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2

3 **Extreme methane clumped isotopologue bio-signatures of aerobic and anaerobic**  
4 **methanotrophy: insights from the Lake Pavin and the Black Sea sediments**

5

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17

18 **Abstract**

19 **Microbial methane oxidation - or methanotrophy - is a key control of the global methane**  
20 **budget on Earth, and perhaps in other planetary systems. Here, we explore the potential role of**  
21 **mass-18 isotopologues of methane, expressed as  $\Delta^{13}\text{CH}_3\text{D}$  and  $\Delta^{12}\text{CH}_2\text{D}_2$  values, in tracking both**  
22 **aerobic and anaerobic methanotrophy in nature. We examine two well documented**  
23 **methanotrophic environments: the Lake Pavin (France) water column, where methane**  
24 **degradation is dominated by aerobic methanotrophy (AeOM), and the Black Sea sediments**  
25 **(offshore Romania), dominated by anaerobic methanotrophy (AOM) coupled to sulfate-reduction.**  
26 **In both settings, lighter isotopologues are preferentially consumed, generating elevated**  
27  **$^{13}\text{CH}_4/^{12}\text{CH}_4$ ,  $^{12}\text{CH}_3\text{D}/^{12}\text{CH}_4$ ,  $^{13}\text{CH}_3\text{D}/^{12}\text{CH}_4$  and  $^{12}\text{CH}_2\text{D}_2/^{12}\text{CH}_4$  ratios. This results in increasing of**  
28  **$\delta^{13}\text{C}$  and  $\delta\text{D}$  values in the residual methane for both settings, as observed commonly in systems**  
29 **dominated by methanotrophy. As a result, AeOM and AOM cannot be easily distinguished by the**  
30 **development of  $\delta^{13}\text{C}$  and  $\delta\text{D}$ . In contrast, the  $\Delta^{13}\text{CH}_3\text{D}$  and  $\Delta^{12}\text{CH}_2\text{D}_2$  (departure from stochastic)**  
31 **values have opposite trajectories, with minimal decreases in the case of the AeOM-dominated**  
32 **system, but dramatic increases in the case of AOM, with  $\Delta^{13}\text{CH}_3\text{D}$  and  $\Delta^{12}\text{CH}_2\text{D}_2$  reaching values**

33 as high as 15.7 ‰ and 76.6 ‰, respectively. This contrasting behavior of clumped isotopologues  
34 signatures illustrates fundamental distinction between the two processes and the way they  
35 segregate methane isotopologues. These data demonstrate that both AeOM and AOM have  
36 distinctive kinetic isotope effects in natural settings, consistent with preliminary laboratory work.  
37 In particular, we find that  $\gamma$ -values (which measure the deviation to the product of ‘normal’ bulk  
38 isotope fractionation factors) are close to unity in the case of AeOM (i.e. a negligible clumped  
39 isotope effect), but significantly below unity in the case of AOM (i.e. strong clumped isotope effect).  
40 In addition, our data also illustrate how AOM under low-sulfate conditions may promote methane  
41 isotopologue equilibration. Taken together, we suggest these data and apparent isotopologue  
42 fractionation factors extrapolated from these two environments may help refine the potential bio-  
43 signatures of methane affected by methanotrophy.

44

## 45 1. Introduction

46 Methane (CH<sub>4</sub>) is the simplest reduced form of carbon and is presumably involved in  
47 biological reactions nearly since life appeared on our planet (Ciccarelli *et al.*, 2006; Weiss *et al.*, 2016;  
48 Russell and Nitschke, 2017). Although there are abiotic pathways for both production and degradation  
49 of methane, it is considered that most of methane cycling on Earth is biologically-mediated. Microbial  
50 methanogenesis (*i.e.* methane production) is an ubiquitous process and has been recognized in diverse  
51 range of anoxic environments, from wetlands and lakes (Borrel *et al.*, 2012; Lopes *et al.*, 2015), to marine  
52 and terrestrial sediments (*e.g.* Oremland and Taylor, 1978, Schoell, 1988; Martini *et al.*, 1996; Whiticar,  
53 1999, Inagaki *et al.*, 2015), to deep within cratonic basement rocks (*e.g.* Sherwood Lollar *et al.*, 1993,  
54 Ward *et al.*, 2004). Microbial methanogenesis is performed by a diverse group of Archaea referred to as  
55 methanogens, and is often the final step of organic matter degradation. On the other hand, microbial  
56 methanotrophy (*i.e.* methane degradation) may also be regarded as a terminal step of organic matter  
57 degradation, to the extent methane being produced is eventually oxidized into CO<sub>2</sub> or assimilated into  
58 cellular biomass. Methanotrophy is also a ubiquitous process, mediated by distinct communities of  
59 Bacteria and Archaea. The Aerobic Oxidation of Methane (AeOM) occurs by activity of a group of  
60 obligatory methanotrophic bacteria utilizing O<sub>2</sub> as the terminal electron acceptor (McDonald *et al.*, 2008;  
61 Trotsenkoto and Murrell, 2008) as follows:

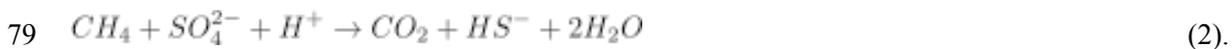
62



64

65 Aerobic methanotrophy is the dominant pathway for methane oxidation in environments where oxygen  
66 is available such as in marine or lacustrine water columns (Schubert *et al.*, 2016; Lopes *et al.*, 2011;  
67 Oswald *et al.*, 2016) or in soils and wetlands (Chowdhury and Dick, 2013). The Anaerobic Oxidation of  
68 Methane (AOM) is performed in anoxic conditions, mostly by Anaerobic Methane-Oxidizing Archaea  
69 (ANME). These microorganisms can catalyze AOM by reversing the CO<sub>2</sub> reducing methanogenesis  
70 pathway (Hallam *et al.*, 2004), and may utilize, in syntrophy with other microbial partners, a variety of  
71 terminal electron acceptors including sulfate (Boetius *et al.*, 2000; Orphan *et al.*, 2001), nitrate  
72 (Raghoebarsing *et al.*, 2006; Harron *et al.*, 2013) or metal oxides (Beal *et al.*, 2009; Cai *et al.*, 2018; Leu  
73 *et al.*, 2020). The sulfate-dependent AOM was first to be recognized in shallow marine sediments, in  
74 pronounced Sulfate-Methane Transition Zones (STMZ), (Barnes and Goldberg, 1976; Iverson and  
75 Jørgensen, 1985; Alperin *et al.*, 1988; Hoehler *et al.*, 1994; Hinrichs *et al.*, 1999) and is now considered  
76 to be a major methane sink in the oceans (Hinrichs and Boetius, 2002; Reeburgh, 2007). There, ANME  
77 thrive in syntrophy with sulfate-reducing bacterial partners, which result in the following net reaction:

78



80

81 The role of AOM mediated by microbial consortia has now been proposed and/or identified in many  
82 other environments such as in sedimentary gas reservoirs (Martini *et al.*, 2003; Meng *et al.*, 2017; Giunta  
83 *et al.*, 2019), in lake sediments (Martinez-Cruz *et al.*, 2017; Weber *et al.*, 2017), in fracture fluids from  
84 cratonic rocks (Simkus *et al.*, 2016; Lau *et al.*, 2016; Warr *et al.*, 2021) or in certain lakes as well as in  
85 marine water columns (Wakeham *et al.*, 2003; Eller *et al.*, 2005; Lopes *et al.*, 2011). AOM is also  
86 considered as a plausible metabolism for life in the subsurface of Mars or Titan as well (Norman, 2011;  
87 Marlow *et al.*, 2014; House *et al.*, 2022). Overall, both AeOM and AOM metabolisms are considered  
88 critical processes for regulating the global methane budget on Earth. Therefore, it is crucial to develop  
89 tools to better identify and constrain the magnitude of these processes in the various natural settings  
90 where they might be at play.

91 Bulk carbon and hydrogen stable isotopes are commonly used to explore methane-based  
92 metabolisms. The methane bulk isotopic compositions for <sup>13</sup>C/<sup>12</sup>C and D/H are expressed in ‰ relative  
93 to international standards according to the classic δ-notation:

94

$$\delta^{13}C = \frac{(^{13}C/^{12}C)_{Sample}}{(^{13}C/^{12}C)_{VPDB}} - 1 \quad (3)$$

96

97 and

$$\delta D = \frac{(D/H)_{Sample}}{(D/H)_{VSMOW}} - 1 \quad (4)$$

99

100 where VPDB is the Vienna Pee Dee Belemnite, and VSMOW is Vienna Standard Mean Oceanic Water.  
 101 An empirical knowledge of ‘typical’ methanogenic or methanotrophic isotopic signatures as well as  
 102 comparisons of isotope fractionation factors with those measured in laboratory cultures have been  
 103 commonly used to explore both sources and sinks of methane in nature (*e.g.* Bernard *et al.*, 1976; Schoell,  
 104 1988; Whiticar, 1999; Milkov and Etiope, 2018). However, in many instances, the co-existence of various  
 105 sources of methane (whether biotic or abiotic) and of metabolisms at play can blur the isotopic signatures  
 106 of these sources and sinks of methane, making interpretation of the bulk isotope ratios challenging (*e.g.*  
 107 Miller *et al.*, 2016; Etiope, 2017). Furthermore, because the  $\delta^{13}C$ -CH<sub>4</sub> and  $\delta D$ -CH<sub>4</sub> crucially depend on  
 108 the isotopic signatures of the carbon and hydrogen precursors, which are not necessarily identified nor  
 109 accessible for sampling, the diagnosis of methane’s origin remains challenging, especially in deep  
 110 biosphere environments. What is more, isotope fractionations observed in nature often differ from those  
 111 in laboratory studies (see Okumura *et al.*, 2016 and reference therein).

112 Recently, advances in high-resolution mass-spectrometry and in laser adsorption spectroscopy  
 113 allowed the measurement of the methane, ‘clumped’, doubly-substituted isotopologues <sup>13</sup>CH<sub>3</sub>D (Stolper  
 114 *et al.*, 2014a; Ono *et al.*, 2014; Young *et al.*, 2016) and <sup>12</sup>CH<sub>2</sub>D<sub>2</sub> (Young *et al.*, 2016; Eldridge *et al.*,  
 115 2019; Gonzales *et al.*, 2019). This novel approach allows the investigation of isotope bond ordering in  
 116 methane molecules. At thermodynamic equilibrium these relative abundances of multiply-substituted  
 117 isotopologues depend on the temperature. The ‘clumped’ isotopologue compositions are reported versus  
 118 a stochastic distribution (Wang *et al.*, 2004), representing a perfectly random distribution of all of  
 119 isotopes comprising all isotopologues that would occur at an infinite temperature, and expressed in per  
 120 mil using the capital delta notation:

121

$$\Delta^{13}CH_3D = \frac{(^{13}CH_3D/^{12}CH_4)_{Sample}}{(^{13}CH_3D/^{12}CH_4)_{Stochastic}} - 1 \quad (5)$$

123

124 and

125

$$\Delta^{12}\text{CH}_2\text{D}_2 = \frac{(^{12}\text{CH}_2\text{D}_2/^{12}\text{CH}_4)_{\text{Sample}}}{(^{12}\text{CH}_2\text{D}_2/^{12}\text{CH}_4)_{\text{Stochastic}}} - 1 \quad (6)$$

126

127 In the ‘clumped’ systematic,  $\Delta$  values are internally referenced to the stochastic distribution of the sample.

128 This formalism for reporting the abundances of  $^{13}\text{CH}_3\text{D}$  and  $^{12}\text{CH}_2\text{D}_2$  has the advantage that it depends

129 solely on the composition of methane itself. At equilibrium, these abundances reflect intra-species

130 thermometers. So far, measurements of  $\Delta^{13}\text{CH}_3\text{D}$  and  $\Delta^{12}\text{CH}_2\text{D}_2$  have provided constraints on the

131 temperature history experienced by methane in various geological contexts, from sedimentary

132 environments dominated by thermogenic methane (*i.e.* thermocatalytic breakdown of organic

133 matter)(Stolper *et al.*, 2014b; 2015; 2017; Wang *et al.*, 2015; Douglas *et al.* 2016; Young *et al.*, 2017;

134 Gruen *et al.*, 2018; Giunta *et al.*, 2019; 2021) to abiotic methane in hydrothermal systems (Wang *et al.*,

135 2018; Labidi *et al.*, 2020) or in cold crystalline environments (Wang *et al.*; 2015; Young *et al.*, 2017).

136 They are also useful as a means of tracing methane-based metabolisms. In laboratory cultures, both

137 methanogens and methanotrophs appear to produce substantial disequilibrium signatures that could

138 facilitate the recognition and quantification of these metabolisms (*e.g.* Stolper *et al.*, 2015; Wang *et al.*,

139 2015; Douglas *et al.*, 2016; Wang *et al.*, 2016; Young *et al.*, 2017; Giunta *et al.*, 2019; Ono *et al.*, 2021).

140 These exotic signatures have been recognized in certain natural environments, but there are numerous

141 others where  $\Delta^{13}\text{CH}_3\text{D}$  and  $\Delta^{12}\text{CH}_2\text{D}_2$  instead seem to reflect a near-equilibration of the methane at

142 environmental temperatures, raising questions on how *in situ* substrate availability, growth rates and

143 complex symbiotic ecosystems may affect clumped isotopologue signatures in nature.

144

145 The aim of this study is to further explore the distinction between AeOM and AOM as it

146 manifests in the relative abundances of residual methane isotopologues. In particular, we aim to better

147 characterize the isotopic effects associated with the narrow transition horizon between methanogenesis

148 and methanotrophy. We investigate two well characterized natural settings: the meromictic Lake Pavin

149 in central France, and shallow sediments from the Black Sea. Both settings display methanogenic to

150 methanotrophic transition depth-profiles, though with different regimes of methanotrophy. The Lake

151 Pavin water column is dominated by AeOM (Biderre-Petit *et al.*, 2011; Lopes *et al.*, 2011), whereas the

152 Black Sea sediments show evidence of sulfate-dependent AOM (*e.g.* Jørgensen *et al.*, 2001, 2004;

153 Michaelis *et al.*, 2002; Treude *et al.*, 2005). In this study, we expand on concepts and ideas that have

154 been developed by some recent studies (*e.g.* Yoshinaga *et al.*, 2014; Wang *et al.*, 2016; Ash *et al.*, 2019)  
155 to explore on how methanotrophy may affect isotopic and isotopologue signatures, and how methane  
156 ‘clumped’ isotopologues might be used to identify and quantify the activity of these two metabolisms in  
157 natural environments.

158

## 159 **2. Site description**

### 160 **2.1. Lake Pavin (France)**

161 Lake Pavin is a meromictic crater lake located in the volcanic area of the Massif Central in  
162 France (**Fig. 1**). The crater’s formation is associated with the most recent volcanic activity in the area  
163 (*ca.* 7000 years ago, see Chapron *et al.*, 2010 and references therein) and is mostly composed of basaltic,  
164 trachyandesitic and granitic rocks. The lake is at an altitude of 1197 m above sea level and has a mean  
165 diameter of 750 m (area of 0.445 km<sup>2</sup>) for a maximum depth ( $D_{\max}$ ) of 92 m. This particular geometry  
166 (great depth for limited surface area) has favored the development of permanent stratification (*i.e.*  
167 meromixis) of the lake water-column which is best evidenced by strong physico-chemical gradients with  
168 depth (*e.g.* temperature, conductivity, dissolved O<sub>2</sub>) (Michard *et al.*, 1994; Viollier *et al.*, 1997; Lopes *et*  
169 *al.*, 2011). In particular, the bottom layer of the lake (below ~ 60m depth), also referred as the  
170 monimolimnion, is a dense and strictly anoxic layer that is presumably not affected by the seasonal  
171 mixing impacting the overlying waters (referred as mixolimnion). This deep layer is considered to be at  
172 steady-state (Viollier *et al.*, 1997; Aeschbach-Hertig *et al.*, 2002) and is home for elevated concentrations  
173 of reduced compounds, in particular methane, whose concentrations can reach as high as 4 mM at the  
174 bottom of the lake and up to 8 mM in the underlying sediment (Lopes *et al.*, 2011; Borrel *et al.*, 2012).

