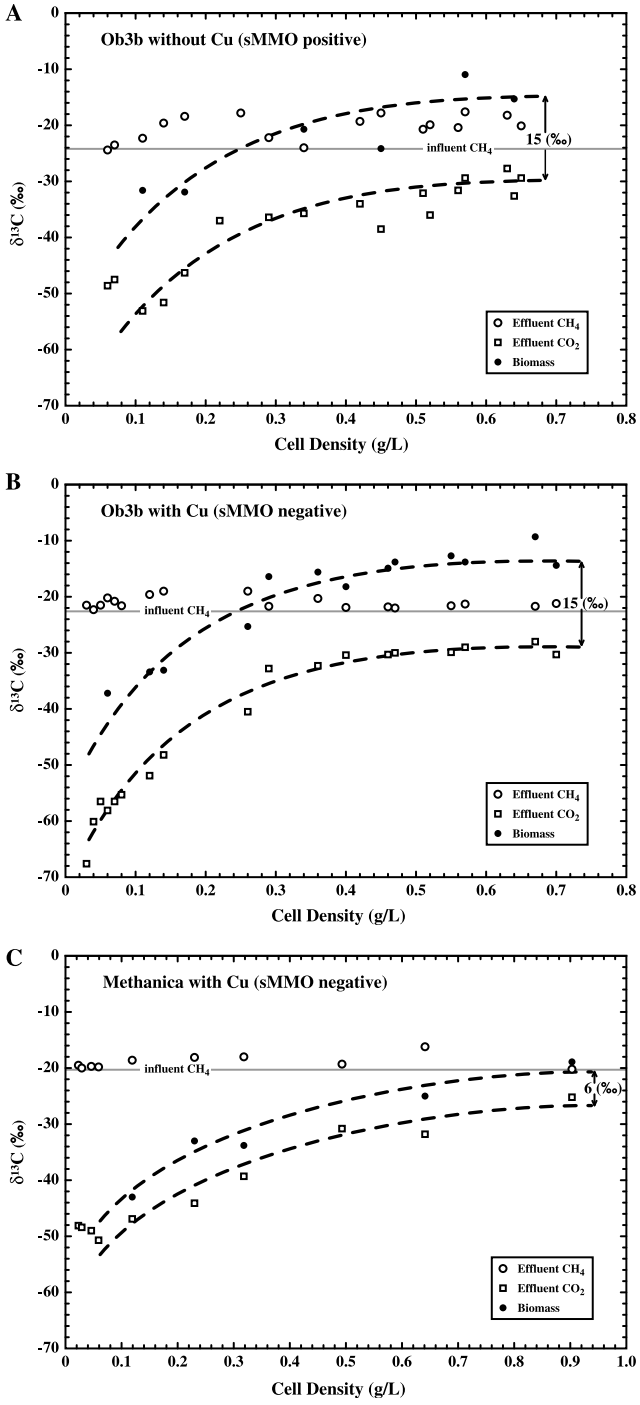

Fig. 3. $\delta^{13}C$ versus cell density (g/L) plot for pure cultures of *M. trichosporium* and *M. methanica* grown in flow-through flasks. (A) *M. trichosporium* without copper, (B) *M. trichosporium* with copper, (C) *M. methanica* with copper. The filled squares represent isotope data for effluent CH_4, the filled circles represent CO_2 data and the empty circles represent biomass data. The upper dashed curves drawn through the biomass data is a hand-drawn, best-fit line. The lower dashed curves are the same as the upper curve shifted down by 15‰ for *M. trichosporium* and 6‰ for *M. methanica*.

a significant source or sink of carbon. The concentrations were as high as 1 and 10 mM, representing a carbon reservoir approximately equivalent to the amount of CO_2 in the effluent gas flushed out of the experiments in 3 h or less. Furthermore, the DIC was in isotopic equilibrium with the CO_2 in the effluent gas (8–9‰ higher than the CO_2) indicating that it did not have a significant effect on the measured isotopic compositions of the CO_2.

In Fig. 3, we have shown the calculated $\delta^{13}C$ value of new biomass produced between each time point ($\delta^{13}C_{newcells}$). The calculated $\delta^{13}C$ values were derived using a mass balance approach where the $\delta^{13}C$ value of the biomass measured at any given time point (i.e., $\delta^{13}C_{t_2cells}$) is derived from the mass weighted average of the $\delta^{13}C$ value of the biomass at the previous time point ($\delta^{13}C_{t_1cells}$) and the $\delta^{13}C$ values of cells newly produced ($\delta^{13}C_{newcells}$). The fraction of the total cells that are newly produced ($f_{newcells}$) is known from the measured increase in optical density between each time point.

$$(\delta^{13}C_{(\text{newcells})} \times f_{(\text{newcells})}) + (\delta^{13}C_{(t_1\text{cells})} \times f_{(t_1\text{cells})}) = \delta^{13}C_{t_2\text{cells}} = \delta^{13}C_{\text{biomass}}, \quad (1)$$

$$f_{(\text{newcells})} = \frac{(OD_{t_2} \times Vol_{t_2}) - (OD_{t1} \times Vol_{t1})}{OD_{t_2} \times Vol_{t_2}}, \quad (2a)$$

$$f_{(t_1\text{cells})} = (1 - f_{(\text{newcells})}). \quad (2b)$$

3.2.1. *Methylosinus trichosporium*

To test whether the results obtained for the mixed culture experiment were typical for Type II methanotrophs, a series of fractionation experiments were conducted with pure cultures of *M. trichosporium* using batch flasks fed a continuous-flow of CH_4 and O_2. To determine if the form of MMO expressed by the bacteria affected the isotopic shifts, experiments were conducted both with copper to promote expression of the pMMO and without copper for expression of the sMMO (Table 2).

