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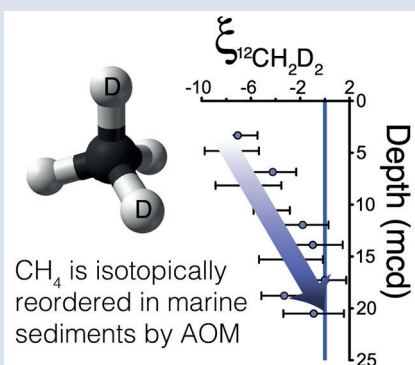
## Exchange catalysis during anaerobic methanotrophy revealed by $^{12}\text{CH}_2\text{D}_2$ and $^{13}\text{CH}_3\text{D}$ in methane

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### Abstract



The anaerobic oxidation of methane (AOM) is a crucial component of the methane cycle, but quantifying its role *in situ* under dynamic environmental conditions remains challenging. We use sediment samples collected during IODP Expedition 347 to the Baltic Sea to show that relative abundances of  $^{12}\text{CH}_2\text{D}_2$  and  $^{13}\text{CH}_3\text{D}$  in methane remaining after microbial oxidation are in internal, thermodynamic isotopic equilibrium, and we attribute this phenomenon to the reversibility of the initial step of AOM. These data suggest that  $^{12}\text{CH}_2\text{D}_2$  and  $^{13}\text{CH}_3\text{D}$  together can identify the influence of anaerobic methanotrophy in environments where conventional bulk isotope ratios are ambiguous, and these findings may lead to new insights regarding the global significance of enzymatic back reaction in the methane cycle.

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### Letter

Quantifying the subsurface carbon cycle by linking biogeochemistry to deep biosphere metabolisms remains a long-standing challenge compounded by slow rates of reaction (Edwards *et al.*, 2012; Marlow *et al.*, 2017). Even in well-characterised environments, determining how methanogens and anaerobic methanotrophs influence organic matter degradation can be challenging (Regnier *et al.*, 2011; Beulig *et al.*, 2018). These challenges are due in part to how methane produced by methanogens inherits characteristics from its substrates (both a range of organic and inorganic carbon) (Conrad, 2005), and they are further complicated when methane is itself the substrate during anaerobic methane oxidation (AOM) (Whiticar, 1999). Our understanding of how anaerobic methanotrophs respond to varying environmental conditions (Holler *et al.*, 2009, 2011; Yoshinaga *et al.*, 2014) and how they might contribute to “cryptic cycles” (the rapid production and consumption of reactive intermediates) in the marine subsurface is constantly evolving (Egger *et al.*, 2018).