175

### 176 **2.2. Black Sea sediment (offshore Romania)**

177 The Black Sea is a marginal sea that bridges the European and Asian continents (**Fig. 1**). It is  
178 indirectly connected to the Atlantic Ocean through the Marmara and the Mediterranean Seas. The Black  
179 Sea was mostly composed of a freshwater lake during last Pleistocene glaciation and its reconnection to  
180 the open sea through the shallow Bosphorus about 9000 years ago resulted in an inflow of (Atlantic)  
181 seawater, leading to its permanent water layer stratification. Freshwater is supplied by three main rivers,  
182 the Danube to the West, and the Dniepr and the Dniester to the North. These rivers deliver an important  
183 amount of organic carbon, the degradation of which has led to the enrichment of the water mass in  
184 hydrogen sulfide, making the Black Sea the largest anoxic basin on Earth. The west and the northwest

185 parts of the Black Sea consist in a vast continental shelf bounded by the Danube and Dniepr river deltas.  
186 This vast sea is characterized by widespread seafloor methane-rich gas emissions from the river mouth  
187 to the deep basin (e.g. Reeburgh *et al.*, 1991; Kessler *et al.*, 2006; Pape *et al.*, 2008). Accordingly,  
188 methane concentrations are generally elevated (up to several mM) in sediments and the water column.  
189 As per in other margin sedimentary environments, organoclastic sulfate reduction and methanogenesis  
190 are the dominant pathways for the degradation of organic matter in the sediment of the Black Sea  
191 (Jørgensen *et al.*, 2001, Egger *et al.*, 2016; Ruffine *et al.*, 2021). The methane, and its links with the sulfur  
192 and iron cycles, among others, has been extensively investigated over the past decades through the help  
193 of classical bulk isotopic tools (e.g. Jørgensen *et al.*, 2001, 2004; Treude *et al.*, 2005; Knab *et al.*, 2009;  
194 Egger *et al.*, 2016).

195

### 196 **3. Methods**

#### 197 **3.1. Sampling procedures**

198 The main objective of this study is to measure the abundances of rare isotopologues of  
199 methane,  $^{13}\text{CH}_3\text{D}$  and  $^{12}\text{CH}_2\text{D}_2$ , throughout vertical profiles where microbial methane oxidation is taking  
200 place. In order to put constraints on isotope fractionation factors associated to such reactions, one needs  
201 to measure isotopic compositions of methane over a large range of methane concentrations. Thus, being  
202 able to measure isotope ratios, especially clumped isotopologue ratios, in the main degradation intervals  
203 is challenging, because methane concentrations are low, necessitating the sampling of large volumes of  
204 water and/or of sediments in order to obtain the minimum amount of  $\text{CH}_4$  required for analyses ( $> 20$   
205  $\mu\text{moles}$ ). Though the sampling procedures were different for the Lake Pavin (sampling of the water  
206 column) than for the Black Sea sediments (sub-sampling of fresh sediment cores), both procedures were  
207 adapted from well-described techniques for sampling methane for methane bulk isotopic ratio  
208 measurements ( $\delta^{13}\text{C}$  and  $\delta\text{D}$ ).

209

210 Lake Pavin sampling: The Lake Pavin water-column was sampled at different depths using a  
211 20 L Niskin bottle from a barge located in the middle of the lake, where it reaches its greatest depth (~  
212 93 m). For each sampled depth, the filled Niskin bottle is recovered on the barge and allowed to overfill  
213 different type of glass bottles, some dedicated to methane concentration analyses, some dedicated to  
214 isotopic analyses. Samples for methane concentration measurements were collected in 160 mL  
215 borosilicate glass serum vials, and were quickly closed with blue butyl rubber stopper and aluminium

216 seal. Depending on expected methane concentrations (based on [Lopes et al., 2011](#)), samples for isotopic  
217 analyses were collected in threaded borosilicate glass bottles of various sizes (from 150 to 2000 mL) and  
218 were quickly closed with butyl rubber stoppers and open screw caps. All water samples were then  
219 poisoned with 1 to 5 mL of saturated HgCl<sub>2</sub> solution, which was added through septa with a syringe and  
220 a connected open needle to allow purging an equivalent volume of sample. In laboratory, a helium head-  
221 space corresponding to ~20% of the total volume was generated in each glass bottle. Meanwhile, the lake  
222 sediment was also sampled by using a UWITEC corer (D9 # L60 cm). The muddy sediment recovered  
223 from the core was then sub-cored between 40-44 cm and 48-52 cm depth with a 50 mL pre-cut plastic  
224 syringe and added to a 150 mL threaded glass bottle, then closed with massive butyl septa and open screw  
225 cap. Note that these sediment samples therefore had a head-space consisting of air.

226

227 *Black Sea sampling:* The Black Sea sediments were sampled during Envri Methane cruise  
228 (April 2019, <https://campagnes.flotteoceanographique.fr/campagnes/18001189/>) on board the R/V *Mare*  
229 *Nigrum* operated by GeoEcoMar Romania. The aim of the Envri Methane cruise was to develop a joint  
230 pilot experiment to measure methane transfer from the seafloor to the atmosphere ([Grilli et al., 2021](#)).  
231 Fresh sediments were sampled by gravity core at a water depth of *ca.* 110 m (**Fig 1a**). Gravity cores GC-  
232 01 and GC-02 allowed the recovery of 139 and 142 cm of fresh sediment, respectively. Both cores were  
233 dedicated to the investigation of methane isotopologues in the pore-water column. On board, each core  
234 was cut into several sections, allowing sub-sampling of the sediment on fresh cut surfaces using 50 mL  
235 pre-cut plastic syringes. For each depth interval, a volume of 200 cm<sup>3</sup> of sediment or of 400 cm<sup>3</sup> was  
236 collected and rapidly put into a 500 mL or a 1000 mL borosilicate glass bottle (see **Fig. S1** for more  
237 details). Samples were subsequently all poisoned with addition of 100 or 200 mL of NaOH (2M) solution,  
238 before sealing the glass bottles with butyl rubber stopper and an open screw caps. Each glass bottle was  
239 then stored upside down at 4 °C. In the meantime, additional samples were also collected for analyses of  
240 dissolved species concentration in the pore-water, as well as for measuring the methane concentration.  
241 The sampling of pore waters for solute concentration analyses was achieved by using Rhizon samplers  
242 that were placed in the middle of each core section. Rhizon samplers consist of a hydrophilic porous  
243 polymer tube with an inner diameter of 2.5 mm and a length of 50 mm ([Seeberg-Elverfeldt et al., 2005](#)).  
244 The recovered pore-water, whose volume was generally between 5 and 15 mL, was then filtered (0.2µm)  
245 and collected into a Nalgene bottle that was pre-acidified with 0.5 mL of HNO<sub>3</sub> (16N). Meanwhile,  
246 sediment samples for the determination of the methane concentration as well of δ<sup>13</sup>C values with Picarro

247 analyzer were also collected, by adapting the standard IODP procedure (Andr n *et al.*, 2015). Fresh  
248 sediment was sub-sampled with a 3 mL pre-cut plastic syringe and added into a 20 mL glass serum vial  
249 together with 8 mL of NaOH (2M) solution. The serum vial is then rapidly sealed with a septum and an  
250 aluminum seal and stored upside down. The sediment sub-sampling for methane concentration  
251 measurement was done on fresh cut surfaces between each section (*i.e.* together with larger sub-sampled  
252 volumes for rare isotopologue analyses, see Supp.), whereas for GC-02, the sub-sampling was done at  
253 the same depth where the Rhizon samplers were installed.

254

### 255 **3.2. Concentration measurements**

256 Methane analysis were carried out by gas chromatography (Perichrom® Pr2250 and Pr2100)  
257 equipped with an FID detector and with manual injections for Lake Pavin samples and by autosampler  
258 (Dani HSS 86.50) for the Black Sea samples. Methane samples were left to equilibrate during at least 2  
259 hours at room temperature according to the procedure of Abril and Iversen, (2002) before analysis. Sam-  
260 ples from the Lake Pavin were then quickly injected in the GC Perichrom Pr2250 using a Hamilton  
261 gastight sample lock 100 µL syringe. Head space samples from the Black Sea were heated to 60° C  
262 during 10 min with the DANI autosampler and then injected in the GC Pr 2100. For both types of sam-  
263 ples, methane was separated on a Perichrom® Porapak Q (80/100 mesh) packed column with a length of  
264 2m and an internal diameter of 1/8 inch. The retention time for methane is 1.4 min. Once eluted, methane  
265 was burned inside the FID in a hydrogen / air flame at 250°C. Analyses were recorded using the Winilab  
266 software. Methane concentrations were calculated using the solubility coefficients for methane from the  
267 literature (Yamamoto *et al.* 1976). The repeatability of measurements is 3%. The limit of quantification  
268 for methane is of *ca.* 5 nmol.L<sup>-1</sup>.

269 The dissolved oxygen (DO) profiles in the Lake Pavin were acquired with a multiparameter  
270 CTD probe (EXO 2), equipped with an optical O<sub>2</sub> sensor (optode). Calibration was performed for local  
271 saturation of 100% O<sub>2</sub> in water vapor saturated air at lake surface pressure and response for 0% sat. was  
272 checked in a 10%wt solution of Na<sub>2</sub>SO<sub>3</sub>. The detection limit for dissolved O<sub>2</sub> is *ca.* 0.1% or about 0.3 ±  
273 0.2 µM.

274

### 275 **3.3. Methane pre-concentration and purification**

276 For samples collected for clumped isotope analyses, the methane was essentially ‘diluted’ in  
277 rather large head-space volumes dominated by He for the lake Pavin samples, or dominated by air for

278 the Black Sea sediments. This required quantitative pre-concentration of the methane in smaller volumes  
279 for shipping and future analyses of clumped isotopes ratios at UCLA. We developed a vacuum line  
280 interfaced with a gas-chromatograph (Agilent 7890A) equipped with a PorapakQ of 2m length and a  
281 TCD at Ifremer. The sample is introduced onto the purification line via a purge-and-trap method. In this  
282 method, the entire volume of the head-space is flushed with a He carrier-gas (flow-rate of 80 mL/min)  
283 into a liquid nitrogen trap filled with active charcoal. The trap was designed in a U-shape using a stainless  
284 steel tube measuring 30 cm in length with a ¼ inch outside diameter. This rather large trapping volume  
285 was chosen to allow for quantitative trapping of methane, having in mind that air (N<sub>2</sub> + O<sub>2</sub>) in head-space  
286 volumes of Black Sea samples would also partially condensate on active charcoal at liquid nitrogen  
287 temperature. Also, in order to avoid carrying too much moisture that could potentially form ice that would  
288 plug the line, the liquid nitrogen trap was preceded by a cylindrical volume filled with Drierite®. After  
289 10 min of purging the sample head-space, the trap is isolated from the rest of the line and the He-excess  
290 is pumped. The trap is then warmed up to 80 °C and a secondary He carrier gas is used to flush (flow-  
291 rate of 25 mL/min) the released towards GC which is warmed up at a constant temperature of 35 °C. The  
292 GC allows relatively good separation of air and methane. The peak of air starts eluting after 2 min and is  
293 flushed away, then the peak of methane is released after 5 min and is collected at the exit of the GC on a  
294 second liquid nitrogen trap (same design than first one) for 10 min. After isolation and pumping of He-  
295 excess in the second trap, the gas was finally transferred into an evacuated glass vial of 10 cc volume  
296 one third filled with active charcoal. Note however that for the Black Sea samples, the air content was  
297 too elevated for quantitative separation of methane and air in one single pass through the GC (air still  
298 being released at the time of methane elution), thus necessitating a second pass through the GC. In such  
299 case, the content of the second trap (mixture of air and methane) was thus transferred back to the first  
300 trap and then re-processed through the GC, allowing for complete separation of air and methane prior to  
301 collection of the methane in the evacuated glass tube of filled with active charcoal. At UCLA, these pre-  
302 concentrated (and purified) gas samples were purified once again on the lab vacuum line prior to  
303 introduction on the *Panorama*, following the standard UCLA method already outlined in [Young et al.,](#)  
304 [\(2017\)](#).

305

### 306 **3.4. Isotopologue ratio measurements**

307 *Picarro* ( $\delta^{13}\text{C}$ ):  $\delta^{13}\text{C}$ -CH<sub>4</sub> analysis were carried out with a G2201-i Cavity Ring-Down Spectrometer  
308 (CRDS) from Picarro (Santa Clara, USA) equipped with a Small Sample Isotope Module (SSIM) for low

309 gas injections (between 25 to 100  $\mu\text{L}$ ). The instrument was used with the High Range (HR) mode and  
310 the gas volume was adjusted to have a methane concentration ranges between 10 and 1000 ppm at the  
311 CRDS cavity. Environmental gas samples analyzed in this study were injected directly inside the SSIM  
312 port using a gas-tight syringe (Brandily *et al.*, 2021). Three certified  $\delta^{13}\text{C}$ - $\text{CH}_4$  gas standards of -23.9, -  
313 38.3 and -66.5 ‰ were used for the isotopic calibration of the CRDS analyzer (Isometric Instruments,  
314 Victoria, Canada). These gas standards are composed of methane diluted in hydrocarbon-free air. The  
315 CRDS analyzer was calibrated by injecting 5 replicates of 5 mL of certified  $\delta^{13}\text{C}$ - $\text{CH}_4$  gas standards with  
316 the help of gastight syringes (Hamilton) and Tedlar® sampling bags (Restek). The residual standard  
317 deviation for the  $\delta^{13}\text{C}$ - $\text{CH}_4$  is less than 0.4%.

318

319 *Panorama* ( $\delta^{13}\text{C}$ ,  $\delta\text{D}$ ,  $\Delta^{13}\text{CH}_3\text{D}$  and  $\Delta^{12}\text{CH}_2\text{D}_2$ ): Methane gas samples were analyzed at UCLA to obtain  
320 methane isotopologue abundances. The ratios  $^{13}\text{CH}_3\text{D}/^{12}\text{CH}_4$ ,  $^{12}\text{CH}_2\text{D}_2/^{12}\text{CH}_4$ ,  $^{13}\text{CH}_4/^{12}\text{CH}_4$ , and  
321  $^{12}\text{CH}_3\text{D}/^{12}\text{CH}_4$  are measured with the prototype Nu Instruments Panorama, a high-resolution gas-source  
322 double-focusing mass spectrometer at UCLA. In order to measure  $^{12}\text{CH}_4^+$ ,  $^{13}\text{CH}_4^+$ ,  $^{12}\text{CH}_3\text{D}^+$ ,  $^{13}\text{CH}_3\text{D}^+$   
323 and  $^{12}\text{CH}_2\text{D}_2^+$  ion currents, the *Panorama* mass spectrometer is set to a mass resolving power equal to or  
324 greater than 40000. This allows resolving of the two mass-18 isotopologues ( $^{13}\text{CH}_3\text{D}$  and  $^{12}\text{CH}_2\text{D}_2$ ), both  
325 measured on the axial collector with an electron multiplier. Meanwhile, mass-16 and mass-17  
326 isotopologues are measured on Faraday collectors with amplifier resistors of  $10^{11} \Omega$ . Sample and  
327 reference bellows are adjusted so that ion current intensities are balanced. The current intensities are  
328 rebalanced after each measurement cycle. At first, the magnet is set to measure simultaneously  
329  $^{12}\text{CH}_3\text{D}^+/^{12}\text{CH}_4^+$  and  $^{12}\text{CH}_2\text{D}_2^+/^{12}\text{CH}_4^+$  ratios, with  $^{12}\text{CH}_2\text{D}_2^+$  (18.04385 amu) being measured on the axial  
330 collector. In a second setting, the magnet is set to measure  $^{13}\text{CH}_3\text{D}^+$  (18.04090 amu) on the axial collector,  
331 and  $^{13}\text{CH}_4^+/^{12}\text{CH}_4^+$  and  $^{13}\text{CH}_3\text{D}/^{12}\text{CH}_4^+$  ratios are measured simultaneously. Overall, the external  $1\sigma$  error  
332 ( $n=5$ ) including both the accuracy and the reproducibility is estimated to be  $\pm 0.1$  ‰ for  $\Delta^{13}\text{CH}_3\text{D}$ ,  
333  $\pm 0.8$  ‰ for  $\Delta^{12}\text{CH}_2\text{D}_2$ ,  $\pm 0.1$  ‰ for  $\delta^{13}\text{C}$ , and of approximately  $\pm 0.3$  ‰ for  $\delta\text{D}$ .