There were several significant differences between the conditions for these experiments and the mixed culture experiment. The influent gas flow to the *M. trichosporium* cultures (per liter of media) was only 25–50% of the amount given the mixed culture. This resulted in lower cell concentrations and a larger fraction of the oxidized CH_4 being incorporated into biomass (30–50% versus 10–20% for the mixed culture). This coupled with the infinite liquid residence time led to biomass concentrations that increased from 0.015 g/L to between 0.6 and 0.7 g/L during the experiment. The influent CH_4 concentration was held at approximately 13.5% for these experiments except near the end of the experiments when the influent CH_4 concentration dropped briefly to 7.8% for the sMMO expressing flasks when the CH_4 tank ran out, resulting in effluent CH_4 concentrations of 3.9%. The CO_2 concentrations in the effluent were 0.1–0.2% initially, increasing to as high as 5% at the end of the experiments.

The carbon isotope data for *M. trichosporium* are plotted versus cell density in Fig. 3. For the experiments with added copper (Fig. 3A), the colorimetric assay showed that sMMO was not expressed. The $\delta^{13}C$ value of the influent CH_4 was $-22.5‰$. The increase in the $\delta^{13}C$ values of the effluent CH_4 due to partial oxidation of the CH_4 was $<4‰$. The largest shift was observed for cell densities between 0.1 and 0.3 g/L. The $\delta^{13}C$ values of CO_2 in the effluent gas increased from $-55‰$ to $-60‰$ at low cell density to $-28‰$ to $-30‰$ at high cell density. The $\delta^{13}C$ values of the biomass (corrected for new biomass using Eqs. (1), (2a), (2b), (3)), ranged from $-37‰$ in the early samples to as high as $-10‰$ in the later samples. There is considerable scatter in the data, as expected given the uncertainties in calculating the amounts of new versus old biomass, but the difference between the $\delta^{13}C$ values of the CO_2 and the biomass remained relatively constant at approximately $15‰$ (identical to the difference observed for the mixed culture). For the *M. trichosporium* cultures without copper (Fig. 3B), sMMO was expressed (although we cannot rule out the possibility that there was also some pMMO expressed). These cultures grew somewhat faster, but the isotopic results were essentially identical to those for the experiments with copper.

3.2.2. *Methylomonas methanica*

Isotopic fractionation experiments with and without copper were conducted with pure cultures of *M. methanica* to test whether the carbon isotopic shifts caused by Type I methanotrophs are similar to those produced by Type II methanotrophs (Table 3). The *M. methanica* culture without copper had very limited growth. The cell densities were less than 0.4 g/L and the amount of CO_2 in the effluent gas fluctuated between 0.1% and 0.4% with $\delta^{13}C$ values of between $-38‰$ and $-45‰$ with no discernible trend. The average $\delta^{13}C$ of the CO_2 was $-42.3‰$ and was approximately $20‰$ lower than the average $\delta^{13}C$ value of the effluent CH_4 ($-22.7‰$). The $\delta^{13}C$ values of 7 biomass samples were also analyzed. The $\delta^{13}C$ values of those samples decreased from $-37.0‰$ for the earliest sample to $-44.1‰$ for the last sample.

As expected, the copper-amended *M. methanica* experiment exhibited more robust growth than the copper-free experiment. The cell densities increased from 0.02 to 0.90 g/L. No sMMO activity was detected with the naphthalene oxidation assay, indicating that pMMO activity predominated. The amount of CO_2 in the effluent gas increased from near background (0.04%) to 1.8% and the $\delta^{13}C$ values of the CO_2 increased from $-50‰$ during the early stages of growth to $-25‰$ at the highest cell density (Fig. 3C). The decrease in carbon isotope fractionation between CH_4 and CO_2 ($\Delta^{13}C_{CH_4-CO_2}$) at higher cell densities is similar to the trend observed for the mixed Type II and *M. trichosporium* experiments.

Measurements of the total flow of carbon for *M. methanica* with copper show that 55% of the oxidized CH_4 was incorporated in biomass and 45% was dissimilated to CO_2. The $\delta^{13}C$ composition of the new biomass was higher than the CO_2 produced at the same time by an average of $6‰$ (Fig. 3C), which is comparable to the difference observed for the *M. methanica* experiment without copper. However, it is less than half of the difference between the $\delta^{13}C$ values of the biomass and CO_2 produced from in the experiments with the Type II organisms ($15‰$).

