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Fractionation of the methane isotopologues $^{13}CH_4$, $^{12}CH_3D$, and $^{13}CH_3D$ during aerobic oxidation of methane by *Methylococcus capsulatus* (Bath)

David T. Wang [a,b,*], Paula V. Welander [c], Shuhei Ono [a]

[a] *Department of Earth, Atmospheric and Planetary Sciences, Massachusetts Institute of Technology, Cambridge, MA 02139, USA*
[b] *Marine Chemistry and Geochemistry Department, Woods Hole Oceanographic Institution, Woods Hole, MA 02543, USA*
[c] *Earth System Science Department, Stanford University, Stanford, CA 94305, USA*

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Abstract

Aerobic oxidation of methane plays a major role in reducing the amount of methane emitted to the atmosphere from freshwater and marine settings. We cultured an aerobic methanotroph, *Methylococcus capsulatus* (Bath) at 30 and 37 °C, and determined the relative abundance of $^{12}CH_4$, $^{13}CH_4$, $^{12}CH_3D$, and $^{13}CH_3D$ (a doubly-substituted, or "clumped" isotopologue of methane) to characterize the clumped isotopologue effect associated with aerobic methane oxidation. In batch culture, the residual methane became enriched in ^{13}C and D relative to starting methane, with D/H fractionation a factor of 9.14 ($^{D}\varepsilon/^{13}\varepsilon$) larger than that of $^{13}C/^{12}C$. As oxidation progressed, the $\Delta^{13}CH_3D$ value (a measure of the excess in abundance of $^{13}CH_3D$ relative to a random distribution of isotopes among isotopologues) of residual methane decreased. The isotopologue fractionation factor for $^{13}CH_3D/^{12}CH_4$ was found to closely approximate the product of the measured fractionation factors for $^{13}CH_4/^{12}CH_4$ and $^{12}CH_3D/^{12}CH_4$ (i.e., $^{13}C/^{12}C$ and D/H). The results give insight into enzymatic reversibility in the aerobic methane oxidation pathway. Based on the experimental data, a mathematical model was developed to predict isotopologue signatures expected for methane in the environment that has been partially-oxidized by aerobic methanotrophy. Measurement of methane clumped isotopologue abundances can be used to distinguish between aerobic methane oxidation and alternative methane-cycling processes.

Keywords: Methane; Clumped isotopologues; Aerobic methane oxidation; Methanotrophic cultures; Isotopic fractionation

1. INTRODUCTION

Methane is an important long lived (well-mixed) greenhouse gas whose atmospheric concentration has more than doubled (~720 ppb to >1800 ppb) since pre-industrial time (Wahlen, 1993; IPCC, 2013). Important sources of atmospheric methane include natural wetlands (up to one-third of emissions), agriculture (including paddy rice fields and ruminant animals), and fossil fuel usage (Bousquet et al., 2006; Dlugokencky et al., 2011). Methanogenic archaea are responsible for the majority of emissions, with thermogenic sources accounting for most of the remainder. The primary methane sink in the atmosphere is reaction with tropospheric hydroxyl radicals (OH). Despite rigorous bottom-up accounting and top-down estimates based on remote sensing data and high-frequency measurements, the flux of methane from sources and to sinks remains poorly constrained (e.g., Kirschke et al., 2013).

Emissions from natural and human-made wetlands and other aquatic environments account for nearly two-thirds

* Corresponding author at: Department of Earth, Atmospheric, and Planetary Sciences, Massachusetts Institute of Technology, 77 Massachusetts Ave, Bldg. E25-645, Cambridge, MA 02139, USA.
E-mail address: dtw@mit.edu (D.T. Wang).

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of all methane sources, though substantial uncertainty is associated with source strength estimates (Kirschke et al., 2013). Methanotrophic processes consume over half of the methane produced in aquatic environments prior to emission into the atmosphere (Reeburgh, 2007). It is estimated that a large fraction of methane produced in freshwater sediments, as much as 90% at some sites (Oremland and Culbertson, 1992), is removed via the aerobic oxidation of methane. In addition, soil-dwelling aerobic methanotrophs are responsible for oxidation of a small fraction (~2%) of methane from the atmosphere (Kirschke et al., 2013). Furthermore, activity of methanotrophic bacteria with high affinity for atmospheric methane in Arctic soils has been reported (Lau et al., 2015). Thus, understanding the magnitude and dynamics of methanotrophic sinks is important for global methane cycle budgets and constraining inputs to climate simulations.