334

### 335 **3.5. Reactive transport modeling**

336 In order to infer isotope fractionation factors associated with methanotrophy, we developed a  
337 simple reactive transport model. We assume that at steady-state, the vertical distribution of the bulk  
338 methane concentration or of any of its isotopologue ( $^{12}\text{CH}_4$ ,  $^{13}\text{CH}_4$ ,  $^{12}\text{CH}_3\text{D}$ ,  $^{13}\text{CH}_3\text{D}$  or  $^{12}\text{CH}_2\text{D}_2$ ) can be  
339 described by the general second order differential equation:

340

341 
$$D_i^* \cdot \frac{\partial^2 C_i}{\partial x^2} + v \cdot \frac{\partial C_i}{\partial x} + R_i = 0$$
, (7)

342

343 where  $C_i$  is the methane isotopologue concentration of interest,  $D_i^*$  its effective diffusion coefficient  
344 taking into account the porosity structure and/or the tortuosity if diffusing in a porous medium like  
345 sediments (Boudreau, 1997) or eddy diffusivity in the water columns,  $v$  is the effective vertical velocity  
346 term, and  $R_i$  is the reaction rate for that isotopologue (source or sink). For modeling each methane  
347 isotopologue concentration with depth, we assume the reaction rates in the two settings can be described  
348 by a second-order rate equations. For the Lake Pavin waters we have

349

350 
$$R_i = k_i \cdot C_i \cdot C_{O_2}$$
, (8)

351

352 and for the Black Sea sediments,

353

354 
$$R_i = k_i \cdot C_i \cdot C_{SO_4^{2-}}$$
. (9)

355

356 In these formulations, the rate constants  $k_i$  are the only free parameters to be optimized in order to fit the  
357 data, and should be regarded as phenomenological rate constants for oxidation rather than as the constants  
358 for the elementary reaction steps comprising reactions (1) and (2). Reactive transport modeling is  
359 generally aimed at better quantifying and deciphering the various reactions rates affecting the methane  
360 and/or other dissolved compounds within the water or the sediment column (e.g. Lopes *et al.*, 2011;  
361 Egger *et al.*, 2016; 2017), and thus can require a more rigorous physical description of all potential  
362 reactions involving methane. However, the goal of the modeling performed here is to extract the effective  
363 isotope fractionation factors for these two distinct environments. Thus, simplifying the sinks of methane  
364 to a single, net oxidation reaction, is sufficient for this purpose. In each setting, the Eq. 7 is solved  
365 numerically for all isotopologues, using a finite difference method developed with a Python code. Two  
366 boundary conditions are defined as the two concentrations at the limits of the depth domain. At first, an  
367 effective rate constant for  $^{12}\text{CH}_4$  (i.e.  $^{12}\text{CH}_4 k$ ) is determined through least-square minimization between  
368 the model and the methane concentration profile where it is assumed that  $^{12}\text{CH}_4$  concentrations are equal  
369 to bulk methane concentrations (a valid assumption given natural isotopic abundances). Then, the

370 effective rate constants for singly- and doubly-substituted isotopologues are optimized through least-  
371 square minimization between the model and the isotopic data, making use of the results for  $^{12}\text{CH}_4$  and  
372 solving for the isotope fractionation factors according to  $^i k = \alpha \cdot ^{13}\text{CH}_4 k$ .

373

## 374 4. Results

### 375 4.1. Lake Pavin water column and sediment

376 The water column was sampled at several discrete depths, from below the lake surface down  
377 to 90 m water depth (near the bottom of the lake). From the bottom, the methane concentration decreases  
378 across the monimolimnion over a 35 m depth profile and with minimal value of of 15 nM at measured at  
379 51 m water depth. This methane concentration profile is consistent both in shape and amplitude with  
380 methane profiles published in previous studies (Lopes *et al.*, 2011; Jézéquel *et al.*, 2016). This indicates  
381 that the sampling protocol used here, which was required for collecting large volumes of water, did not  
382 yield measurable methane loss through degassing compared to more conservative syringe sampling  
383 methods (Lopes *et al.*, 2011; Jézéquel *et al.*, 2016). This is likely due to the low temperatures of the  
384 collected water ( $\sim 4\text{-}5^\circ\text{C}$ ), allowing methane to remain mostly soluble upon transfer from the Niskin  
385 bottle to the glass bottles. In contrast, the poor sediment cohesivity recovered through coring at the  
386 bottom of the lake did not allow precise measurement of the dissolved methane concentration in the  
387 sediment. Borrel *et al.*, (2012) have reported concentrations ranging between 6 and 9 mM, thus  
388 significantly above the maximum concentration reported the water column.

389 In the water column, the methane  $\delta^{13}\text{C}$  increases from  $-61\text{‰}$  at 90 m depth ( $[\text{CH}_4]=4.08\text{ mM}$ )  
390 to  $-46\text{‰}$  at 52 m water depth ( $[\text{CH}_4]=0.001\text{ mM}$ ). With the exception of the sample LP-90,  
391 measurements of the  $\delta^{13}\text{C}$  performed on the *Panorama* (together with other methane isotopologues) are  
392 all in good agreement with those performed on the *Picarro* analyzer (Fig. 2b). For sample LP-90  
393 however, there is a shift of nearly  $6\text{‰}$  between the two measurement techniques. The reason for this  
394 poor agreement is unknown. However, given the relative homogeneity of the data measured on the  
395 *Picarro* for LP-90, LP-80, LP-70 and LP-60 (all with  $\delta^{13}\text{C}$  near  $-60\text{‰}$ ), and the good agreement with  
396 LP-70 and LP-60 measured on the *Panorama*, we suspect that for LP-90 sample, the methane may have  
397 undergone fractionation upon incomplete transfer on the purification line prior to measurement on the  
398 *Panorama*. Because it was not possible to replicate this measurement, the *Panorama* measurement for  
399 PV-90 must be considered with the caveat that it may be in error.

400 Measurements for methane  $\delta D$ ,  $\Delta^{13}CH_3D$  and  $\Delta^{12}CH_2D_2$  were performed on the *Panorama*  
401 IRMS. The methane  $\delta D$  appears relatively stable at water depths of 90 m, 70 m and 60 m (despite the  
402 dubious measurement for PV-90), averaging  $-307 \pm 3$  ‰, and increases to  $-251$  ‰ at 54 m water depth  
403 (conc. = 0.005 mM) (**Fig. 2c**). Meanwhile,  $\Delta^{13}CH_3D$  and  $\Delta^{12}CH_2D_2$  are rather stable at 60 and 70 m water  
404 depth, averaging  $2.4 \pm 0.4$  ‰ and  $-17.6 \pm 0.1$  ‰ respectively, and decrease to  $0.7$  ‰ and  $-20.3$  ‰ at 55 m  
405 water depth. Note that sample collected at 90 m shows the most positive  $\Delta^{13}CH_3D$  and  $\Delta^{12}CH_2D_2$  values  
406 in the water column, of  $2.8$  ‰ and  $-12.3$  ‰ respectively. However, given the poor agreement for this  
407 sample between *Picarro* and *Panorama* measurements of  $\delta^{13}C$ , it is unclear how reliable these data are  
408 for this specific sample. The two samples collected from the lake sediment yield nearly identical methane  
409 isotopologue signatures, with  $\delta^{13}C$  of  $-65$  ‰,  $\delta D$  of  $-250$  ‰ and  $\Delta^{13}CH_3D$  and  $\Delta^{12}CH_2D_2$  of about  $5$  ‰  
410 and  $7.9$  ‰ respectively. Such methane isotopic composition in the sediment pore-waters is thus distinct  
411 from the methane collected in the water-column.

412

#### 413 4.2 Black Sea sediments

414 The dissolved methane from Black Sea shallow sediments was analyzed at a few discrete  
415 depths in two distinct cores of 139 cm and 142 cm length. In both cores, the dissolved methane  
416 concentration is observed to decrease from bottom to top, with concentration reaching up to 2.2 mM in  
417 GC01 and 3.9 mM in GC02 at depth (**Fig. 3a**). Though generally consistent with previous studies on  
418 Black Sea shallow sediments (*e.g. Jørgensen et al., 2001; Egger et al., 2016*), our maximum  $CH_4$   
419 concentrations data are above methane saturation of *ca.* 1.3 mM at atmospheric pressure (calculated at  
420  $20$  °C and salinity of 22 g/L following *Mogollón et al., 2013*) and yet below methane saturation at *in situ*  
421 subsurface condition (*ca.* 10 mM at 6 bars and  $10$  °C). Therefore, it is difficult to evaluate how much *in*  
422 *situ* dissolved methane may have been lost and to what extent the  $CH_4$  concentrations presented here  
423 could be underestimated. The sulfate concentration profiles are nearly identical in the two cores,  
424 decreasing from *ca.* 12 mM at 15 cm depth, down to *ca.* 0.5 mM in the deepest sampled horizon of each  
425 cores. Thus, albeit limited in overall recovered lengths, the two cores collected for this study are  
426 successfully capturing the SMTZ, occurring at *ca.* 40-100 cm depth.

427 The two cores share similar methane isotopic features. Measurements on the *Picarro* analyzer  
428 reveal relatively homogeneous  $\delta^{13}C$  below *ca.* 60 cm depth in each cores, averaging  $-74.2 \pm 0.7$  ‰ ( $n=3$ )  
429 in GC01 and  $-71.4 \pm 0.6$  ‰ ( $n = 3$ ). The  $\delta^{13}C$  then increases to  $-67.1$  ‰ at 33 cm in GC01 and to  $-62.3$  ‰  
430 at 25 cm in GC-02. The *Panorama* measurements of  $\delta^{13}C$  on 6 samples collected at similar depth intervals

431 all yielded consistent values (**Fig. 3b**), with perhaps one exception for the sample collected at the 50-  
432 60 cm depth interval in GC02. There,  $\delta^{13}\text{C}$  is different by 4.6 ‰ compared to the sample collected at  
433 50 cm depth and measured on the *Picarro* analyzer. We suggest this apparent discrepancy is due to the  
434 nature of the sampling. The sample for clumped isotopes integrates all methane contained in the sediment  
435 pore water over a 10 cm length (*i.e.* the length of the 50 cc syringe used for sediment sub-sampling, see  
436 **Fig. S1**). Therefore, given the overall  $\text{CH}_4$  concentration profile (*i.e.* increasing with depth) and the fact  
437 that  $\delta^{13}\text{C}$  values measured below this depth interval all point towards  $\delta^{13}\text{C}$  values in the vicinity of -  
438 71 ‰, we suggest the measured  $\delta^{13}\text{C}$  value simply represents the isotopic composition of the methane  
439 integrated between 50 and 60 cm depth. Unfortunately, our sampling resolution – which was primarily  
440 dictated by the need to collect enough methane – does not allow to establish how sharp the isotopic  
441 transition is at the location of sampling. Overall, the apparent consistency of other  $\delta\text{D}$ ,  $\Delta^{13}\text{CH}_3\text{D}$  and  
442  $\Delta^{12}\text{CH}_2\text{D}_2$  data between the two cores (see below) suggests that the bulk and clumped isotope values for  
443 the sample collected at the 50-60 cm depth interval in GC02 are valid.

444 The *Panorama* measurements of  $\delta\text{D}$ ,  $\Delta^{13}\text{CH}_3\text{D}$  and  $\Delta^{12}\text{CH}_2\text{D}_2$  further demonstrate the  
445 similarity of the methane isotopic profiles between the two cores. The  $\delta\text{D}$  increases from -230 ‰ at the  
446 base of the SMTZ in the two cores (*ca.* 130 cm depth) to -121 ‰ in GC01 at the 55-75 cm depth interval  
447 and to -104 ‰ in GC02 at the 50-60 cm depth interval. Note this range of variations for  $\delta\text{D}$  in the Black  
448 Sea sediments is consistent with the data reported by [Egger \*et al.\*, \(2016\)](#) in the Western part of the Black  
449 Sea. In both cores,  $\Delta^{13}\text{CH}_3\text{D}$  and  $\Delta^{12}\text{CH}_2\text{D}_2$  show limited variations in the deepest sampled intervals,  
450 averaging  $7.2 \pm 0.6$  ‰ and  $19.4 \pm 4$  ‰ respectively, and exhibit dramatic increases in the shallowest  
451 intervals, with values as high as 15.7 ‰ and 74.6 ‰ respectively for GC01, and as high as 14.6 ‰ and  
452 61.1 ‰ respectively for GC02.

453

### 454 **4.3. Near-equilibrium and disequilibrium signatures among microbially-mediated methane**

455 When a sample reflects thermodynamic equilibrium, both mass-18 isotopologue yield  
456 concordant temperatures and the gas sample plots on the equilibrium curve in the  $\Delta^{13}\text{CH}_3\text{D}$ - $\Delta^{12}\text{CH}_2\text{D}_2$   
457 space. In natural samples, whether an equilibrium temperature reflects a methane formation or re-  
458 equilibration is currently a subject of investigation. On the other hand, samples plotting off the  
459 equilibrium curve may reflect kinetics and/or reservoir effects associated with methane synthesis, or  
460 perhaps other secondary processes such as mixing, diffusion, or partial degradation of methane.

461 On the basis of  $\Delta^{13}\text{CH}_3\text{D}$  and  $\Delta^{12}\text{CH}_2\text{D}_2$  signatures, we identified three groups with specific

462 patterns. A first group of samples, coming from the sediment of the Lake Pavin and from the lower part  
463 of the SMTZ in the Black Sea sediments, are near-equilibrium low-temperature region, and bear striking  
464 resemblance to other methane-based ecosystems found in marine or continental sediments (**Fig. 4**). The  
465 second group of samples is composed of the four samples from Lake Pavin water-column, with negative  
466  $\Delta^{12}\text{CH}_2\text{D}_2$  values down to -22 ‰. These signatures reflect disequilibrium and are similar to microbial  
467 methane produced in the laboratory (e.g. Young *et al.*, 2017; Giunta *et al.*, 2019) and methane of microbial  
468 origin from boreal lakes (Young, 2019) (**Fig. 4**). Lastly, a third group of samples is defined by the two  
469 samples collected in the SMTZ horizon of the Black Sea sediments, and show extremely enriched  
470  $\Delta^{13}\text{CH}_3\text{D}$  and  $\Delta^{12}\text{CH}_2\text{D}_2$  values. These samples also reflect disequilibrium, and represent to date, the  
471 most positive values ever reported for clumped isotopologues in a natural setting.

472

## 473 **5. Discussion**

### 474 **5.1. The ambiguity of ‘pristine’ signatures of methanogenesis**

475 In both settings investigated here, the methane found at the bottom of each depth-profile is  
476 deriving from microbial methanogenesis. In Lake Pavin, methane is produced both at the bottom of the  
477 water-column through hydrogenotrophic methanogenesis and in the sediment through a mix of  
478 hydrogenotrophic and acetoclastic methanogenesis (Biderre-Petit *et al.*, 2011; Lopes *et al.*, 2011). Yet,  
479 these two presumed production reservoirs show very distinct bulk and clumped isotope signatures. The  
480 large offsets from  $\Delta^{13}\text{CH}_3\text{D}$ - $\Delta^{12}\text{CH}_2\text{D}_2$  equilibrium observed in the lower part of the water-column  
481 resemble those measured on methane produced by hydrogenotrophic methanogens in the laboratory  
482 (Young *et al.*, 2017; Giunta *et al.*, 2019; **Fig. 4**). It is worse noting that bulk isotope fractionation between  
483  $\text{CH}_4$ - $\delta\text{D}$  and water- $\delta\text{D}$  (ranging from ca. -50 to -56 ‰, Gal *et al.*, 2015) would also illustrate such an  
484 isotope fractionation typical of methanogenic growth (e.g. Wang *et al.*, 2015; Gruen *et al.*, 2016). Taken  
485 together, these observations support genome-based studies suggesting that the hydrogenotrophic pathway  
486 is the main production route for methane in the water-column (Biderre-Petit *et al.*, 2011). In sharp  
487 contrast, the two samples collected in the Pavin sediment, are plotting much closer to the equilibrium  
488 line, at odds with expectations based on laboratory cultures for a system dominated by microbial  
489 methanogenesis. In the Black Sea sediments, the production of methane is thought to primarily derive  
490 from hydrogenotrophic methanogenesis. The four samples from the lower part of the SMTZ show  
491 signatures close to clumped isotopologue equilibrium, with average  $\Delta^{13}\text{CH}_3\text{D}$  and  $\Delta^{12}\text{CH}_2\text{D}_2$  values of

492  $7.2 \pm 0.6$  ‰ and  $19.4 \pm 4$ ‰. These values translate in apparent temperatures of  $-14_{-14}^{+15}$  °C and  $26_{-24}^{+29}$  °C,  
493 statistically indistinguishable from *in situ* temperature of *ca.* 10 °C.