4. Discussion

4.1. Isotopic fractionation during conversion of CH_4 to CH_3OH

The $\delta^{13}C$ value of residual CH_4 remaining after incomplete oxidation by methanotrophic organisms is determined by the first irreversible oxidation step (CH_4 dissolved $\rightarrow CH_3OH$) catalyzed by methane monooxygenase. The CH_3OH is then used either catabolically (CO_2 production) or anabolically (biomass formation). Accordingly, the combined, mass-weighted $\delta^{13}C$ values of the CO_2 and biomass generated must balance the shift in the $\delta^{13}C$ value

of the residual CH_4. The $\delta^{13}C$ value of the CO_2 and biomass may differ from each other due to subsequent fractionation after the branch point in the carbon flow (i.e., at formaldehyde) and/or due to steps in the carbon-assimilation pathway which may involve the assimilation of CO_2, such as the uptake of HCO_3^- by PEP carboxylase for type II methanotrophs using the serine pathway (Fig. 1).

If the isotopic fractionation factor associated with bacterial CH_4 oxidation is constant, then it is only necessary to monitor the concentrations and $\delta^{13}C$ values of either the reactants (in this case CH_4) or the products (CO_2 and biomass) in order to isolate the fractionation effects caused by conversion of CH_4 to CH_3OH. However, if fractionation is not constant, as was clearly observed in this study, it is necessary to measure the concentrations and $\delta^{13}C$ values of the residual CH_4 and of the CO_2 and biomass produced in order to gain a full understanding of the fractionation effect.

4.1.1. Evidence for variable carbon isotope fractionation associated with the conversion of CH_4 to CH_3OH

Fig. 4 is a plot of the fraction of CH_4 oxidized versus the isotopic compositions of the residual CH_4 (open circles) and the combined, weighted average $\delta^{13}C$ value of effluent CO_2 plus biomass (open boxes). Data from all of the experiments are plotted. The fraction of CH_4 oxidized was determined by normalizing the measured concentrations of CH_4 in the gas samples to the concentrations of inert gases (N_2+Ar) in the samples. The fraction oxidized was then calculated by dividing the difference between the normalized influent and effluent CH_4 concentrations by the influent CH_4 concentration. We reiterate that these are continuous-flow system, so we are plotting the fraction of methane oxidized per unit time (e.g., per minute).

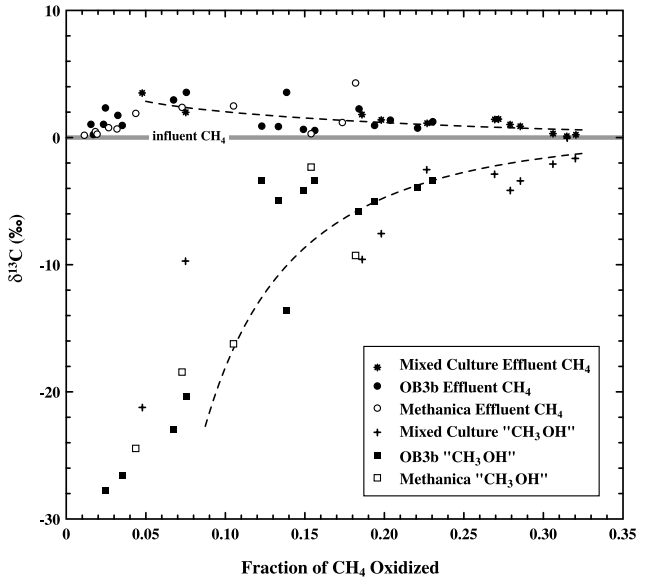

Fig. 4. $\delta^{13}C$ of residual CH_4 and calculated CH_3OH (derived from a weighted average of the CO_2 and biomass data) versus fraction of CH_4 oxidized. Filled squares are CH_4 data, filled circles are calculated CH_3OH compositions. The data includes all of the experiments with pure Type I and Type II and mixed Type II methanotrophs. Also plotted are a best-fit line through the effluent CH_4 data (upper dashed line) and a line representing the calculated isotopic compositions of CH_3OH that would offset the observed shifts in the effluent CH_4, using the full-chemostat experiment data.

The $\delta^{13}C$ value of the "CH_3OH" was calculated using the following formula:

$$\delta^{13}C_{(CH_3OH)} = \frac{\delta^{13}C_{CO_2} \times \text{moles}_{CO_2} + \delta^{13}C_{\text{biomass}} \times \text{moles}_{\text{biomass}}}{\text{moles}_{CO_2} + \text{moles}_{\text{biomass}}}. \quad (3)$$

The upper dashed line on Fig. 4 is an exponential best-fit curve through the effluent CH_4 $\delta^{13}C$ data from one of the experiments, the full-chemostat with a mixed Type II culture. The lower dashed curve is the isotopic composition of the oxidized fraction of the CH_4 calculated from the upper curve. The fit of the lower curve with the $\delta^{13}C$ data of the "CH_3OH" is good considering the uncertainties in calculating the amount of CH_4 oxidized and the combined CO_2 + biomass $\delta^{13}C$ value (especially at low oxidation levels). The $\delta^{13}C$ values of the Type II biomass represent the most significant uncertainty in the isotope data. These organisms assimilate 30–50% of carbon from HCO_3^- (Hanson and Hanson, 1996). At low respired CO_2 concentrations, the background contribution from the CO_2 in the influent gas and/or DIC in the starting media could cause a shift in the $\delta^{13}C$ of the biomass of several per mil. Given all the unknowns (exact fraction of biomass derived from HCO_3^-, amount of new biomass produced at any given time-step, etc.), it was not possible to correct for this effect. Nevertheless, the agreement between the lower curve and the trend of the data demonstrates that isotopic mass balance was kept throughout this experiment. That a good isotope mass balance was maintained indicates that CH_4 was the only major carbon source. It also means that biomass and CO_2 were the only significant carbon sinks and there was no accumulation of intermediary byproducts (e.g., CH_3OH, CHCO) or loss due to leakage in the experimental apparatus.