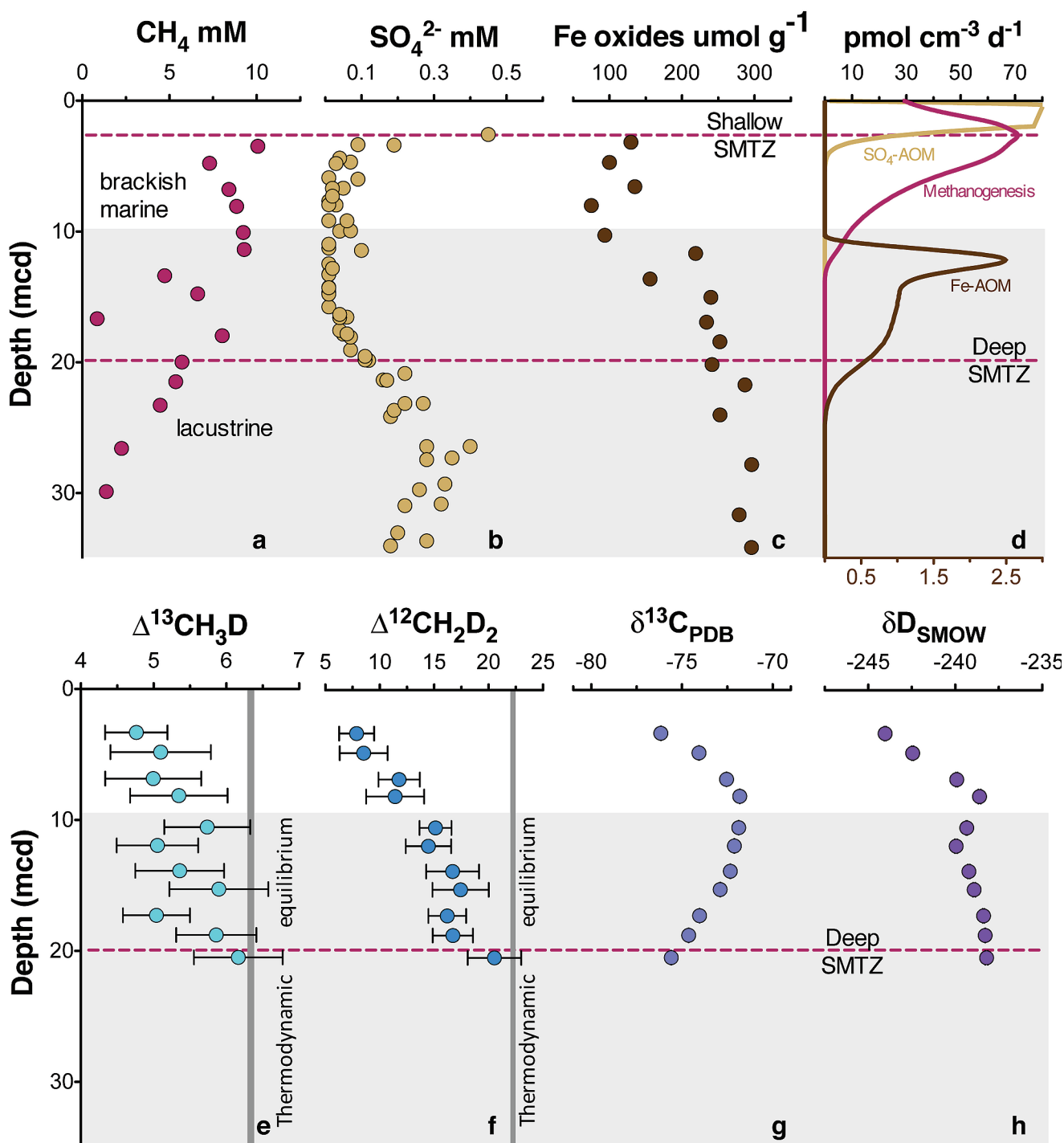
AOM is thought to be carried out by a consortium of methane oxidising archaea and sulphate reducing bacteria performing the net reaction  $\text{CH}_4 + \text{SO}_4^{2-} \rightarrow \text{HCO}_3^- + \text{HS}^- + \text{H}_2\text{O}$

(Boetius *et al.*, 2000). Evidence suggests that these processes can be decoupled (Milucka *et al.*, 2012; Scheller *et al.*, 2016) and metal oxides may also act as terminal electron acceptors (Beal *et al.*, 2009; Cai *et al.*, 2018). In all cases, the first step of AOM involves C-H bond activation by a modified methyl-coenzyme M reductase (Mcr), the terminal enzyme in methanogenesis (Scheller *et al.*, 2010). Anaerobic methanotrophs use enzymes homologous to those used by methanogens (Krüger *et al.*, 2003; Shima *et al.*, 2012), prompting questions regarding the directionality of these metabolisms (Lloyd *et al.*, 2011; Beulig *et al.*, 2019).

Coupling exploration of methane enzymatic chemistry with isotope geochemistry can provide powerful insights. Here, we interrogate subsurface methane metabolisms by precisely determining concentrations of molecules containing two heavy isotopes, referred to as “clumped” isotopologues (Eiler, 2007). The relative proportions of  $^{13}\text{CH}_3\text{D}$  and  $^{12}\text{CH}_2\text{D}_2$  (reported as  $\Delta^{13}\text{CH}_3\text{D}$  and  $\Delta^{12}\text{CH}_2\text{D}_2$  values relative to a random distribution of isotopes among all  $\text{CH}_4$  isotopologues; see Supplementary Information) have known temperature sensitive equilibrium concentrations and thus are sensitive indicators of reversibility in reactions (Piasecki *et al.*, 2016). For instance, axenic cultures of methanogens produce methane