494 Our data illustrate the complexity of the information captured by the of clumped signatures  
495 for microbial methanogenesis in nature. In many environments where microbial methanogenesis is  
496 presumed to be the dominant methane-based metabolism, the clumped isotopologues appear to reflect  
497 near-equilibration production of the methane, at odds with disequilibrium signatures systematically  
498 observed in laboratory cultures, regardless of the pathway involved. This near-equilibration behavior  
499 appears especially visible among what Okumura *et al.*, (2016) call ‘aged methane’: meaning in  
500 hydrogeological environments that are isolated enough to accumulate methane over geological time, for  
501 instance in deep sea sediments (Wang *et al.*, 2015; Douglas *et al.*, 2016; Inagaki *et al.*, 2015; Ijiri *et al.*,  
502 2018) or in some continental sedimentary gas reservoirs (Stolper *et al.*, 2015). The isotopic discrepancy  
503 between ‘aged methane’ and laboratory methane may reflect the rate of methanogenesis (Wang *et al.*,  
504 2015; Stolper *et al.*, 2015, 2017; Douglas *et al.*, 2016, 2020; Gropp *et al.*, 2022). Accordingly, large  
505 substrate availability (and thus free energy) in laboratory cultures would dominate kinetic effects for  
506 methane isotopologues, yielding disequilibrium abundance of isotopologues, whereas lower substrate  
507 availability, perhaps typical of the deep biosphere, would result in lower rates of methanogenesis and  
508 thereby in isotopic signatures that are controlled by thermodynamic equilibrium rather than kinetics. In  
509 line with this hypothesis, isotopologue disequilibrium at the bottom of the Pavin water-column could  
510 reflect the expression of *fast* methane production (perhaps promoted by continuous delivering of fresh  
511 labile organic matter into the lake), whereas near-equilibrium signatures in the Pavin sediment or from  
512 the lower part of the SMTZ in the Black Sea would reflect *slower* methanogenesis resulting from *more*  
513 *limited* nutrient availability. Note this hypothesis currently still lacks a clear experimental validation.  
514 Experiments under low hydrogen (H<sub>2</sub>) partial pressures suggest that microbial methane could form closer  
515 to thermodynamic equilibrium, but still far from what is observed in nature on ‘aged’ methane (Okumura  
516 *et al.*, 2016; Douglas *et al.*, 2020). This may reflect the challenge of reproducing the (presumably)  
517 extremely low methanogenesis rates of the deep biosphere in the laboratory.

518 An alternative hypothesis for explaining these near-equilibrium signatures is that they instead  
519 reflect the interplay of different metabolisms in natural ecosystems. In particular, it has been proposed  
520 that micro-organisms involved in AOM may in energy-limited environments contribute to reprocess  
521 microbial methane towards thermodynamic equilibrium (Yoshinaga *et al.*, 2014; Young *et al.*, 2017,  
522 2019; Giunta *et al.*, 2019; Ash *et al.*, 2019; Warr *et al.*, 2021, Wegener *et al.*, 2021; Ono *et al.*, 2021,

523 2022; Gropp et al., 2022). Note this hypothesis has some experimental validation: AOM coupled to  
524 sulfate-reduction appears to at least partially drive the residual methane towards isotopic equilibrium  
525 (Yoshinaga et al., 2014; Young, 2019; Wegener et al., 2021). The exact interplay of methanogenesis and  
526 AOM and the net resultant on isotopic signatures in conditions representative of the deep biosphere is  
527 still under investigation however (Ono et al., 2022). In the Black Sea sediments, the widely documented  
528 presence of ANME (e.g. Michaelis et al., 2002; Knittel et al., 2005; Treude et al., 2006) may argue in  
529 favor of this *reprocessing* hypothesis for explaining the near-equilibrium values observed at the base of  
530 the SMTZ (we will explore this further in section 5.3.3). In the Lake Pavin sediment however, 16S rRNA  
531 analyses identified the presence of known methanogens (*Methanosaetaceae* and *Methanomicrobiales*)  
532 but not of ANME nor of related lineages (Lehours et al., 2007; Borrel et al., 2012). Yet, genomic analyses  
533 have also revealed that the archeal community in Lake Pavin sediment is largely dominated (>70 %) by  
534 uncultured archeal lineages of the Marine Benthic Groupe-D (MBG-D). The metabolisms of the MBG  
535 representatives are mostly unknown, but their ubiquitous presence in methane-rich environments suggest  
536 their potential role in methane cycling (Beal et al., 2009; Zhou et al., 2019). In particular, the presence  
537 of MBG members in habitats where AOM occurs, whether in wetlands (Valenzuela et al., 2017), in lakes  
538 (Schubert et al., 2011) or in marine sediments (Inagaki et al., 2006; Beal et al., 2009; Webster et al.,  
539 2011), suggest they may be involved in methanotrophy. Whether they could contribute, as ANME, to  
540 (partial) re-equilibration of microbial methane is currently unknown, but could offer an explanation for  
541 near-equilibrium signatures of the methane in the sediment of Lake Pavin.

542

## 543 **5.2. The effects of methanotrophy on methane isotopologues**

544 Both settings exhibit typical methanotrophic transitions, with methane concentrations  
545 decreasing from *ca.* 4 mM at depth to near 0 mM, consistent with the methane being progressively  
546 consumed as it is transported upward. In both settings, the bulk  $\delta^{13}\text{C}$  and  $\delta\text{D}$  values of the methane  
547 increase along with the decrease in methane concentration, which is *a priori* consistent with either AeOM  
548 or AOM processes, both kinetically favoring the degradation of lighter isotopologues (e.g. Coleman et  
549 al., 1981; Alperin and Reeburgh, 1988; Holler et al., 2011; Feisthauer et al., 2011). However, the rare  
550 isotopologues behave differently for the two settings. The  $\Delta^{13}\text{CH}_3\text{D}$  and  $\Delta^{12}\text{CH}_2\text{D}_2$  values decrease with  
551 lower methane concentrations in the Lake Pavin water column dominated by AeOM, whereas they show  
552 dramatic increases in the Black Sea sediments dominated by AOM (Fig. 2 and 3). Importantly, our data  
553 from the Black Sea suggest that AOM is capable of producing strong disequilibrium effects on the

554 residual methane, as evidenced by the extraordinarily elevated  $\Delta^{13}\text{CH}_3\text{D}$  and  $\Delta^{12}\text{CH}_2\text{D}_2$  values measured  
555 here. This observation is consistent with first experimental observations (Ono *et al.*, 2021), but contrast  
556 with recent field-based observations in the Baltic sea where AOM was interpreted to produce equilibrium  
557 among methane clumped isotopologues (Ash *et al.*, 2019).

558 Taken together, these data illustrate how AeOM and AOM yield vastly different methane  
559 isotopologue signatures. Methane isotopologue signatures could provide useful constraints on the type  
560 of methane consumption operative in nature.

561

### 562 **5.3. Inferring *in situ* fractionation factors of methanotrophy**

563 The expression of contrasting behaviors of methane isotopologues during methanotrophy must  
564 reflect different isotope fractionation factors at play in these environments. In the case of methanotrophy,  
565 isotope fractionation factors correspond to the relative rate constant of a heavy isotopologue (whether  
566  $k^{13}\text{CH}_4$ ,  $k^{12}\text{CH}_3\text{D}$ ,  $k^{13}\text{CH}_3\text{D}$  or  $k^{12}\text{CH}_2\text{D}_2$ ) to the rate constant of the light, non-substituted, isotopologue  
567 (*i.e.*  $k^{12}\text{CH}_4$ ). In the following, fractionation factors for bulk carbon isotopes and for bulk hydrogen  
568 isotopes, as well as for ‘clumped’ isotopologues are referred as  $^{13}\alpha$ ,  $^{\text{D}}\alpha$ ,  $^{13\text{D}}\alpha$  and  $^{\text{D}2}\alpha$  respectively. Basic  
569 kinetic considerations suggest that  $\alpha$  values should be less than 1 for methanotrophy in which there is a  
570 preferential consumption of the lighter  $^{12}\text{CH}_4$  isotopologues. In laboratory cultures or incubations of  
571 methanotrophic organisms, derived fractionation factors vary considerably, with  $^{13}\alpha$  ranging between  
572 0.971 and 0.997, and  $^{\text{D}}\alpha$  ranging from 0.680 to 0.906 (*e.g.* Coleman *et al.*, 1981; Templeton *et al.*, 2006;  
573 Kinnaman *et al.*, 2007; Feisthauer *et al.*, 2011; Rasigraf *et al.*, 2012; Ono *et al.*, 2021). Note that when  
574 extrapolated from natural environments, kinetic isotope fractionation factors often tend to be closer to 1,  
575 representing less of a kinetic fractionation. For example,  $^{13}\alpha$  values of 0.996 and  $^{\text{D}}\alpha$  values of 0.952 have  
576 been reported (*e.g.* Happell *et al.*, 1994).

577 Fractionation factors for ‘clumped’ isotopologues of methane are less constrained, in particular  
578 for  $^{12}\text{CH}_2\text{D}_2$ . In the limit where isotopes distribute stochastically across isotopologues, the fractionation  
579 factors for doubly-substituted isotopologues would simply be the product of the bulk fractionation  
580 factors, following the “rule of the geometric mean” (Bigeleisen, 1955). This is the result at  
581 thermodynamic equilibrium at high temperatures where the isotope exchange equilibrium constants are  
582 simply the ratios of symmetry numbers. However, the few studies on methane clumped isotopologues  
583 have shown that this is not the case in general, especially in the oxidation reactions where bond rupture  
584 is involved (Whitehill *et al.*, 2017; Haghnegahdar *et al.*, 2017; Ono *et al.*, 2021). This may lead to

585 fractionation factors for clumped isotopologues that deviate significantly from the product of bulk isotope  
 586 fractionation factors. The  $\gamma$  coefficient has been introduced as a means of quantifying this departure from  
 587 the rule of the geometric mean (Wang *et al.*, 2016) and to define  $^{13D}\alpha$ , the fractionation factor for  
 588  $^{13}\text{CH}_3\text{D}/^{12}\text{CH}_4$  as  
 589

$$590 \quad ^{13D}\alpha = \frac{k_{13\text{CH}_3\text{D}}}{k_{12\text{CH}_4}} = ^{13}\alpha \cdot D_\alpha \cdot ^{13D}\gamma, \quad (10)$$

591

592  
 593 where  $^{13D}\gamma$  is an empirical parameter that can be adjusted to fit experimental or field data and would  
 594 reflect the intrinsic ‘clumped’ effect for  $^{13}\text{CH}_3\text{D}$  isotopologue. Similarly,  $^{D2}\alpha$ , the fractionation factor for  
 595  $^{12}\text{CH}_2\text{D}_2/^{12}\text{CH}_4$ , can be described as  
 596

$$597 \quad ^{D2}\alpha = \frac{k_{12\text{CH}_2\text{D}_2}}{k_{12\text{CH}_4}} = D_\alpha \cdot D_\alpha \cdot ^{D2}\gamma, \quad (11)$$

598

599 where  $^{D2}\gamma$  is an empirical parameter reflecting the intrinsic ‘clumped’ effect for the  $^{12}\text{CH}_2\text{D}_2$   
 600 isotopologue. When  $\gamma$  coefficients equal unity, the fractionation factor for the multiply-substituted  
 601 isotopologue simply corresponds to the product of bulk isotope fractionation factors. So far, laboratory  
 602 cultures of *Methylococcus capsulatus* performing AeOM have revealed an absence of an intrinsic  
 603 ‘clumped’ isotope effect with  $^{13D}\gamma \sim 1$  (Wang *et al.*, 2016), whereas recent AOM experiments by ANME  
 604 (Ono *et al.*, 2021) have revealed  $^{13D}\gamma$  values significantly lower than unity. Note that the magnitude of  
 605 the  $\gamma$  exerts a fundamental control on whether  $\Delta^{13}\text{CH}_3\text{D}$  and  $\Delta^{12}\text{CH}_2\text{D}_2$  values of a methane experiencing  
 606 methanotrophy become more positive or more negative. Below, we derive apparent isotope fractionation  
 607 factors through simple 1-D reactive transport modeling of these two settings.  
 608

### 609 **5.3.1. Lake Pavin water-column, AeOM methanotrophy**

610 In the Lake Pavin, the methane oxidation occurs at a water depth ranging between 50 and 60 m  
 611 (Lopes *et al.*, 2011). Below 60 m, the distribution of methane concentration and isotopologue ratios must  
 612 be essentially controlled by turbulent diffusion. Contrasting with a pure diffusion process, turbulent or  
 613 eddy diffusion is “non-fractionating” for entrained isotopologues. In the bottom part of the Lake Pavin  
 614 water column, the eddy diffusivity is low, inferred to be in the order of 1–20  $\text{m}^2\cdot\text{yr}^{-1}$  (Aeschbach-Hertig

615 *et al.*, 2002), which is only one to two order of magnitudes greater than typical diffusion coefficients of  
616 a dissolved substance in water (in the range of  $0.1 \text{ m}^2 \cdot \text{yr}^{-1}$ ), so it is conceivable that molecular diffusion  
617 could be expressed.

618 In order to infer the apparent fractionation factors associated with methanotrophy in Lake  
619 Pavin, we modeled the concentration of all methane isotopologues as described in section 4.5. Our model  
620 fits the Lake Pavin water column concentration and isotopic profiles (**Fig. 5**) with net degradation rates  
621 that are consistent with *Lopes et al.*, (2011), showing a peak near 55 m depth of *ca.*  $0.4 \text{ mM} \cdot \text{yr}^{-1}$ . The  
622 model carbon and hydrogen bulk isotope fractionation factors are of rather low magnitude, with  $^{13}\alpha =$   
623  $0.9978$  (or *ca.* 2.2 ‰) and  $^D\alpha = 0.9610$  (or *ca.* 33.9 ‰), placing them at the lower end of all fractionation  
624 factors reported so far for AeOM whether in laboratory cultures or in field studies (**Fig. 6**). Note that  
625 accounting for a diffusion isotope effect would change each magnitude of fractionation factors by about  
626 0.003 (*i.e.* 3 ‰) (**Fig. S3**). Fractionation factors for clumped isotopologues are of small magnitude as  
627 well, with  $^{13D}\alpha = 0.9608$  and  $^{D2}\alpha = 0.9261$ . For comparison, pure AeOM in laboratory cultures have  
628 resulted in  $^{13D}\alpha$  values ranging between 0.7804 and 0.8847 (*Wang et al.*, 2016, note there was no available  
629  $^{12}\text{CH}_2\text{D}_2$  data in this study). Importantly, these fractionation factors for clumped isotopologues are  
630 indistinguishable from the product of bulk isotope fractionation factors, with  $^{13D}\gamma = 1.0021 \pm 0.0026$  and  
631  $^{D2}\gamma = 1.0029 \pm 0.0037$ . This observation is consistent with the results of *Wang et al.*, (2016) on  $^{13}\text{CH}_3\text{D}$   
632 isotopologue, and could be a fingerprint of AeOM metabolisms in nature.