The $\delta^{13}C$ values of CH_4 and calculated "CH_3OH" for the Type I pure culture (*M. methanica*) and Type II pure cultures (*M. trichosporium*) with copper (sMMO negative) are also plotted versus the fraction of CH_4 consumed on Fig. 4. Despite large differences in the $\delta^{13}C$ values of biomass and CO_2 produced by *M. methanica* and *M. trichosporium*, the magnitude of carbon isotope fractionation caused by the conversion of dissolved CH_4 to CH_3OH follows an identical trend for the two organisms. These trends are comparable to those observed for the Type II mixed culture (sMMO positive). This and the similarity between the results for *M. trichosporium* with and without sMMO (Fig. 3), indicates that the form of MMO utilized by these organisms does not affect the magnitude of carbon fractionation during oxidation of dissolved CH_4 to CH_3OH.

The results of all of the experiments during which significant cell growth occurred clearly show that the fractionation factor for CH_4 oxidation was not constant for the growing population. The maximum shift in the $\delta^{13}C$ of the residual methane occurred at the lowest cell densities, even though only the smallest fraction of CH_4 was oxidized. This was regardless of the type of organism or the form of MMO utilized to oxidize the CH_4. This is the reverse of what would be expected for a simple Rayleigh distillation process using a constant fractionation factor, where the residual CH_4 should be more fractionated when more CH_4 has been consumed. These data indicate that the total active enzyme concentration and increased diffusion-controlled limitation of CH_4 at high cell densities determines the magnitude of carbon isotope fractionation caused by microbial oxidation of CH_4 (e.g. Zyakun et al., 1987).

These findings differ somewhat from the interpretations of two previous studies of carbon isotope fractionation by methanotrophic bacteria (Summons et al., 1994; Jahnke et al., 1999). In a series of experiments designed to measure the carbon isotope shifts expected for methanotrophic biomarker compounds, Summons et al. (1994) observed that the $\Delta^{13}C_{CH_4-biomass}$ fractionation for *Methylococcus capsulatus*, a Type X methanotroph, decreased from 31‰ to 16‰ during growth. They also noted that increased sMMO activity was measured at higher cell densities. By adding higher concentrations of copper to the cultures, they were able to eliminate both the production of sMMO and the shift in the carbon isotope fractionation. To explain these observations, they hypothesized that sMMO results in a smaller carbon isotope fractionation than pMMO. However, they also measured smaller shifts when they reduced the amount of CH_4 supplied to the cultures (from 47% to 5% in the influent gas), leading them to suggest that CH_4 limitation may also be a factor. In a subsequent set of experiments, Jahnke et al. (1999) measured a systematic difference of approximately 10‰ in the $\delta^{13}C$ of biomass produced by *M. capsulatus* when the cultures were induced to express sMMO versus pMMO. This difference was relatively constant despite differences in the concentrations and growth rates of the cultures produced by varying the amount of supplied O_2. All of these experiments were done with high CH_4 concentrations (47% in the influent) in order to ensure that CH_4 limitation would not be a factor. However, they did not measure the concentrations and $\delta^{13}C$ values of either the effluent CH_4 or CO_2, so it is not possible to confirm that there was not a significant shift in the $\delta^{13}C$ values of the CH_4 or whether there were differences in the relative amounts or $\delta^{13}C$ values of the CO_2 produced when the organisms were expressing either pMMO or sMMO. This is particularly important as *M. capsulatus* utilizes a somewhat different RuMP pathway for synthesizing biomass than Type I methanotrophs. In addition, it has been shown that *M. capsulatus* is capable of assimilating low levels of CO_2 using the ribulose bisphosphate carboxylase oxygenase (Rubisco) pathway and possibly the serine pathway (Taylor et al., 1981). These characteristics could lead to significant differences in carbon assimilation for Type X versus Type I methanotrophs that could result in differences between the carbon isotope compositions of biomass versus CO_2 under copper limited conditions.

Jahnke et al. (1999) also conducted a set of fractionation experiments with *M. trichosporium* expressing sMMO and pMMO. For these experiments, they supplemented the influent gas with varying amounts of CO_2 (0.3% and 4%) as a substrate for carbon assimilation by the serine pathway. They also supplied the cultures with differing amounts of CH_4 (3% and 37%) in the influent gas. In all cases, the $\delta^{13}C$ values of the biomass in cultures given less CH_4 were significantly higher than those given more CH_4. Furthermore, with one exception, at higher influent CO_2 concentrations the $\delta^{13}C$ values of the produced biomass were higher than at low CO_2 concentrations. This is because a higher proportion of the CO_2 assimilated into the biomass was derived from the added CO_2, which had a higher $\delta^{13}C$ value than the CO_2 produced by the organisms. The exception was the case with the highest biomass $\delta^{13}C$ values. Assuming the $\delta^{13}C$ values of the CO_2 produced by the cultures was approximately 15‰ lower than the biomass (as observed during our experiments), the microbial CO_2 produced from dissimilation of CH_4 for that experiment would have had about the same $\delta^{13}C$ value as the added CO_2. Consequently, the results of Jahnke et al. (1999) for *M. trichosporium* are consistent with the results presented here suggesting that there is no significant difference in the isotopic shift associated with pMMO versus sMMO.