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**Figure 1** Geochemical profiles ( $\pm 2 \sigma$ ) from Bornholm Basin from 3-35 MCD. Horizontal grey bar denotes sediments deposited during lacustrine conditions overlain with sediments deposited during brackish marine conditions. Samples were not recovered above the shallow SMTZ. Pink dashed lines denote a shallow (3.3 MCD) and deep (19.8 MCD) SMTZ; only the shallow SMTZ is shown in the bottom panel for clarity. Dark grey vertical bars in e and f denote equilibrium isotopologue compositions for the subsurface temperature of  $7.8 \pm 0.6$  °C. Modelled  $\text{CH}_4$  production, Fe-AOM and  $\text{SO}_4$ -AOM rates are shown in panel d (Dijkstra *et al.*, 2018).  $\text{CH}_4$  production (purple) and  $\text{SO}_4$ -AOM (gold) rates correspond with the upper x axis and Fe-AOM rates (brown) correspond with the lower x axis.  $\text{SO}_4$ -AOM rates above the upper SMTZ are calculated using bottom water  $\text{SO}_4$  concentrations of 15.0 mM. Additional porewater data shown in Supplementary Information.

with depletions in clumped isotopes relative to the random distribution that reflect kinetic processes (Stolper *et al.*, 2015; Wang *et al.*, 2015; Douglas *et al.*, 2016; Young *et al.*, 2017; Gruen *et al.*, 2018). In some cases, the clumped isotope composition of environmental microbial methane is consistent with the axenic cultures, but in others it displays a clumped isotope composition reflective of equilibration within the environment of formation (Wang *et al.*, 2015; Young *et al.*, 2017). This span in clumped isotope distributions suggests that enzymatic

reactions associated with methanogenesis and methanotrophy are capable of ranging from reversible to kinetic. Previous work using one rare isotopologue ( $\Delta^{13}\text{CH}_3\text{D}$ ) suggests that the rate of methanogenesis controls the degree of molecular isotopic equilibrium (Stolper *et al.*, 2015; Wang *et al.*, 2015). However, the inability to produce equilibrated methane from axenic cultures has led to speculation that AOM may be responsible for equilibrated environmental methane (Stolper *et al.*, 2015; Wang *et al.*, 2016; Young *et al.*, 2017; Gruen *et al.*, 2018). The use

of a second rare isotopologue,  $^{12}\text{CH}_2\text{D}_2$ , aids in interpreting the causes of intra-methane disequilibrium and suggests that microbial methanogenesis does not lead to isotopologue equilibrium among methane molecules.

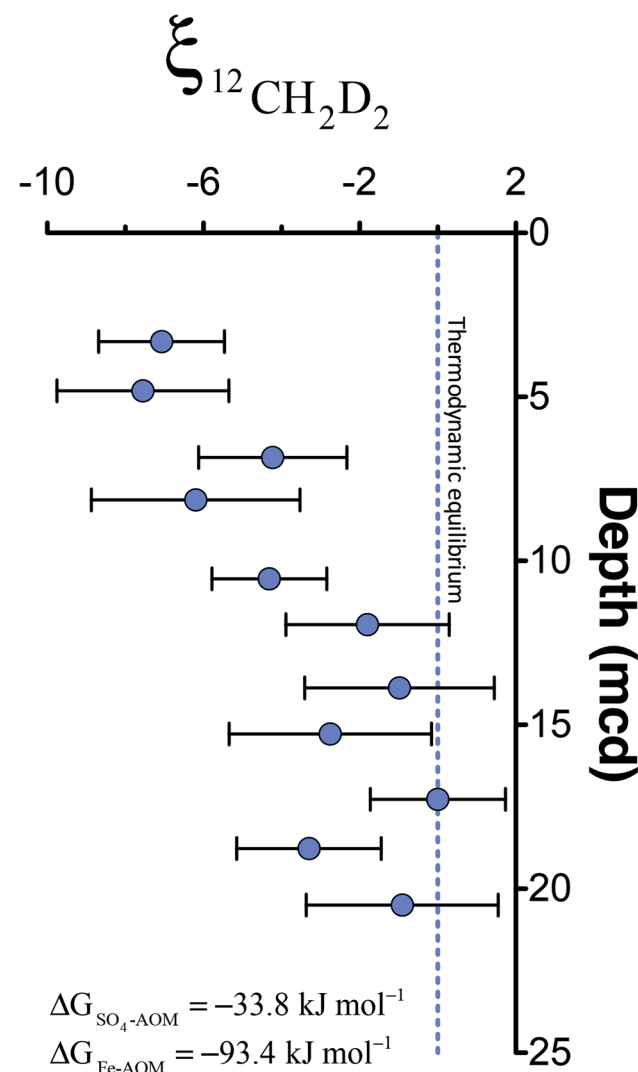
Here, we examine microbial methane from the Bornholm Basin (Baltic Sea; see Supplementary Information) to test the hypothesis that methane remaining after anaerobic oxidation is in thermodynamic equilibrium due to reversibility of the initial step of AOM. We compare methane clumped isotope composition to geochemical profiles (Andr n *et al.*, 2015; Egger *et al.*, 2017) and present day rates of methanogenesis and methanotrophy calculated with a multicomponent diagenetic model (Dijkstra *et al.*, 2018) to determine parameters controlling the isotopic composition of methane (Fig. 1a-d). Methane  $\delta^{13}\text{C}$  and  $\delta\text{D}$ ,  $\delta^{13}\text{C}_{\text{DIC}}$ ,  $\delta^{13}\text{C}_{\text{TOC}}$  and  $\delta\text{D}_{\text{H}_2\text{O}}$  vary non-monotonically over 30 metres below seafloor (Fig. 1g,h; Fig. S-1) while  $\Delta^{13}\text{CH}_3\text{D}$  and  $\Delta^{12}\text{CH}_2\text{D}_2$  values increase with depth (Fig. 1e,f).