633 It is difficult to categorically explain the small magnitude of fractionation factors obtained  
634 from the Lake Pavin data, although we note it is generally consistent with the fact that  $\alpha$  values  
635 determined from the field are often *smaller* than in the laboratory (**Fig. 6**). In a reactive-transport model,  
636 transport parameters such as diffusion coefficients and/or advection rates (treated here as input  
637 parameters) may in principle affect the derived model isotope fractionation parameters. Sensitivity  
638 analyses performed for a range of  $D$  and  $v$  values did not yield significantly different results, however  
639 (**Fig. S3**). The values derived here may thus accurately capture the *in-situ* fractionation factors for this  
640 setting. Experimental works suggest that the absolute magnitude of isotope fractionation upon AeOM  
641 may be controlled by  $\text{CH}_4$  transport across cell membranes or by the form of the enzyme (the methane  
642 monooxygenase - MMO) the  $\text{CH}_4$  is binding to (*e.g.* *Summons et al.*, 1994; *Templeton et al.*, 2006;  
643 *Feisthauer et al.*, 2011). Importantly, chemostat experiments by *Templeton et al.*, (2006) clearly  
644 demonstrated that a single type of methanotrophic bacteria may produce apparent  $^{13}\alpha$  values varying  
645 widely, from 0.997 (*ca.* 3 ‰) to 0.962 (*ca.* 38 ‰), as a function of cell density. Accordingly, under high

646 cell density and high concentration of MMO enzymes, the diffusion of methane across cell membranes  
647 may become a rate-determining step. This would mask the intrinsic fractionation factor associated with  
648 oxidation of methane by MMO enzymes and result in *smaller* apparent fractionation factors (Templeton  
649 *et al.*, 2006). In the Lake Pavin water-column, the important methane flux contributes to elevated  
650 concentrations of methanotrophic bacteria, with cell densities as high as  $10^5$  cell.mL<sup>-1</sup> at *ca.* 50–60m  
651 depth (Lehours *et al.*, 2007). Thus, although it seems difficult to evaluate how the chemostat experiments  
652 would scale (in terms of cell densities and absolute rates) to the Lake Pavin water column, the mechanism  
653 proposed by Templeton *et al.*, (2006) may offer a partial explanation for the rather low fractionation  
654 factors derived from the natural data here. Future studies that include carbon isotope signatures of the  
655 associated biomass and DIC may help to validate this hypothesis with additional mass balance  
656 constraints.

657

### 658 **5.3.2. Black Sea sediments, AOM methanotrophy**

659 Solving the reactive transport equation in the Black Sea sediments is more challenging because  
660 of the large uncertainties regarding the dissolved methane concentration profiles. This is partly due to  
661 the poor sampling resolution, a direct consequence of sampling large volumes of sediments for clumped  
662 isotope analyses. It is also likely that dissolved methane has escaped from the samples between the time  
663 of the coring and the onboard sub-sampling of fresh sediment into the head-space vials. For comparison,  
664 complete bio-geochemical modeling of the methane concentration profile in the Romanian sector of the  
665 Black Sea point towards CH<sub>4</sub> concentrations of about 4 to 5 mM below the SMTZ (at sulfate  
666 concentration equivalent to those measured here at the base of each core), and even up to 15 mM in  
667 deeper sections of the sediments (Egger *et al.*, 2016). Here, we tested two different scenarios with distinct  
668 boundary conditions for modeling the CH<sub>4</sub> concentration profiles (see details in **Fig. 7**). Independently  
669 of which scenario is used, our model results in maximum degradation rates located between 80 and  
670 100 cm depth in the two cores; only the amplitude of the degradation rate varies (**Fig. 7b**). Importantly,  
671 inferred fractionation factors are quite insensitive to the scenario chosen and nearly identical in the two  
672 cores (**Fig. S4 and S5**), all resulting in largely positive  $\Delta^{13}\text{CH}_3\text{D}$  and  $\Delta^{12}\text{CH}_2\text{D}_2$  values such as measured  
673 here. Bulk isotope fractionation factors extrapolated are of higher magnitude than those extrapolated in  
674 the Lake Pavin, but still on the low end of values reported so far in the literature for microbial AOM  
675 processes, especially for carbon isotopes (**Fig. 6**). For <sup>13</sup>CH<sub>3</sub>D isotopologue, we infer <sup>13D</sup> $\alpha$  values of  
676 0.8676 and 0.8817, which compare positively in terms of magnitude with those determined in AOM

677 cultures, ranging between 0.764 and 0.869 (Ono *et al.*, 2021). Lastly, we note that the  $^{13D}\gamma$  and  $^{D2}\gamma$  that  
678 we retrieved from our models are persistently below unity, with values of 0.9929 (GC01) and  
679 0.9939 (GC02), and 0.9462 (GC01) and 0.9546 (GC02), respectively. Despite the large uncertainties  
680 associated with these numbers, this observation is consistent with the  $^{13D}\gamma$  values of 0.9872 and 0.9952  
681 in AOM incubations (Ono *et al.*, 2021, note that  $^{12}CH_2D_2$  was not measured in their study). We note  
682 however that this reactive transport modeling does not achieve a perfect fit with the isotopic profiles. In  
683 particular, the model fails to produce the isotopic homogeneity observed at the bottom of each profile  
684 (**Fig. 7c, d, e and f**). Taken at face value, the observed homogeneous isotopic signatures would instead  
685 suggest that there is limited methane oxidation. This seems to be in contradiction with the modeled  $CH_4$   
686 concentrations and the resulting degradation rate profiles (**Fig. 7b**) instead suggesting that methane is  
687 already being consumed at the base of each sediment profiles. This apparent discrepancy between  
688 modeled isotopic data and net degradation rates may simply result from our poor sampling resolution  
689 and incorrect evaluation of the *true*  $CH_4$  concentration profile in the sediment. Yet for exploratory  
690 purposes, we speculate that in the context of sulfate-dependent AOM, the observed behavior could reflect  
691 the co-occurrence of methane re-cycling, along with methane degradation.

692

### 693 **5.3.3 Reversibility of the AOM pathway in the Black Sea sediments?**

694 At the base of the SMTZ,  $\Delta^{13}CH_3D$  and  $\Delta^{12}CH_2D_2$  values yield apparent temperatures of  
695  $-14_{-14}^{+15}$  °C and  $26_{-24}^{+29}$  °C, thus consistent with *in situ* temperatures of *ca.* 10 °C. This may suggest a  
696 mechanism for re-equilibrating the methane isotopologues in these sedimentary horizons. Under low  
697 sulfate concentrations, it is speculated that methanotrophic Archaea may promote partial reversibility of  
698 the AOM reaction, or at least of some of its key enzymatic steps. This would imply that a substantial  
699 amount of methane could be re-formed or “re-bonded” during the process (Holler *et al.*, 2011, Yoshinaga  
700 *et al.*, 2014; Timmers *et al.*, 2017; Marlow *et al.*, 2017; Beulig *et al.*, 2019; Young, 2019; Wegener *et al.*,  
701 2021). The concept of reversibility during AOM suggests that the isotopic evolution of products and  
702 substrates may be partly controlled by equilibrium effects instead of being solely controlled by kinetic  
703 effects. From our data, the fact that bottom  $\Delta^{13}CH_3D$  and  $\Delta^{12}CH_2D_2$  values are close to equilibrium values  
704 at *in situ* temperatures argue in favor of such a mechanism.

705 To explore the potential role of reversibility on methane isotopologue distribution, we adapted  
706 our reactive transport model following Burdige *et al.*, (2016) and Chuang *et al.*, (2019) (see  
707 Supplementary Material). In this simple model, the degree of reversibility  $r$  is set to control the extent to

708 which kinetic or equilibrium effects are expressed among methane isotopologues. The reversibility is  
709 defined as the ratio between the backward and forward reaction rates, and may range between 0  
710 (irreversible reaction) and 1 (fully reversible reaction). The forward reaction rate corresponds to the  
711 methane oxidation rate, and is associated with a kinetic fractionation factor  $\alpha_F$  such as derived in the  
712 previous sections. The backward reaction rate corresponds to the rate of methane re-forming during the  
713 reaction (*i.e.* yielding to a methane back-flux), and is related to the forward rate constant through the  
714 equilibrium fractionation factor, such that  $\alpha_B = \alpha_F \times \alpha_{Eq}$ , where  $\alpha_{Eq}$  is the equilibrium fractionation  
715 factor, generally well known either from theoretical calculations or from experiments. Several additional  
716 assumptions were made for this modeling:

- 717 1. At thermodynamic equilibrium (for  $r = 1$ ), it is assumed that the relative distribution of  
718 clumped isotopologues must be that of isotope-bond equilibrium at local temperature and  
719 that the bulk methane  $^{13}\text{C}/^{12}\text{C}$  and D/H are in isotope equilibrium with DIC or water,  
720 respectively;
- 721 2. The reversibility of the AOM reaction was parameterized in our model to vary as a step  
722 function of a threshold sulfate concentration (following *e.g.* [Yoshinaga et al., 2014](#); [Chuang](#)  
723 [et al., 2016](#)). This threshold concentration was set to 0.55 mM for GC01, corresponding to  
724 the sulfate concentration at 98 cm depth. This is where we estimate from observation of  $\delta\text{D}$ ,  
725  $\Delta^{13}\text{CH}_3\text{D}$  and  $\Delta^{12}\text{CH}_2\text{D}_2$  profiles that the *change* of reversibility must occur. Note this value  
726 is consistent with the 0.5 mM threshold sulfate concentration inferred from AOM  
727 incubations ([Yoshinaga et al., 2014](#)). Thereby, for sulfate concentrations higher than the  
728 threshold concentration, the reversibility of AOM was fixed to  $r = 0.05$ , a minimal  
729 reversibility value determined under high sulfate concentrations ([Holler et al., 2011](#)). For  
730 concentrations below that threshold value, the reversibility was treated as a fitting parameter  
731 and left for optimization on the data.
- 732 3. Considering the small and shallow depth interval investigated here (<150 cm), we  
733 assumed that the isotopic composition of the porewater was homogeneous, with  
734  $\delta\text{D}_{\text{water}} = -15\text{‰}$  (a typical value for bottom seawater in the Black Sea, [Swart, 1991](#); [Dubinin](#)  
735 [et al., 2014](#)).

736 With the aforementioned assumptions, our model may solve the reactive transport equations for  $\delta\text{D}$ ,  
737  $\Delta^{13}\text{CH}_3\text{D}$  and  $\Delta^{12}\text{CH}_2\text{D}_2$  with for each, two parameters left for optimizations: the value of  $r$  for sulfate  
738 concentrations below 0.55 mM (*i.e.* for depths greater than 98 cm depth), the kinetic fractionation factor

739  $\alpha_F$  of AOM. For modeling bulk  $\delta^{13}\text{C}$ , we assumed a  $r$  value similar than that optimized on bulk  $\delta\text{D}$  data,  
740 and left  $\delta^{13}\text{C}_{\text{DIC}}$  as a free parameter to be optimized on the  $\delta^{13}\text{C}_{\text{CH}_4}$  data.

741 For GC01, the best fits with  $\delta\text{D}$ ,  $\Delta^{13}\text{CH}_3\text{D}$  and  $\Delta^{12}\text{CH}_2\text{D}_2$  are obtained with, for each profile,  
742 a reversibility of the AOM reaction as high as *ca.* 0.98 for sulfate concentrations below 0.55 mM (**Fig.**  
743 **8b** and **Fig. S4**). This suggests that AOM is operating close to thermodynamic equilibrium at the base of  
744 the SMTZ, consistent with  $\Delta^{13}\text{CH}_3\text{D}$  and  $\Delta^{12}\text{CH}_2\text{D}_2$  data being close to intra-molecular equilibrium at  
745 environmental temperatures. Though preliminary, this conclusion is in line with several recent studies,  
746 all suggesting that under low sulfate concentrations, a strong reversibility of the AOM pathway or at least  
747 of some of its key intermediate reactions is expected. For instance, incubations of ANME with their  
748 sulfate-reducing bacterial partners point to a reversibility of the AOM pathway as high as 0.75 under  
749 sulfate concentrations of 0.5 mM (Yoshinaga *et al.*, 2014). Likewise, modeling of the carbon cycle in  
750 sediments offshore Taiwan allow estimating a reversibility of the AOM pathway ranging between 0.91  
751 and 0.99 for sulfate concentrations ranging between 5 and 0.05 mM (Chuang *et al.*, 2016). The recent  
752 study by Wegener *et al.*, (2021) provided some more nuanced conclusions. In their incubation  
753 experiments with only 0.05 mM sulfate, they concluded that the overall reversibility is no more than  
754 0.15. Through sophisticated biochemical modeling of key intermediate reactions of the AOM pathway,  
755 they identified some intermediate steps operating close to equilibrium (*i.e.* *high* reversibility), and some  
756 other such as the terminal Formyl-methanofuran dehydrogenase (Fmd)-catalyzed reaction, being  
757 essentially controlled by kinetics (*i.e.* non-reversible). As a result on methane isotopic compositions, it  
758 is expected that the linear chained-reaction for carbon isotope equilibration between methane and DIC  
759 may rarely be achieved, as this would require all intermediate steps to be operating close to equilibrium.  
760 By contrast, hydrogen atoms are subtracted from the carbon through a branched reaction at several  
761 intermediate steps during the AOM pathway. Thus the high reversibility of the first metabolic step (*i.e.*  
762 activation of HS-CoB intermediate by Mcr enzyme) may allow sufficient hydrogen exchange with intra-  
763 cellular water to promote the equilibration of the methane  $\delta\text{D}$  as well as of the  $\Delta^{13}\text{CH}_3\text{D}$  and  $\Delta^{12}\text{CH}_2\text{D}_2$   
764 (Wegener *et al.*, 2021). Accordingly, it is probably not accurate to model the  $\delta^{13}\text{C}$  data with the same  
765 reversibility value than the one inferred on  $\delta\text{D}$  data like we did here. In our model, overestimating the  
766 reversibility associated with the carbon isotope exchange would result in overestimating the  $\delta^{13}\text{C}$  of the  
767 DIC. It will not affect the extrapolation of the  $^{13}\alpha$  value however. Here we infer  $\delta^{13}\text{C}_{\text{DIC}} = -6 \text{ ‰}$ , a rather  
768 *high* value for system presumably controlled by AOM. This may support the idea that the reversibility  
769 value (*i.e.* of 0.98) applied to carbon isotopes in our model is indeed overestimated. Regardless of the

770 magnitude of the reversibility, our model also suggests that the depth at which the *change* of reversibility  
771 occurs may not be the same for carbon and for hydrogen exchanges. From both the model and the data,  
772 the  $\delta D$ ,  $\Delta^{13}CH_3D$  and  $\Delta^{12}CH_2D_2$  values start increasing at 65 cm depth, which can be understood as  
773 kinetics outpacing equilibrium effects at sulfate concentrations higher than 0.55 mM. Yet at this depth,  
774 measured  $\delta^{13}C$  are at their minimal value. This observation is compelling as it suggests that the bulk  
775 carbon isotopes are still partially controlled by equilibrium at this depth, whereas hydrogen isotopes  
776 would be dominantly controlled by kinetics. We note that such apparent “delay” between the  $\delta^{13}C$  and  
777  $\delta D$  data seems to occur as well in the few other SMTZ datasets where  $\delta D$  was investigated with  $\delta^{13}C$   
778 (Alperin *et al.*, 1988; Martens *et al.*, 1999; Kessler *et al.*, 2006; Egger *et al.*, 2016). For GC01  
779 specifically, a better fit of the model with  $\delta^{13}C$  data can be obtained if setting the threshold sulfate  
780 concentration to 2.2 mM (**Fig. 8a**). This observation probably further illustrates the limitation of  
781 considering microbial AOM as a single reaction with a unique reversibility profile applying to both  
782 carbon and hydrogen isotopes.

783 Overall, taking into account the reversibility of the AOM reaction persistently results in  
784 isotope fractionation of larger magnitude for all isotopologues, with  $^{13}\alpha = 0.9928$  (*ca.* 7.2 ‰),  
785  $^D\alpha = 0.8180$  (*ca.* 182 ‰);  $^{13D}\alpha = 0.7942$  and  $^{D2}\alpha = 0.6018$ . The bulk isotope fractionation factors  
786 extrapolated with this revised model appear more consistent with those extrapolated from laboratory  
787 incubations (**Fig. 6**), though the fractionation factor for bulk carbon is again of limited magnitude. An  
788 underestimation of the  $^{13}\alpha$  may well result from how we simplified the concept of reversibility in our  
789 model. Note that setting the threshold sulfate concentration to 2.2 mM for carbon isotope yields slightly  
790 higher magnitude of fractionation, with  $^{13}\alpha = 0.9911$  (*ca.* 8.9 ‰). As an important result, the intrinsic  
791 clumped isotope effects are found to be significantly below unity, with  $^{13D}\gamma = 0.9867 \pm 0.0019$  and  
792  $^{D2}\gamma = 0.9152 \pm 0.0019$ . This particular deviation from product of bulk isotope fractionation factors may  
793 be crucial in identifying AOM in natural environments (see section 6.5). The same modeling can be  
794 applied to GC02, yielding to similar results (see Supplementary). Sensitivity analyses performed for  
795 several of the input parameters have almost negligible influence on the determination of fractionation  
796 factors (see Supplementary), so we suggest our extrapolations are relevant to natural environments where  
797 methane degradation is dominated by sulfate-dependent AOM.