4.1.2. Predicting changes in the isotopic fractionation of CH_4 during growth

Several previous studies have suggested that fractionation factors smaller than the maximum could be observed for enzyme-catalyzed reactions when the diffusion of the substrate is the rate-limited step. One intriguing observation relevant to this study was the finding that at high (e.g., millimolar) NH_4^+ concentrations, the fractionation associated with algal NH_4^+ oxidation was often diminished due to slow passive diffusion across the cell membrane (Fogel and Cifuentes, 1993). In surprising contrast, maximum fractionations are observed at lower (micromolar) NH_4^+ concentrations, due to active-transport mechanisms that remove this diffusion-limitation.

Thus, to examine the interrelationship between diffusion and rate of CH_4 oxidation on the carbon isotope fractionations expressed by methanotrophs, a simple model is used to demonstrate how a change in the cell-density could affect the overall $\Delta^{13}C_{CH_4-CO_2}$ and $\Delta^{13}C_{CH_4-biomass}$. In this model, the initial, irreversible partitioning of carbon between gaseous CH_4 and the cellular carbon pool controls the isotopic fractionation of CH_4 during partial utilization by methanotrophic organisms. This includes (1) dissolution of CH_4 into the liquid phase and (2) uptake and subsequent oxidation of the CH_4 by MMO to form CH_3OH as the first intermediate in the oxidative pathway. Eq. 4 outlines the transformation of CH_4 involved in the initial irreversible

oxidation process and identifies three important rate constants, k_1, k_2, and k_3, that determine which step controls the magnitude of the isotope fractionation expressed by the methanotrophs.

$$CH_4(g) \underset{k_2}{\overset{k_1}{\leftrightarrow}} CH_4(aq) \underset{MMO}{\overset{k_3}{\leftrightarrow}} CH_3OH(cell) \quad (4)$$

The rate constants k_1 and k_2 are related to dissolution and volatilization of CH_4 (i.e., the rate of transfer across the air–water interface). The term k_3 is a rate constant for oxidation of dissolved CH_4 by MMO. Since this is an enzyme-catalyzed reaction, it is dependent upon the number of active enzyme molecules present. Thus, k_3 is a second-order rate constant and the rate of formation of $CH_3OH_{(cell)}$ is dependent on the concentration of $CH_4(aq)$ and the total enzyme concentration $[E_T]$, as well as the magnitude of k_3. In this approach, the active enzyme concentration $[E_T]$ is a changing parameter proportional to the density of the cells present during exponential growth.

If the steps in Eq. 4 are in thermodynamic equilibrium, the fractionation associated with each step will be additive. However, for all the steps where equilibrium has not been reached, the isotopic fractionation will be kinetic and the overall fractionation will in large part be determined by the magnitude of the fractionation associated with the rate-determining step. For example, if k_1 and k_2 are sufficiently large relative to k_3 to establish equilibrium, the oxidation step will be rate-determining and the overall isotope fractionation will be determined by the maximum $^{13}C/^{12}C$ discrimination expressed by the MMO enzyme. On the other hand, if the active enzyme concentration increases significantly during exponential growth, the relationship between the oxidation rate constant ($k_3[E_T]$) and the rate constant for dissolution of CH_4 (k_2) could shift. The rate constants for the diffusion and oxidation steps could become almost equal, so that neither step is entirely rate limiting. In this situation, the overall isotope fractionation will become an average of the intrinsic fractionation factors associated with each step.

To calculate the effective fractionation factor for the conversion of CH_4 gas to CH_3OH, the treatment outlined by O'Leary (1981) for the fixation of CO_2 by Rubisco in C_3 plants has been modified to produce the following equations:

$$F_1 = \frac{k_1^{12}}{k_1^{13}}, F_2 = \frac{k_2^{12}}{k_2^{13}}, F_3 = \frac{k_3^{12}}{k_3^{13}}, \quad (5)$$

$$\frac{k^{12}}{k^{13}}\text{overall} = F_1\left[\left(\frac{F_3}{F_2} + \frac{k_3 E_T}{k_2}\right) \Big/ \left(1 + \frac{k_3 E_T}{k_2}\right)\right], \quad (6)$$

where F_1 and F_2 are the fractionation factors for dissolution and volatilization of CH_4 in water, respectively, and F_3 is the fractionation factor resulting from the enzymatic conversion of dissolved CH_4 to CH_3OH by MMO. They are equal to the ratio of the rate constants for each reaction for $^{12}CH_4$ versus $^{13}CH_4$. The difference between this approach and that of O'Leary (1981) is that $k_3[E_T]/k_2$ is not a constant value since $[E_T]$ must increase during growth as a function of the increasing cell densities observed in these experiments. As discussed by O'Leary (1981), the ratio k_3/k_2 (or $k_3[E_T]/k_2$ in this study) is a partitioning factor for the intermediate dissolved gas phase (i.e., $CH_{4(aq)}$) and is critical in determining the rate controlling step.