The bacterium *Methylococcus capsulatus* (Bath), an obligate aerobic methanotroph, is a model organism for studies of the genetics, physiology, and geomicrobiology of aerobic methane oxidation in sediments and water columns (Whittenbury et al., 1970; Bowman, 2014). This organism uses the enzymes soluble methane monooxygenase (sMMO) and particulate methane monooxygenase (pMMO) to oxidize methane to methanol, which is further oxidized to CO_2 as an end product (Hanson and Hanson, 1996). Carbon derived from methane can also be assimilated into cellular biomass. The overall reaction is thus described by the stoichiometry:

$$CH_4 + 2O_2 \rightarrow b\ C_{cell} + (1-b)CO_2 + 2H_2O \quad (1)$$

where C_{cell} represents cellular carbon and b is the fraction of carbon assimilated into biomass.

In experiments with pure and enrichment cultures, microbes utilizing this pathway have been shown to generate large and correlated carbon ($^{13}C/^{12}C$) and hydrogen (D/H) isotope fractionations during aerobic methane oxidation (Coleman et al., 1981; Kinnaman et al., 2007; Powelson et al., 2007; Feisthauer et al., 2011). Measurements of $^{13}C/^{12}C$ and D/H ratios in environmental methane samples can be used to assess whether they have experienced partial oxidation (Hornibrook et al., 1997; Chanton et al., 2005).

Recently, methods were developed to determine the abundance of multiply-substituted "clumped" isotopologues (e.g., $^{13}CH_3D$) in methane samples to sub-permille precision (Ono et al., 2014; Stolper et al., 2014b; Young et al., 2016). Measurements of the abundance of multiply-substituted isotopologues are of geochemical interest because of their potential for use as an isotopic geothermometer that can be accessed via analyses of a single compound (Wang et al., 2004; Eiler, 2007). Furthermore, clumped isotopologue data provide another dimension for probing kinetic and equilibrium isotope effects and for constraining isotope exchange processes in natural settings (e.g., Eiler and Schauble, 2004; Yeung et al., 2012, and Yeung, 2016). For example, the isotope exchange reaction

$$^{13}CH_4 + {}^{12}CH_3D \rightleftharpoons {}^{13}CH_3D + {}^{12}CH_4 \quad (2)$$

has an equilibrium constant K that varies between ~1.007 at 0 °C to 1.000 at temperatures approaching infinity (at which isotopes are randomly distributed amongst all possible isotopologues, i.e., the stochastic distribution) (see Wang et al., 2015, and references therein for details regarding calculations from which K is obtained).

Subsequent surveys of methane in the environment revealed that in methane of microbial origin produced in both natural settings and pure cultures, the reaction quotient (Q, see also Section 2.2) of Reaction (2) varies between 0.997 and 1.007 (Stolper et al., 2014a, 2015; Inagaki et al., 2015; Wang et al., 2015; Douglas et al., 2016), a range that is much larger than that expected for thermodynamic equilibrium (ca. 1.004 to 1.007) at temperatures at which microbial life is possible (~0 to 120 °C; Takai et al., 2008) (Wang et al., 2015). The nonequilibrium isotope signatures were attributed to intrinsic clumped isotopologue effects expressed during biological methanogenesis under conditions of low reversibility (Stolper et al., 2015; Wang et al., 2015). Using inferences based on $\delta^{13}C$ and δD data, methane oxidation was excluded as a significant origin of the nonequilibrium isotope signals (Wang et al., 2015). However, experimental constraints on the fractionation of $^{13}CH_3D$ during biological methane oxidation are lacking in the clumped isotope literature.

In this paper, we report experimental measurements of the fractionation of $^{13}CH_3D$ during aerobic methane oxidation by cultures of *M. capsulatus* (Bath). It is demonstrated that aerobic methanotrophy affects the abundance of $^{13}CH_3D$ in a predictable fashion relative to $\delta^{13}C$ and δD; the directionality and magnitude of these effects depend on whether oxidation occurs in a closed or open system. We present simple models to illustrate the expected shifts in $^{13}CH_3D$ abundance under different scenarios, and review available environmental clumped isotopologue data in light of the new experimental constraints.

2. METHODS

2.1. Cultures

M. capsulatus strain Bath cultures were grown in 10 ml of nitrate mineral salts medium supplemented with 5 μM $CuSO_4$ (Welander and Summons, 2012). Serum bottles (160 cm^3) were inoculated with 2%(v/v) inoculum from a starter culture that had grown for ca. 30 h, stoppered and sealed without removing ambient air, and injected with 20 cm^3 SATP (~810 μmol) of methane from commercially-sourced cylinders using a gas-tight syringe. Tests indicated that the starting gas compositions were consistent within analytical error (±5%) between serum bottles. Multiple serum bottles were inoculated for each of the two experimental temperatures (Table 1). Cultures were incubated at 30 or 37 °C while shaking at 225 rpm and sacrificed at given times by adding 1 ml of 1 M hydrochloric acid. Each row in Table 1 shows the composition of one serum bottle at the time at which the experiment was stopped. Experimental timepoints were selected based on monitoring of growth during preliminary incubations of starter cultures (by tracking optical density, see Supplementary Fig. 1). However, to minimize puncturing of the serum bottles during the isotopic fractionation experiments, optical densi-