798

799

#### 5.4. Implications for the identification of the methane bio-signatures

800 By measuring the resolved abundances of  $^{13}\text{CH}_3\text{D}$  and  $^{12}\text{CH}_2\text{D}_2$ , our study identifies  
801 fundamental distinctions between two main types of biological degradation pathways, in line with recent  
802 laboratory studies on  $^{13}\text{CH}_3\text{D}$  (Wang *et al.*, 2016; Ono *et al.*, 2021). Our data suggest that in nature,  
803 AeOM likely occurs with negligible intrinsic ‘clumped’ isotope effect. This means that the isotope  
804 fractionation factors associated to the degradation of rare methane isotopologues is expected to be close  
805 to the product of bulk isotope fractionation factors ( $\gamma \approx 1$ ). By contrast, sulfate-dependent AOM appears  
806 to be associated with significant clumped isotope effects, with fractionation factors for  $^{13}\text{CH}_3\text{D}$  and  
807  $^{12}\text{CH}_2\text{D}_2$  being significantly lower than product of bulk isotope fractionation factors ( $\gamma < 1$ ). This can  
808 produce extremely positive  $\Delta^{13}\text{CH}_3\text{D}$  and  $\Delta^{12}\text{CH}_2\text{D}_2$  values, as high as 16 and 75 ‰, respectively. The  
809 fundamental mechanism for explaining this difference between the two pathways is currently unknown,  
810 but may reflect different geometry of transition states between the enzyme involved in AeOM (soluble  
811 methane monooxygenase, sMMO) and enzyme involved in AOM (methyl coenzyme M reductase,  
812 Mcr)(Ono *et al.*, 2021).

813 Our study demonstrates that above a certain sulfate concentration, the AOM reaction does  
814 overprint strong kinetic effects on residual methane isotopologues, including  $^{13}\text{CH}_3\text{D}$  and  $^{12}\text{CH}_2\text{D}_2$ . The  
815 observation is consistent with laboratory studies (Ono *et al.*, 2021), but at odd with postulates that in  
816 nature AOM would mostly promote equilibration of clumped isotopologues, even at high sulfate  
817 concentrations. In sediments of the Baltic Sea, Ash *et al.*, (2019) observed a slight increase of  $\Delta^{13}\text{CH}_3\text{D}$   
818 and  $\Delta^{12}\text{CH}_2\text{D}_2$  values along with decreasing of  $\text{CH}_4$  concentrations in sediments of the Baltic Sea. This  
819 observation is *per se* consistent with the expression kinetic isotope effects as observed in our data from  
820 the Black Sea sediments. Yet, because the sample with the lowest concentration measured in the Baltic  
821 Sea sediments was plotting on the equilibrium curve, the authors interpreted the  $\Delta^{13}\text{CH}_3\text{D}$  and  $\Delta^{12}\text{CH}_2\text{D}_2$   
822 profiles as reflecting a progressive re-equilibration of the methane alongside to net methane degradation  
823 (Ash *et al.*, 2019). Another possibility is that this sample-set from the Baltic Sea was not covering a range  
824 of methane concentration large enough to observe to full expression of kinetic effects among  $\Delta^{13}\text{CH}_3\text{D}$   
825 and  $\Delta^{12}\text{CH}_2\text{D}_2$ , and perhaps that the sample measured with the lowest concentration (only ~50 % of  
826 maximum measured concentration) was only coincidentally plotting on the equilibrium line.  
827 Accordingly, one could suspect that in the Baltic Sea sediments, samples with even lower  $\text{CH}_4$   
828 concentrations (*i.e.* more degraded) would show the more positive values indicative of kinetic  
829 fractionation. Note that in our modeling effort of the Black Sea sediment methane, we do suggest that  
830 AOM could promote equilibration of methane isotopologues (as a result of higher reversibility of the

831 reaction), but only in low sulfate concentrations zones. Thus, although net methane oxidation may be  
 832 occurring in these low-sulfate horizons, it can be speculated that overall, most of methane loss through  
 833 AOM would be associated to kinetic isotope effects and result in large positive excursions of both  
 834  $\Delta^{13}\text{CH}_3\text{D}$  and  $\Delta^{12}\text{CH}_2\text{D}_2$ .

835

#### 836 **5.4.1. Closed-system versus open-system methanotrophy**

837 The manner in which a ‘primary’  $\Delta^{13}\text{CH}_3\text{D}$ - $\Delta^{12}\text{CH}_2\text{D}_2$  signature of a methane may be modified  
 838 by a secondary process such as methanotrophy depends crucially on the magnitude of clumped isotope  
 839 fractionation factors ( $^{13D}\alpha$  or  $^{D2}\alpha$ ), and their deviations from the product of bulk isotope fractionation  
 840 factors ( $^{13D}\gamma$  and  $^{D2}\gamma$ ). Another fundamental aspect is whether the system in which the methanotrophy  
 841 takes place is considered to be closed or open (Wang *et al.*, 2016; Haghnegahdar *et al.*, 2017; Ono *et al.*,  
 842 2021). In a closed system, the methane reservoir is being degraded without being replenished. The  
 843 evolution of a given isotopic ratio may be described by the classic Rayleigh equation  $R = R_{ini} \times f^{\alpha-1}$ ,  
 844 where  $R_{ini}$  is the initial isotopic ratio and  $f$  is the remaining fraction of methane. After some  
 845 simplifications, the evolution of  $\Delta^{13}\text{CH}_3\text{D}$  and  $\Delta^{12}\text{CH}_2\text{D}_2$  may simply be described as (Wang *et al.*, 2016):

846

$$847 \Delta^{13}\text{CH}_3\text{D} = \Delta^{13}\text{CH}_3\text{D}_{ini} + \text{Ln}(f) \cdot ({}^{13D}\gamma \cdot {}^{13}\alpha \cdot {}^D\alpha - {}^{13}\alpha - {}^D\alpha + 1) \quad (12)$$

848

849 and

850

$$851 \Delta^{12}\text{CH}_2\text{D}_2 = \Delta^{12}\text{CH}_2\text{D}_2_{ini} + \text{Ln}(f) \cdot ({}^{D2}\gamma \cdot {}^D\alpha^2 - 2 \cdot {}^D\alpha + 1). \quad (13)$$

852

853 These simple formulations show that, depending on the  $\gamma$  value,  $\Delta^{13}\text{CH}_3\text{D}$  and  $\Delta^{12}\text{CH}_2\text{D}_2$  may either way  
 854 evolve towards more negative or more positive values for a methanotrophic reaction occurring in a closed  
 855 system. The threshold conditions are met for  ${}^{13D}\gamma = ({}^{13}\alpha + {}^D\alpha - 1)/({}^{13}\alpha \cdot {}^D\alpha)$  and  ${}^{D2}\gamma = (2 \cdot {}^D\alpha - 1)/{}^D\alpha^2$ .  
 856 Accordingly, for any  $\gamma$  values sufficiently closed to unity such as the AeOM reaction,  $\Delta^{13}\text{CH}_3\text{D}$  and  
 857  $\Delta^{12}\text{CH}_2\text{D}_2$  are predicted to evolve towards more *negative* compositions (**Fig. 9**). By contrast, the  ${}^{13D}\gamma$  and  
 858  ${}^{D2}\gamma$  values of 0.9867 and 0.9152 determined in this study for AOM are both below unity and most  
 859 importantly, below threshold conditions (calculated here of 0.9984 and 0.9504, respectively). Thus,  
 860 AOM reaction in closed system should promote evolution towards more *positive* values of the residual  
 861 methane (**Fig. 9**). Abiotic oxidation of methane with  $\bullet\text{OH}$  or  $\bullet\text{Cl}$ , the two main methane sinks in the

862 atmosphere, were predicted through *ab initio* calculations to result in  $^{13}\text{D}\gamma$  and  $^{\text{D}2}\gamma$  values similar to those  
 863 determined for AOM (Whitehill *et al.*, 2017; Haghnegahdar *et al.*, 2017). However, given the  $^{13}\alpha$  and  $^{\text{D}\alpha}$   
 864 values associated with these abiotic reactions (*i.e.* of greater magnitude than for AOM),  $\gamma$  values are  
 865 above the threshold conditions yielding to  $\Delta^{13}\text{CH}_3\text{D}$  and  $\Delta^{12}\text{CH}_2\text{D}_2$  decreases for these two processes. As  
 866 a consequence, this leaves AOM the sole methane oxidation process identified so far to promote  
 867  $\Delta^{13}\text{CH}_3\text{D}$  and  $\Delta^{12}\text{CH}_2\text{D}_2$  enrichments of the residual methane.

868 In the case of an open system however, these perspectives may change radically. In a reservoir  
 869 where a source of methane persists alongside to methanotrophy as a sole sink of methane, the basic mass  
 870 balance equation applies:  $dn_i/dt = S_i - k_i n_i$ , where  $n_i$  is the moles of isotopologue  $i$  in the system,  $S_i$   
 871 its source term and  $k_i$  its oxidation rate. At steady-state ( $dn_i/dt = 0$ ), the clumped isotopologue  
 872 compositions may be simply derived in the form of (Whitehill *et al.*, 2017; Haghnegahdar *et al.*, 2017):

$$874 \Delta^{13}\text{CH}_3\text{D} = \Delta^{13}\text{CH}_3\text{D}_{\text{source}} - \text{Ln}(^{13}\text{D}\gamma) \quad (14)$$

875  
 876 and

$$878 \Delta^{12}\text{CH}_2\text{D}_2 = \Delta^{12}\text{CH}_2\text{D}_2_{\text{source}} - \text{Ln}(^{\text{D}2}\gamma). \quad (15)$$

879  
 880 Thus, the  $\Delta^{13}\text{CH}_3\text{D}$  and  $\Delta^{12}\text{CH}_2\text{D}_2$  steady-state signatures in an open system do not depend on bulk  
 881 isotope fractionation factors. Accordingly, for a system dominated by AeOM, the clumped signatures are  
 882 expected to remain relatively closed to the source signature (because  $\gamma \approx 1$ ), whereas in a system  
 883 dominated by AOM, they should be more positive (because  $\gamma < 1$ ) by *ca.* 13 and 105 ‰, respectively.  
 884 Note that in the case of  $\bullet\text{OH}$  or  $\bullet\text{Cl}$  oxidation reactions, the steady-state signatures are also expected to  
 885 be more positive than the source signatures, especially for  $\Delta^{12}\text{CH}_2\text{D}_2$ , where the offset would be in the  
 886 vicinity of + 105 ‰ (similar to that of AOM, see **Fig. 9b**).

887 One may expect that in nature, open systems are prevailing, with methane sources and sinks  
 888 eventually reaching a steady-state. In the atmosphere for instance, where oxidation with  $\bullet\text{OH}$  radicals is  
 889 considered the dominant methane sink, the  $\Delta^{12}\text{CH}_2\text{D}_2$  is predicted to be essentially reflecting a steady-  
 890 state signal with values as high as *ca.* 130 ‰ (Haghnegahdar *et al.*, 2017), thus contrasting with more  
 891 *negative* values one would see for oxidation of methane in a closed system. Arguably, lake or sediments  
 892 (pore-)water columns, such as investigated here, may also be considered as open systems since both

893 production and degradation of methane are co-occurring. Note however that for systems where the  
894 characteristic time of the reaction ( $\tau_k$ ) is much smaller than the characteristic time of the source flux ( $\tau_s$ ),  
895 the ‘trajectory’ of the isotopic values towards steady-state in fact closely mimics that of a closed-system  
896 (**Fig. 9b**). One may use the non-dimensional Damköhler number ( $Da = \tau_k/\tau_s$ ) to evaluate the relevance  
897 of the reaction over that of transport. In a system where transport is dominated by diffusion,  $Da$  is best  
898 approximated by  $D/(k \cdot L^2)$ , where  $D$  is the diffusion coefficient and  $L$  is the length of the system.  
899 Therefore, at the scale of geological systems such as those investigated here, we may generally expect  
900  $Da \ll 1$  (e.g. [Bhatnagar et al., 2008](#)). Accordingly, in a lake or sediment undergoing methanotrophic  
901 reaction, the residual methane flux towards the atmosphere (or the open sea water) may well be that of  
902 an open system steady-state, but the “path” towards these signatures may essentially exhibit those of a  
903 closed-system.

904

#### 905 **5.4.2. The ‘grayness’ of microbial methane isotopic fingerprints**

906 Being able to identify the biological production and degradation routes of methane is a major  
907 challenge for a broad range of disciplines, including atmospheric chemistry, oil and gas exploration,  
908 oceanography, and agriculture. This will likely represent a corner-stone for the search for life in our solar  
909 system (e.g. [Oehler and Etiope, 2017](#); [House et al., 2022](#)). To this end, the methane bulk isotope  
910 geochemistry ( $\delta^{13}\text{C}$  and  $\delta\text{D}$ ) is widely used, but the interpretation of these signatures is largely dependent  
911 on the assumptions made regarding carbon and hydrogen precursors and their isotopic compositions, on  
912 the associated fractionation factors, and on the evaluation of (potentially multiple) secondary processes  
913 that may have impacted the methane. The present study further supports the idea that the use of methane  
914 clumped isotopologues may be useful in identifying methane bio-signatures and related metabolisms,  
915 though they may rarely provide a “silver bullet” of a microbial activity.

916 In nature, we may simply distinguish two types of methane samples. Those with  $\Delta^{13}\text{CH}_3\text{D}$  and  
917  $\Delta^{12}\text{CH}_2\text{D}_2$  signatures indicative of equilibrium, and those which are not at equilibrium (**Fig. 10**).  
918 Regardless of the apparent equivalent temperature of a sample, it is still unclear whether equilibrium  
919 signatures are obtained during formation of the methane (thus reflecting actual formation temperature),  
920 or whether they are acquired after (thus potentially reflecting another temperature experienced by the  
921 methane) ([Stolper et al., 2017](#)). Recent studies have advocated for post-formation *equilibration* or *re-*  
922 *equilibration* of the methane isotopologues in hydrothermal systems or sedimentary reservoirs seeping  
923 at the seafloor ([Labidi et al., 2020](#); [Giunta et al., 2021](#); [Beaudry et al., 2021](#)). The exact mechanisms

924 involved for such isotope equilibration are unclear but are speculated to be promoted by surface-  
925 catalyzed exchange onto clay or metal minerals. Importantly, these ‘abiotic’ mechanisms seem to be  
926 restricted to temperatures  $> 90$  °C. Thus, it is reasonable to suggest that a low temperature equilibrium  
927 signal in a natural sample is, to some extent, as simple criteria to support a biological mediation of the  
928 methane. The mechanism by which microbial methane acquires a low temperature equilibrium signal is  
929 debated as well and could result from slow rate of methanogenesis (*e.g.* Wang *et al.*, 2015), or from  
930 methane re-cycling and *equilibration* by organisms involved in AOM (*e.g.* Young *et al.*, 2017). In this  
931 paper, we suggest that near equilibrium signatures at the base of the SMTZ in the Black Sea are promoted  
932 by AOM reaction occurring with a high degree of reversibility due to low sulfate content. This raises the  
933 possibility that such reversibility may persist even deeper in the sediment including in ‘methanogenesis  
934 horizons’. Regardless of the mechanism (*slow* methanogenesis or AOM re-cycling), low temperature  
935 equilibrium signatures point towards a microbial mediation of methane.