The kinetic fractionation associated with the transfer of CH_4 across the air–water interface is small and determined by the rate of molecular diffusion of the light isotope relative to the heavier isotope. In laboratory experiments, the kinetic carbon isotope fractionation factor for CH_4 was reported to be 1.0008 (Inoue and Sugimara, 1985; Knox et al., 1992) and a similar value of 1.0009 was reported for field studies (Happel et al., 1995).

By correlating a change in $k_3[E_T]/k_2$ to the increase in cell density, using the expressions defined in Eqs. (4)–(6) and applying the kinetic isotope effects for each step that are outlined below, we can simulate the reduction in the isotopic fractionation of CH_4 that is observed during all of the growth experiments. Although the approach could be further refined by fully applying Michaelis–Menten kinetics to describe the enzyme-catalyzed oxidation rate, it is not necessary for modeling the data in this study.

The fractionation effect associated with conversion of $CH_{4(aq)}$ to $CH_3OH_{(cell)}$ will be determined by the isotope discrimination expressed by the MMO enzyme. The largest CH_4–CO_2 fractionation factors reported in the literature are approximately 1.031–1.034 (Zyakun and Zakharchenko, 1998). However, the maximum fractionation may be larger than the apparent value since the isotopic value of the CH_4 pool changes during oxidation (O'Leary, 1981), which was a significant effect in previous studies. In the chemostat experiments described here, CH_4 was continuously flushed through the headspace in order to avoid changes in the CH_4 pool, and a fractionation as large as 35‰ was observed during the early stages of growth. Therefore, 1.035 was chosen as an approximate value for the intrinsic fractionation during CH_4 oxidation in order to predict the low $\delta^{13}C$ CO_2 values observed at the lowest cell densities.

Since the magnitudes of the maximum isotope discriminations caused by the dissolution and oxidation steps are so different, a change in the rate-determining step during growth could shift the observed fractionation factor by more than 30‰. Using Eq. (6), the calculated fractionation factor ($\Delta^{13}C_{CH_4\text{–}CH_3OH}$) will decrease from 33.8‰ to 0.8‰ as $k_3[E_T]/k_2$ increases from 0.001 to 1000, while F_3 is held constant at 1.035. The explain the range of ($\Delta^{13}C_{CH_4\text{–}CH_3OH}$) values that were observed in the experiments described here (30–3‰), $k_3[E_T]/k_2$ would only need to vary between 0.15 and 15. For comparison, calculated values of $\Delta^{13}C_{CH_4\text{–}CH_3OH}$ for F_3 of 1.025 are also plotted on Fig. 5. At high values of $k_3[E_T]/k_2$ (e.g., higher cell concentrations), the differences in the fractionation expressed for F_3 of 1.035 versus 1.025 are small, while at low $k_3[E_T]/k_2$ values (e.g., low cell densitites), 9‰ differences in the observed fractionation are predicted. However, no significant

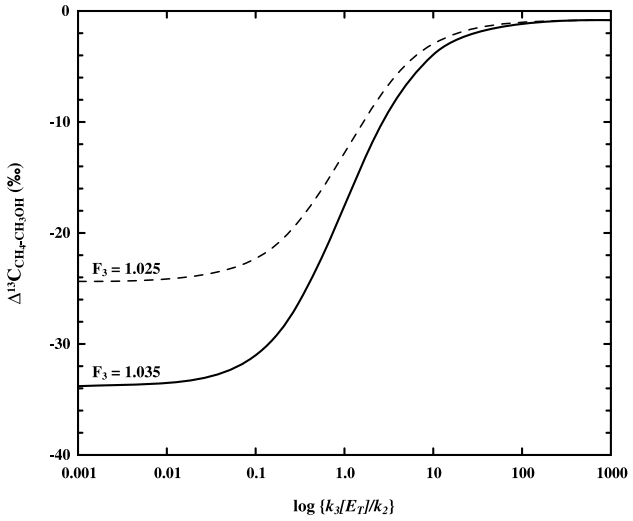

Fig. 5. Plot comparing the $\Delta^{13}C_{CH_4-CH_3OH}$ predicted from our model for two values of F_3 (the isotopic fractionation accompanying the enzymatic conversion of CH_4 to CH_3OH). At low values of $\log\{k_3[E_T]/k_2\}$ (low levels of cells relative to CH_4 supply), the net fractionation approaches a maximum value determined by F_3. At high values of $\log\{k_3[E_T]/k_2\}$ (high levels of cells relative to CH_4 supply), the net fractionation decreases to values governed by the shift caused by dissolution of CH_4 into water. The results of our experiments span the range in values of $\log\{k_3[E_T]/k_2\}$ between 0.1 and 10.

difference in F_3 for oxidation of CH_4 to CH_3OH via pMMO versus sMMO was observed in the experiments in this study, even at low cell densities.