Comparisons of the bulk isotopic composition of methane to carbon and hydrogen reservoirs at this location are complicated by the change from limnic to marine deposition (Egger *et al.*, 2017). For instance, the hydrogen isotopes in methane are seemingly not equilibrated with porewater that changes by >100 ‰ in this interval (Fig. S-1), obscuring the history of this reservoir. Instead, we will focus on the intra-molecular isotopic equilibrium of the methane itself using clumped isotopes, thus sidestepping the complications in bulk isotope ratios. We introduce  $\xi^{12}\text{CH}_2\text{D}_2$ , a parameter that quantifies the degree of thermodynamic disequilibrium for a methane system.  $\xi^{12}\text{CH}_2\text{D}_2$  is analogous to the symbol for the reaction progress variable ( $\xi^{12}\text{CH}_2\text{D}_2 = \Delta^{12}\text{CH}_2\text{D}_2, \text{measured} - \Delta^{12}\text{CH}_2\text{D}_2, \text{equilibrium}$ , see Supplementary Information). Values for  $\xi^{12}\text{CH}_2\text{D}_2$  in upper sediments as low as -7.5 ‰ are significantly different (>6  $\sigma$ ) from 0 ‰ (*i.e.* thermodynamic equilibrium) which is approached with depth (Fig. 2). In order to determine how methanogenesis and methanotrophy influence this transition, we consider evidence for and against reversibility in each metabolism.

Methanogenesis in intra-molecular isotopic thermodynamic equilibrium requires reversibility at the final hydrogen addition step. Trace methane oxidation (TMO) does occur during methanogenesis, but altering the concentration of  $\text{H}_2$  or terminal electron acceptors necessary for oxidising methane does not increase TMO and has never been shown to consume greater than ~3 % of the  $\text{CH}_4$  produced (Moran *et al.*, 2005; Moran *et al.*, 2007). Although some of the first steps of methanogenesis may be reversible (Valentine *et al.*, 2004), the final transfer of the methyl group to coenzyme M and the subsequent final hydrogen addition are believed to be irreversible (G rtner *et al.*, 1994; Thauer, 2011). Work on determining the biochemical pathways of Fe(III) reducing ANME has so far required genetically modifying a methanogen, *M. acetivorans* with the Mcr from an ANME-1 group organism because unmodified *M. acetivorans* are unable to be cultured on methane without the modified Mcr (Soo *et al.*, 2016; Yan *et al.*, 2018).