936           Alternatively, it is clear that a methane sample exhibiting  $\Delta^{13}\text{CH}_3\text{D}$  and  $\Delta^{12}\text{CH}_2\text{D}_2$   
937 disequilibrium signatures could also reflect a microbial activity. Laboratory culture of methanogens have  
938 persistently result in large disequilibrium signatures, often characterized by negative  $\Delta^{12}\text{CH}_2\text{D}_2$  values.  
939 These signatures are recognized in certain ecosystems where microbial methanogenesis is the only  
940 plausible production route, whether in swamps, rumen or in lake water columns such as in the present  
941 study. Yet, it is now established that disequilibrium signatures are not exclusive to microbial production  
942 routes. Both laboratory and computational works now suggest that similar signatures can be obtained  
943 during early stages of thermocatalytic cracking of organic matter (*e.g.* Xie and Gao, 2019; Dong *et al.*,  
944 2021). Likewise, low temperature abiotic generation ( $< 90$  °C) through Sabatier-type reactions may also  
945 produce similar disequilibrium (Young *et al.*, 2017; Cao *et al.*, 2019). These different formation pathways  
946 have in common the fact that hydrogen atoms with disparate D/H ratios can contribute to the assembly  
947 of the product  $\text{CH}_4$  molecules (*e.g.*, Taenzer *et al.* 2020). Taken together, these compelling observations  
948 suggest that in fact, most methane production routes may be associated with significant disequilibrium  
949 among clumped isotopologues. Generally, this prevents one from using clumped isotopologue  
950 disequilibrium as a fingerprint for microbial activity, although the combination of both negative  $\Delta^{13}\text{CH}_3\text{D}$   
951 and  $\Delta\text{CH}_2\text{D}_2$  seems to be a good indicator of microbial methanogenesis. Furthermore, our study further  
952 demonstrates that secondary processes such as methanotrophic reactions could contribute to  
953 disequilibrium. To the degree the system is closed, the kinetic isotope effects associated with AeOM will  
954 produce negative  $\Delta^{13}\text{CH}_3\text{D}$  and  $\Delta^{12}\text{CH}_2\text{D}_2$  offsets, and may thus generate disequilibrium or even

955 “enhance” a pristine disequilibrium signature inherited from the methane’s formation. Microbial AOM  
956 is also associated with kinetic isotopes effects that will contribute to produce disequilibrium through  
957 positive enrichments in  $\Delta^{13}\text{CH}_3\text{D}$  and  $\Delta^{12}\text{CH}_2\text{D}_2$ . We suggest that extreme positive  $\Delta^{13}\text{CH}_3\text{D}$  and  
958  $\Delta^{12}\text{CH}_2\text{D}_2$  values may be a strong argument for microbial activity, as AOM is to date the sole identified  
959 mechanism capable of producing such positives values. Such behavior may in future, constitute a target  
960 for life detection on other planetary bodies such as Mars or Titan, where AOM is considered to be a  
961 thermodynamically viable metabolism for putative organisms (Norman, 2011; Marlow *et al.*, 2014;  
962 House *et al.*, 2022).

963

## 964 **6. Conclusion**

965 We investigated two natural settings where microbial methanotrophy degraded nearly  
966 quantitatively methane being produced by microbial methanogenesis. In the Lake Pavin, where the  
967 methanotrophy is dominated by AeOM, the bulk  $\delta^{13}\text{C}$  and  $\delta\text{D}$  values of the residual methane are  
968 increasing alongside to methanotrophy, whereas  $\Delta^{13}\text{CH}_3\text{D}$  and  $\Delta^{12}\text{CH}_2\text{D}_2$  values are decreasing. By using  
969 a simple 1-D reactive transport model, we extrapolated apparent fractionation factors that are at the low-  
970 end but consistent with the literature, with  $^{13}\alpha = 0.9978$ ,  $^{\text{D}}\alpha = 0.9610$ ,  $^{13\text{D}}\alpha = 0.9608$  and  $^{\text{D}2}\alpha = 0.9261$ .  
971 Importantly, fractionation factors for clumped isotopologues inferred from our model are similar to the  
972 product of bulk isotope fractionation factors. In the Black Sea sediments, where methanotrophy is  
973 dominated by sulfate-AOM, the bulk  $\delta^{13}\text{C}$  and  $\delta\text{D}$  values of the residual methane are increasing alongside  
974 to methanotrophy as per in the Lake Pavin, but  $\Delta^{13}\text{CH}_3\text{D}$  and  $\Delta^{12}\text{CH}_2\text{D}_2$  values are instead showing  
975 extreme positive enrichments, up to 15.7 and 74.6 ‰, respectively. These values are the most positive  
976 values reported so far, and must result from fractionation factors for clumped isotopologues that  
977 significantly deviate from the product of bulk isotope fractionation factors. These contrasting behaviors  
978 between a system dominated by AeOM and a system dominated by AOM illustrate the fundamental  
979 distinction between the two types of metabolisms. We suggest the data presented here, together with the  
980 fractionation factors extrapolated from simple reactive transport modeling, may help to further identify  
981 and constrain these processes, both of them constituting major methane sinks in the global methane  
982 budget on Earth.

983

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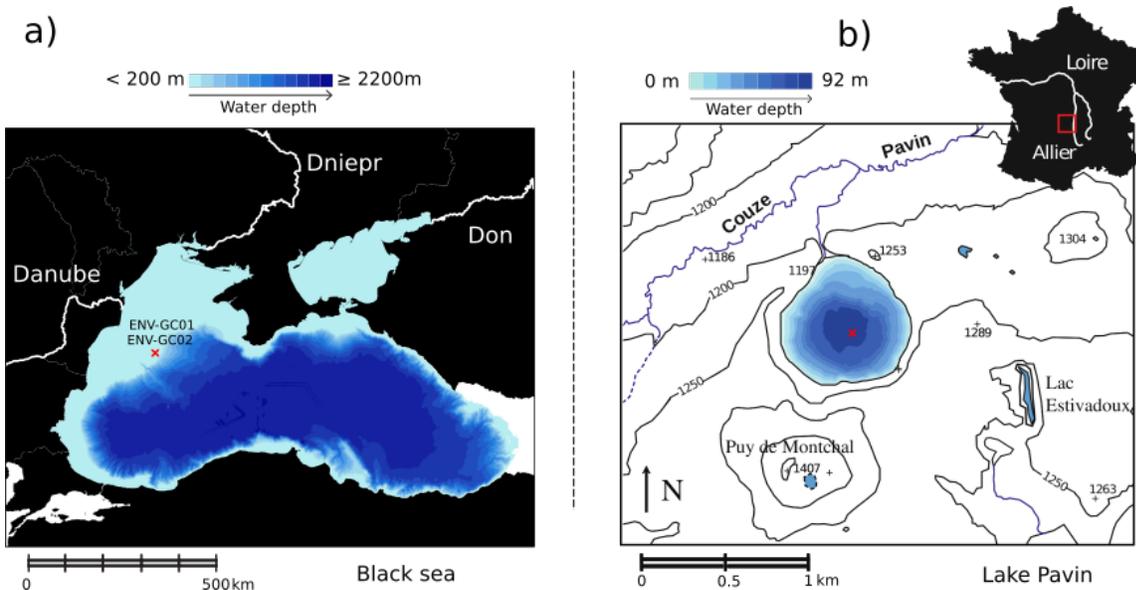
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FIGURES and Captions

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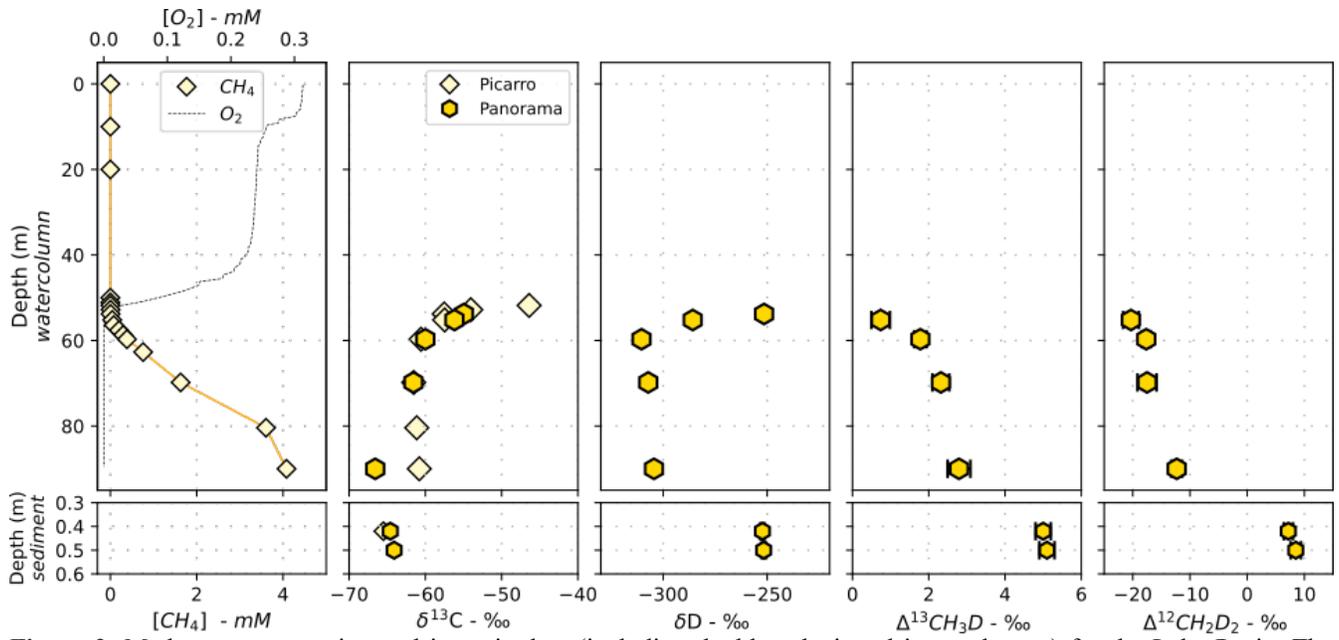


**Figure 1:** a) Bathymetric map of the Black Sea with three of the tributaries and coordinates of the two gravity cores. b) Topographic and bathymetric map of the Lake Pavin in France, modified after Assayag et al., (2008) and Chassiot et al., (2018).

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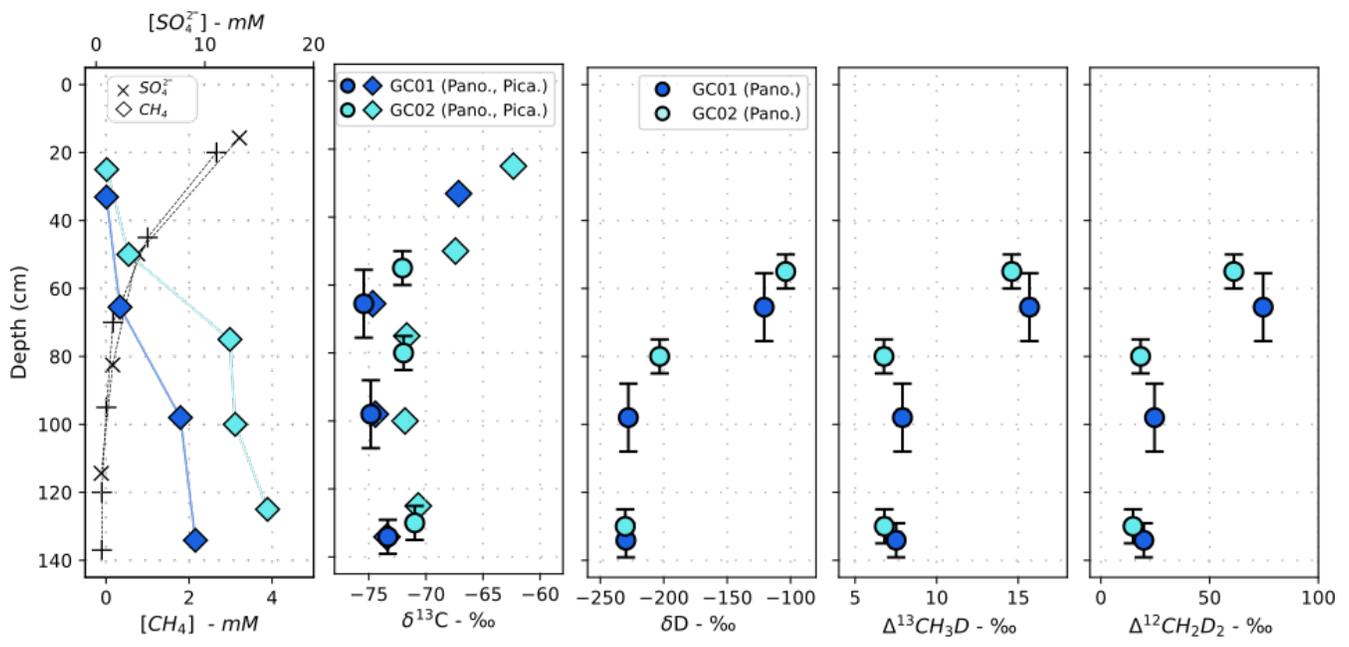


**Figure 2:** Methane concentration and isotopic data (including doubly-substituted isotopologues) for the Lake Pavin. The methane concentration in the sediment was not measured in this study.

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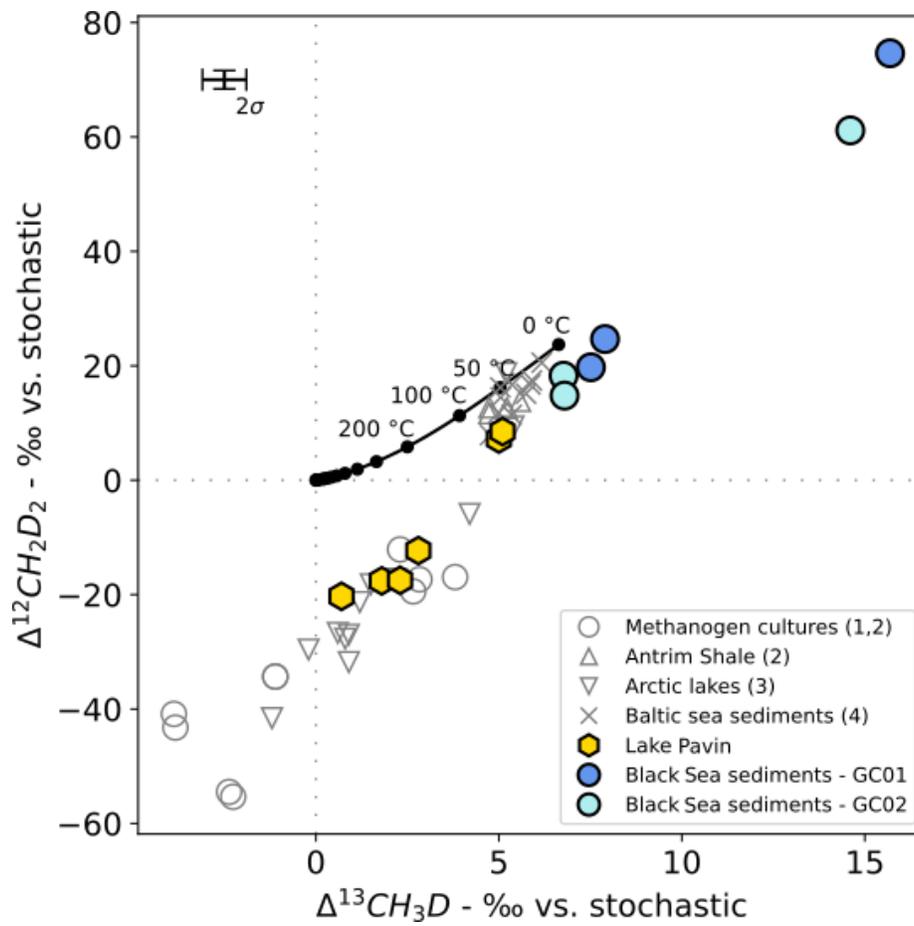


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**Figure 3:** Methane concentration and isotopic data (including doubly-substituted isotopologues) for Black Sea sediments. Errors bars reflect uncertainties of sampling depth (see section 4.1. and Fig. S1)