4.2. Isotopic fractionation during carbon assimilation

In order to evaluate the effects of carbon assimilation pathway on the isotopic composition of biomass produced by methanotrophic bacteria, experimental results can be compared for *M. methanica*, a Type I methanotroph that uses the RuMP pathway for biomass synthesis, and *M. trichosporium*, a Type II methanotroph that uses the serine pathway. The RuMP pathway generates biomass entirely from CH_4, whereas the serine pathway assimilates 50–70% of the cell carbon from CH_4 and 30–50% from CO_2 (Hanson and Hanson, 1996).

For *M. methanica*, the biomass $\delta^{13}C$ values were about 6‰ higher than the $\delta^{13}C$ values of the CO_2 (Fig. 3C). Previous studies that have compared the $\delta^{13}C$ values of biomass and CO_2 produced by Type I methanotrophic bacteria have found similar results (e.g., Zyakun and Zakharchenko, 1998). This indicates that there is a small fractionation associated with the branch point in the metabolic pathway at formaldehyde where some of the carbon is incorporated into biomass and some is oxidized to CO_2 (Fig. 1).

In contrast, the $\delta^{13}C$ values of the biomass produced by *M. trichosporium* were ~15‰ higher than the corresponding CO_2 (Fig. 3). Similar results were obtained for the mixed culture of predominantly Type II methanotrophs grown in the large chemostat experiment (Fig. 2). These results suggest that the $\delta^{13}C$ values of biomass generated by Type I and Type II methanotrophs are consistently different. Furthermore, although previous workers have shown that biomass $\delta^{13}C$ values are typically quite depleted relative to the carbon source, the results here show that the $\delta^{13}C$ values for Type II methanotroph biomass can be equivalent to or higher than the CH_4 substrate when carbon limitation is a factor. Jahnke et al. (1999) also noted this effect for fractionation experiments with *M. trichosporium* when cultures were supplied with limited CH_4.

Since the $\delta^{13}C$ values of CH_3OH produced by the enzymatic oxidation of CH_4 were essentially identical for *M. trichosporium* and *M. methanica*, regardless of the form of MMO expressed (Fig. 4), the differences in the isotopic compositions of the biomass and CO_2 for Type I versus Type II methanotrophs must result from fractionation occurring later in the metabolic pathway. For Type I methanotrophs, the only opportunity for isotopic fractionation is at the formaldehyde branch (Fig. 1). For Type II methanotrophs, however, there could also be a shift associated with the assimilation of CO_2. Any difference in carbon isotope biomass–CO_2 fractionation between Type I and Type II methanotrophs could therefore be due to a difference in the fractionation at the formaldehyde branch and/or a shift associated with assimilation of CO_2 by Type II methanotrophs.

The expected isotopic fractionation pattern for Type II methanotrophs differs depending on whether the shift occurs at the formaldehyde branch point or during CO_2 assimilation. Isotopic fractionation associated with the formaldehyde branch point should produce a constant difference between the biomass and the CO_2 regardless of the fraction of CH_4 consumed. Fractionation resulting from assimilation of CO_2 will vary depending on the ratio of CO_2 to biomass produced by the culture. This is because at higher growth rates, the primary source of CO_2 is biogenic CO_2 produced from the formaldehyde branch point. Since the $\delta^{13}C$ of the CO_2 will vary depending on the proportion of CH_4 oxidized, the $\delta^{13}C$ of the biomass formed from that CO_2 would also vary. This effect is illustrated in Fig. 6, where the difference between the biomass and CO_2 produced by the Type II cultures ($\Delta^{13}C_{biomass-CO_2}$) is predicted for differing degrees of CH_4 oxidation. ($\Delta^{13}C_{biomass-CO_2}$) is calculated for three combinations of α_1 (the carbon isotope fractionation for biomass versus CO_2 at the formaldehyde branch point) and α_2 (the fractionation resulting from assimilation of CO_2 into biomass) assuming that 60% of the biomass carbon comes from formaldehyde and 40% from CO_2. For $\alpha_1 = 1.025$ and $\alpha_2 = 1.000$ (no fractionation), the difference between the $\delta^{13}C$ values of the biomass and CO_2 remain constant at 15‰. For $\alpha_1 = 1.000$ and $\alpha_2 = 1.031$, ($\Delta^{13}C_{biomass-CO_2}$) ranges from 13‰ at 10% carbon going to biomass up to 17.5‰ at 50% carbon going to biomass. The intermediate case ($\alpha_1 = 1.0125$, $\alpha_2 = 1.0155$) produces an intermediate shift in ($\Delta^{13}C_{biomass-CO_2}$).