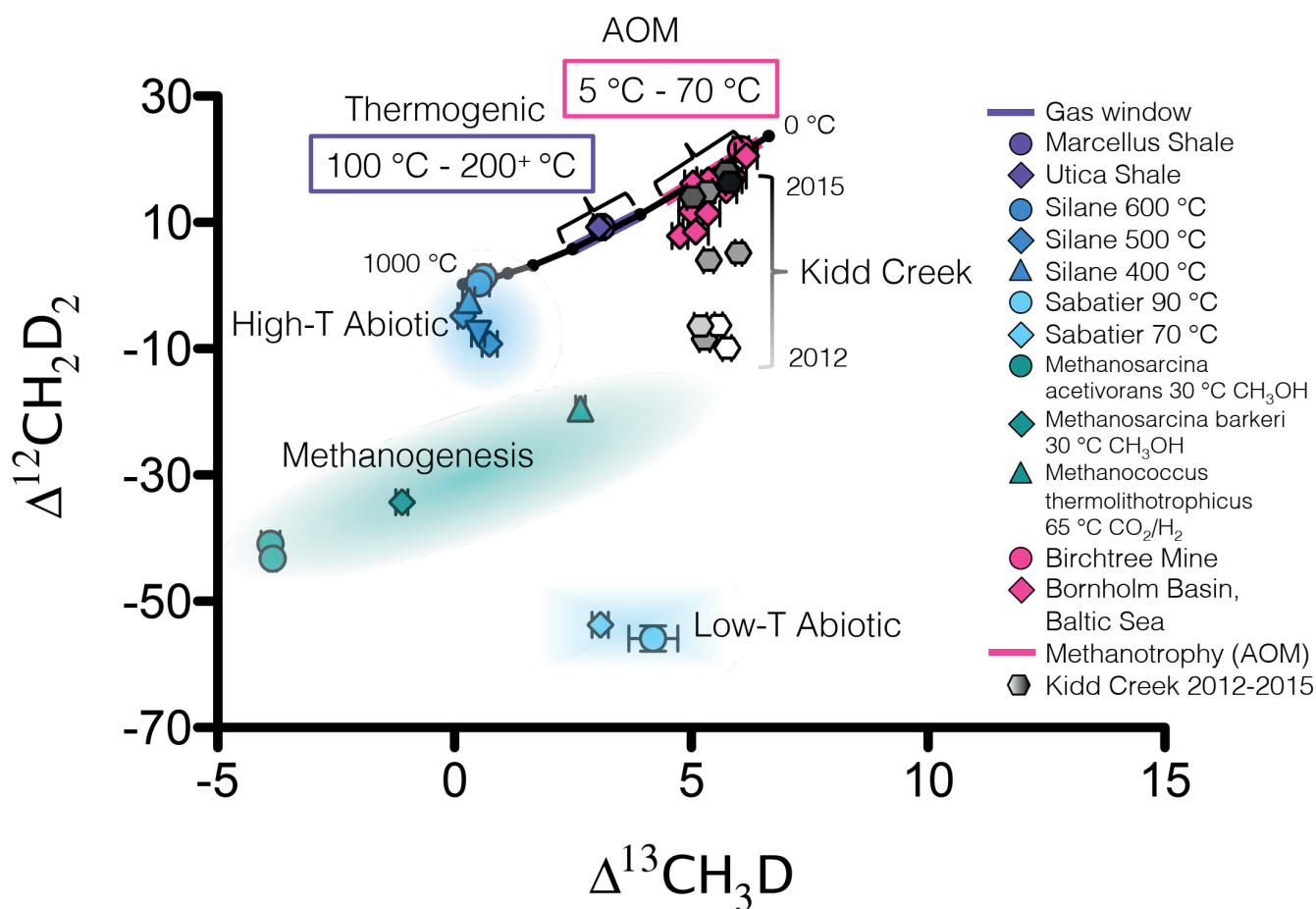
In contrast, AOM is known to have an enzymatic back reaction that suggests the first step of anaerobic methanotrophy is partially reversible, providing a potential mechanism for equilibrating methane isotopologues with environmental temperatures. This back reaction is sensitive to concentrations of the terminal electron acceptor  $\text{SO}_4^{2-}$  (Holler *et al.*, 2011; Yoshinaga *et al.*, 2014). At high concentrations of  $\text{SO}_4^{2-}$ , the back reaction produces 3-7 % of the  $\text{CH}_4$  consumed by AOM, and at low concentrations of  $\text{SO}_4^{2-}$  (below 0.5 mM), back reaction can produce as much as 78 % of the  $\text{CH}_4$  consumed by AOM (Yoshinaga *et al.*, 2014; Timmers *et al.*, 2017). Intra-cellular exposure of methane to Mcr can lead to isotope exchange catalysis during bond rupture and reformation (Marlow *et al.*,

2017), and methane that diffuses back out of the cell could move an environmental reservoir of methane towards thermodynamic equilibrium. Using estimates of AOM rates from Dijkstra *et al.* (2018), we calculate the timescale of this equilibration (see Supplementary Information) to be on the order of  $10^3$ - $10^4$  years, consistent with the estimated age of methane at 20 MCD of <~8000 years (Figs. S-1, S-2). Such timescales would vary in environments with differing rates of AOM.



**Figure 2**  $\xi^{12}\text{CH}_2\text{D}_2$  values ( $\pm 2 \sigma$ ) are the most negative at shallow MCD, indicating the greatest departure from equilibrium and approach zero (violet dashed line) with increasing MCD, indicating an approach towards intra-species thermodynamic equilibrium.  $\Delta G$  values for  $\text{SO}_4$ -AOM and Fe-AOM suggest either metabolism is capable of reversibility (see Supplementary Information).

Our findings suggest that when terminal electron acceptors are limiting, equilibrium bond ordering may be used to identify AOM activity even when the bulk isotopic composition of reservoir material is changing and ambiguous. Methane from Kidd Creek Mine (Ontario, Canada) also shows methane isotopologues transitioning with time from non-equilibrated to equilibrated (Fig. 3). This suggests that as energy-rich fractures open, Mcr exposure during the initial step of AOM catalytically exchanges isotopes leaving behind a reservoir that moves closer to thermodynamic equilibrium (Young *et al.*, 2017).



**Figure 3**  $\Delta^{12}\text{CH}_2\text{D}_2$  is plotted versus  $\Delta^{13}\text{CH}_3\text{D}$ . The solid black line represents theoretical thermodynamic equilibrium abundances (with dots representing 100 °C increments from 0-1000 °C). Methane produced by thermogenesis, high temperature abiotic reactions, microbial methanogenesis and low temperature abiotic reactions inhabit unique zones in double isotopologue space. AOM, equilibrating through exchange catalysis during enzymatic back reaction, also inhabits a unique zone: low temperature intra-species thermodynamic equilibrium. Bornholm Basin data (magenta diamonds) span between the methanogenesis and AOM zones, while data from Kidd Creek Mine (black to white symbols) (Young *et al.*, 2017) span from the abiotic to AOM zone. See Supplementary Information for additional sample description.

Methane remaining after AOM is in low temperature equilibrium due to exchange catalysis by Mcr. Therefore, it has distinct  $\Delta^{13}\text{CH}_3\text{D}$  and  $\Delta^{12}\text{CH}_2\text{D}_2$  concentrations when compared to methane produced through other known mechanisms (thermogenesis, microbial methanogenesis and abiotic methanogenesis) (Fig. 3). AOM metabolisms have been shown to be active from -1 °C to 70 °C (Niemann *et al.*, 2006; Holler *et al.*, 2011), a range of temperatures that is unique and distinguishable from equilibrated thermogenic methane forming in the 100 °C to 250 °C gas window. Abiotic methane may also form at low temperatures, yet even when it is apparently equilibrated in  $\Delta^{13}\text{CH}_3\text{D}$ , it may exhibit large depletions in  $\Delta^{12}\text{CH}_2\text{D}_2$  that distinguish it from methane that has undergone exchange catalysis during AOM. We suggest that when combined (as in the  $\xi_{12\text{CH}_2\text{D}_2}$  parameter),  $\Delta^{12}\text{CH}_2\text{D}_2$  and  $\Delta^{13}\text{CH}_3\text{D}$  values are sensitive indicators of the degree of thermodynamic equilibrium that may be useful in determining the role of enzymatic back reaction during AOM in the global methane cycle.

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## Additional Information

Supplementary Information accompanies this letter at <http://www.geochemicalperspectivesletters.org/article1910>.



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