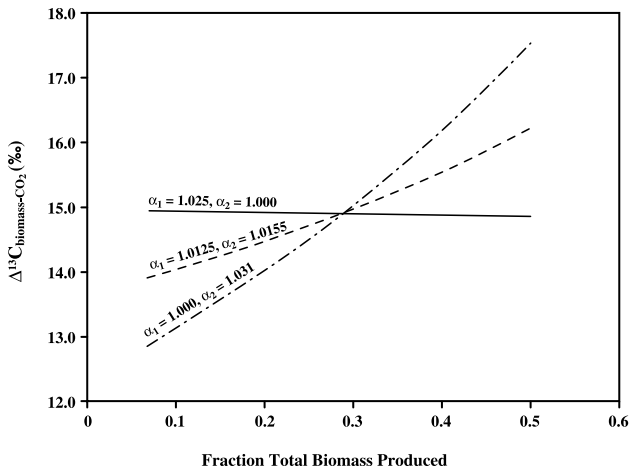

Fig. 6. Calculated differences between the carbon isotopic compositions of biomass and CO_2 ($\Delta^{13}C_{biomass-CO_2}$) for three different combinations of a_1 (the isotopic discrimination between CO_2 and biomass resulting from the formaldehyde branch point) and a_2 (the isotopic discrimination between CO_2 and biomass caused by assimilation of CO_2 to form biomass) plotted against the net fraction of biomass (versus CO_2) produced by the organisms.

The fraction of CH_4 metabolized to produce biomass by the Type II cultures varied during the individual experiments, and from one experiment to another (from approximately 0.1 to 0.5). Since there was a relatively constant difference between the $\delta^{13}C$ values of the biomass and CO_2, the difference between ($\Delta^{13}C_{biomass-CO_2}$) for Type I and Type II methanotrophs must be primarily due to a much larger fractionation associated with the formaldehyde branch point for the serine pathway versus the RuMP pathway. Due to the uncertainties of the measurements, a minor effect resulting from CO_2 assimilation cannot be ruled out, but would likely be relatively small (<10‰).

5. Conclusions and implications for natural systems

The main conclusions derived from the results of the experiments performed for this study include:

1. Residual CH_4 $\delta^{13}C$ values can not be used to determine the fraction of CH_4 oxidized by Type I or Type II bacteria in laboratory or natural systems. Large shifts in CH_4 $\delta^{13}C$ values will likely occur when the cell numbers for methanotrophs are low, because the maximum fractionation is expressed ($\alpha_{CH_4-CH_3OH} \sim 1.035$). In contrast, only small shifts in the residual CH_4 $\delta^{13}C$ values will be observed when a large fraction of the total methane is oxidized because the expressed fractionation factor diminished significantly ($\alpha_{CH_4-CH_3OH} \sim 1.002$) at high cell densities.
2. Neither the type of organism nor the form of MMO utilized in this study had a significant effect on the net fractionation factor resulting from the enzymatic conversion of dissolved CH_4 to CH_3OH.
3. The type of methanotroph (Type I or II) did have a significant effect on the degree of fractionation between biomass and CO_2. The $\alpha_{biomass-CO_2}$ for Type II methanotrophs was consistently ~1.015, regardless of the relative proportions of biomass versus CO_2 produced. The $\alpha_{biomass-CO_2}$ for the one Type I methanotroph studied was much lower at approximately 1.006.

It is clear from the results of these experiments that the magnitude of carbon isotope discrimination by CH_4 oxidizing bacteria can vary significantly depending on environmental conditions. Where there is an excess of CH_4, the carbon isotope fractionation should approach the maximum ($\alpha_{CH_4-CH_3OH} \sim 1.035$). Limited O_2 availability (e.g., in low permeability soils or water-saturated soils) would presumably have a similar effect. This situation would be expected to occur in environments such as flooded rice paddies, marshes or lake sediments. Conversely, in soils with established methanotrophic communities and abundant O_2 supply that are capable of near complete oxidation of any available CH_4, the fractionation effect will be small ($\alpha_{CH_4-CH_3OH} \sim 1.005$). Well-aerated, well-drained landfill covers are a good example of this type of environment.

It is also possible that changing environmental conditions could lead to changes in the degree of carbon isotope fractionation associated with microbial CH_4 oxidation. Conrad et al. (1999) observed large shifts in the $\delta^{13}C$ of soil gas CH_4 (>50‰) during drying of seasonally flooded soils. Once the soils were completely drained, there was no significant shift in the $\delta^{13}C$ of the soil gas CH_4, despite large negative shifts in the $\delta^{13}C$ of soil gas CO_2 indicating a large input from oxidized CH_4. Similar situations could exist in environments such as rice paddies.

The implications of these results for situations with low CH_4 concentrations and low methanotroph populations, such as the consumption of atmospheric CH_4 by soil methanotrophs, are not clear. This particular case is important because it is estimated to represent a sink for ~5% of atmospheric CH_4 (Dörr et al., 1993). The concentration of atmospheric CH_4 is low (currently ~2 ppm) and the distribution of methanotrophic organisms in soils are highly variable. Therefore, the fractionation effect could also be highly variable.

The results of these experiments also imply that it may be inappropriate to estimate carbon isotope fractionation by indigenous methanotroph populations from a site using microcosm experiments unless it can be shown that the conditions in the experiments match those in the field. If high initial concentrations of CH_4 are given to the microcosms, they may yield higher than actual estimates of the carbon isotope fractionation effect. This would lead to erroneously high estimates of the level of CH_4 oxidation from field data. The converse (lower than natural CH_4 concentrations) would also be true. Also, since most of these experiments are done by measuring the $\delta^{13}C$ of the CH_4 at only one time point, the calculated α values for CH_4 oxidation may incorporate a changing degree of fractionation.

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