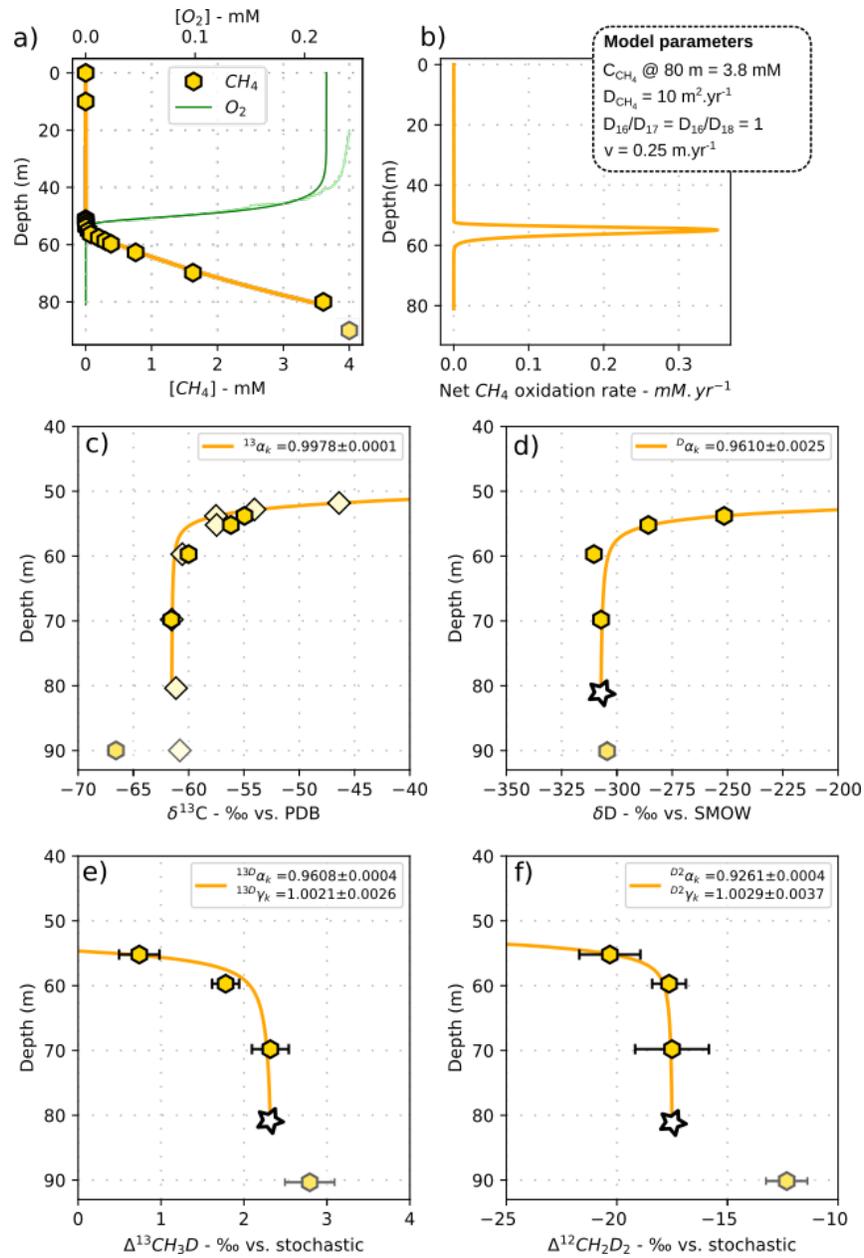
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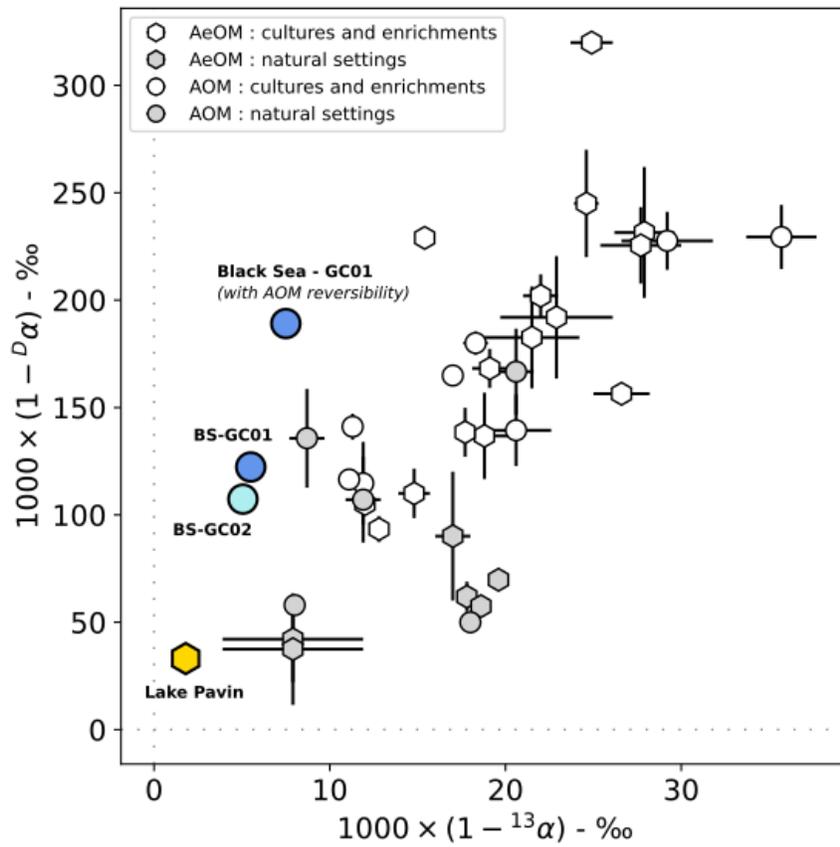


55 **Figure 4:** The thermodynamic equilibrium line (black) is calculated after correlations developed by Young et al., ( 2017). Other reported data are from (1): Young et al., (2016); (2): Giunta et al., (2019); (3): Young, (2019); (4): Ash et al., (2019).

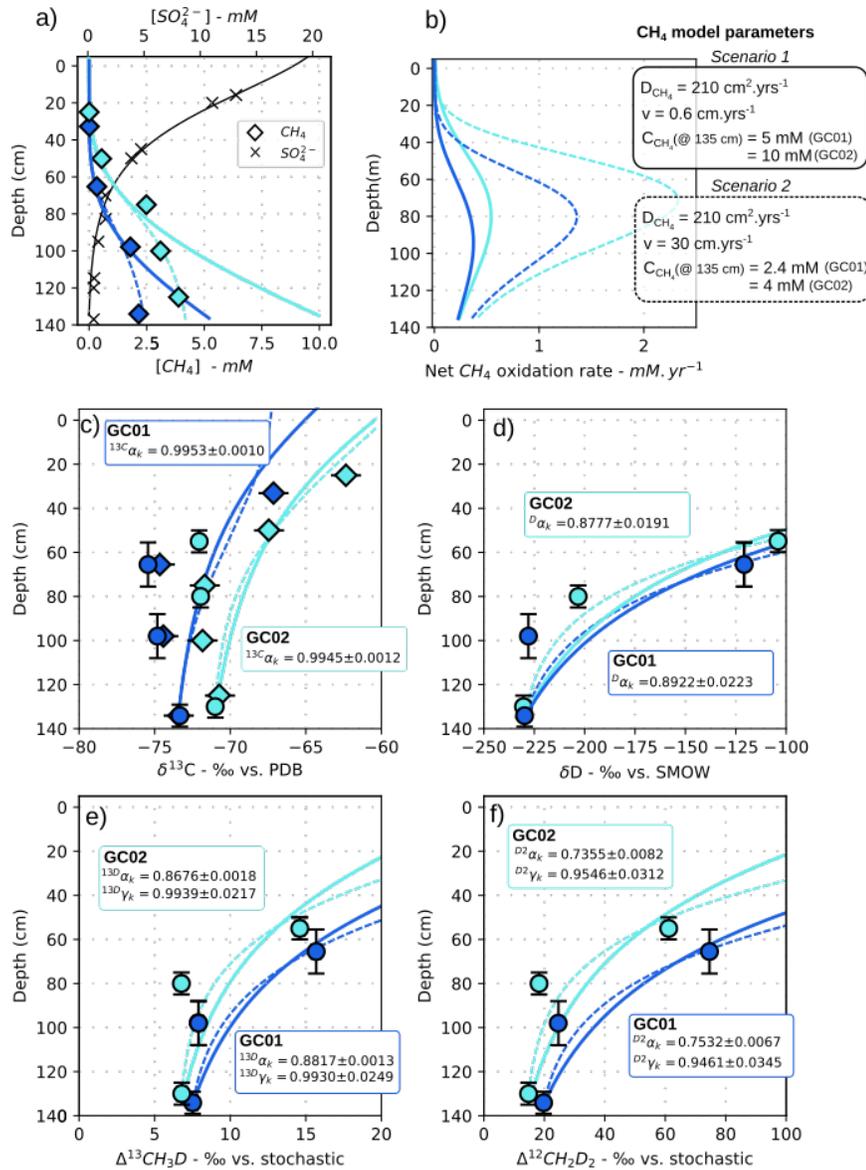
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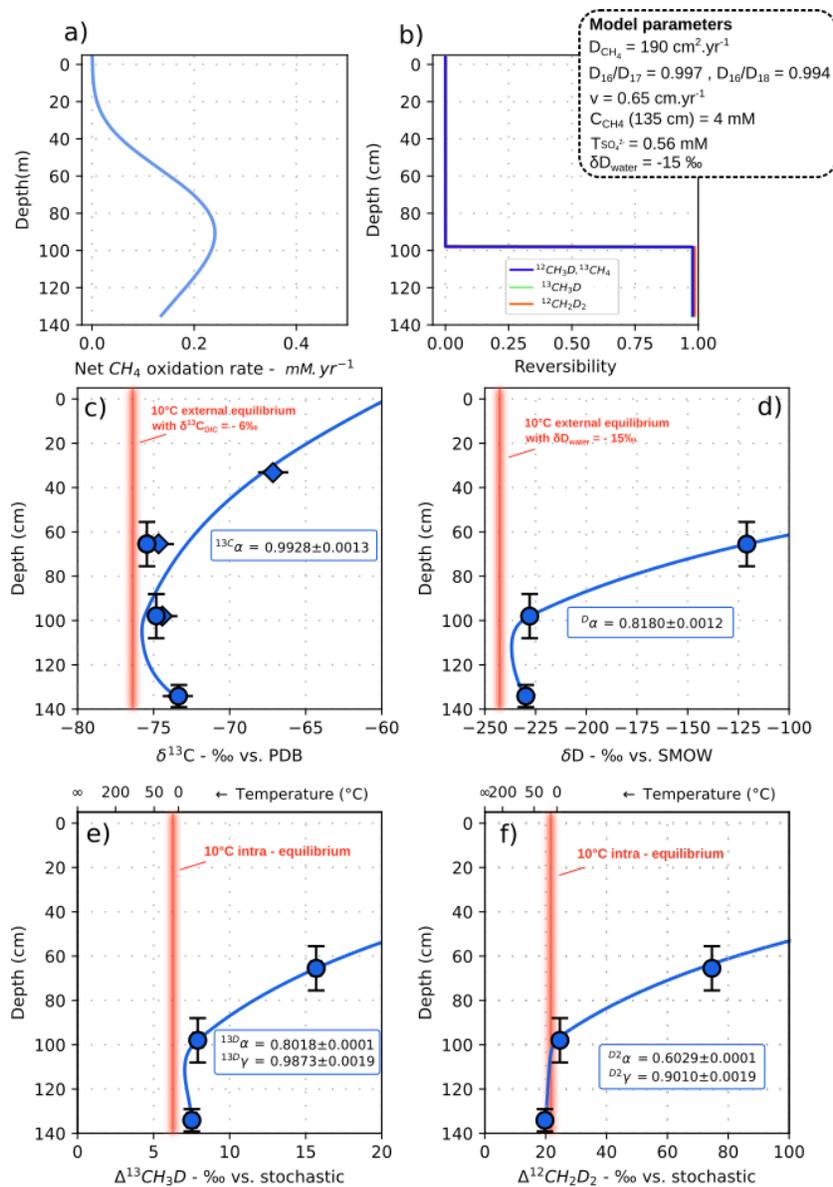
**Figure 5:** Inferring apparent isotope fractionation factors associated to methane degradation in the Lake Pavin water-column with a reactive transport model. In order to avoid considering microbial methane production in the lower part of the water column, the transport-degradation model is solved down to 80 m water depth only. We assumed  $\delta D$ ,  $\Delta^{13}\text{CH}_3\text{D}$  and  $\Delta^{12}\text{CH}_2\text{D}_2$  signatures at 80 m water depth to be identical to 70m depth. A reasonable assumption given that at this depth interval the methane and its isotopologue distributions are solely controlled by non-fractionating transport processes (Lopes et al., 2011). The turbulent diffusion coefficient is set to  $10 \text{ m}^2 \cdot \text{yr}^{-1}$ , an average value between maximum and minimum values inferred for the monimolinion ( $1.6$  to  $19 \text{ m}^2 \cdot \text{yr}^{-1}$ , Aeschbach-Hertig et al., 2002). The vertical fluid flow velocity is set to  $0.25 \text{ m} \cdot \text{yr}^{-1}$ , scaling with the area of the lake at 60 m water depth and an estimated  $1.6 \text{ L} \cdot \text{s}^{-1}$  vertical flow (Assayag et al., 2008).



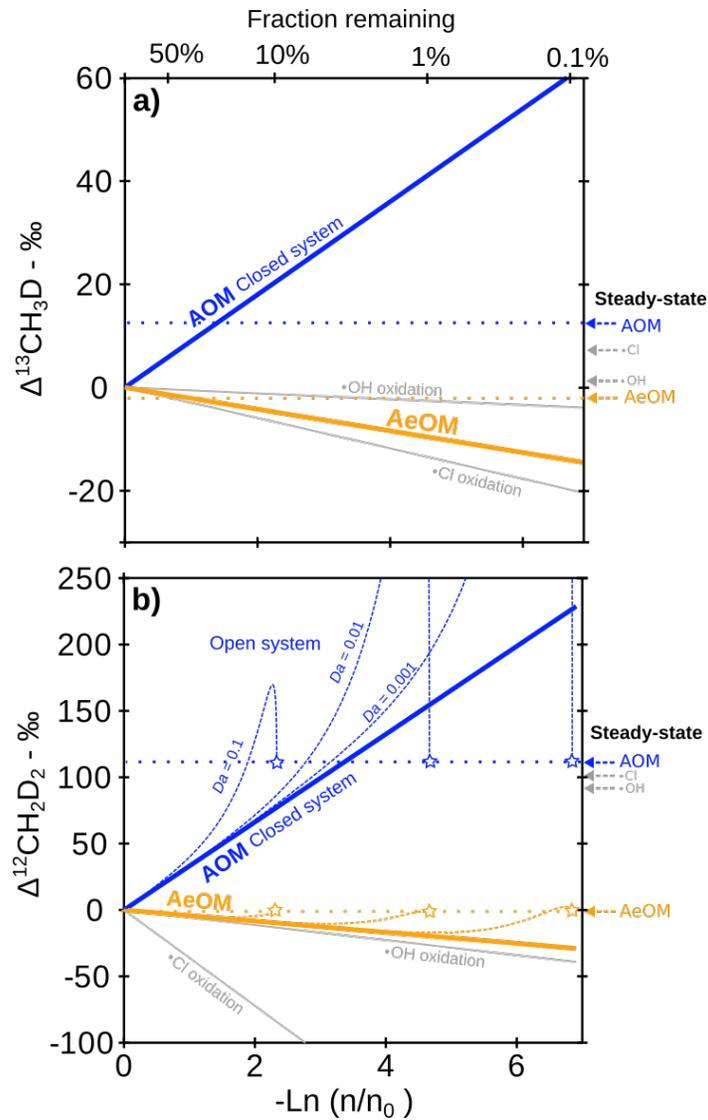
75 **Figure 6:** Comparison of bulk isotope fractionation factors ( ${}^{13}\alpha$  and  ${}^D\alpha$ ) derived from reactive transport modeling, with values extrapolated for AOM or AeOM in laboratory or from field measurements (Coleman et al., 1981; Happell et al., 1994; Kinnaman et al., 2007; Feisthauer et al., 2011; Holler et al., 2011; Rasigraf et al., 2012; Wang et al., 2016; Ono et al., 2021).



**Figure 7:** Inferring apparent isotope fractionation factors of methanotrophy in sediments of the Black Sea. Reactive transport Equation 7 is solved for methane concentration, and subsequently for each isotopologues. Over this small depth interval (<1.5m), the effective diffusion coefficient of methane is considered homogeneous and taken as  $190 \text{ cm}^2 \cdot \text{yr}^{-1}$  (*i.e.* diffusion coefficient of methane in water modulated by a tortuosity coefficient). Diffusion is assumed to fractionate methane isotopologues according to their masses, with  $D_{17}/D_{16} = 0.997$  and  $D_{18}/D_{16} = 0.994$  (see discussion in [Giunta et al., 2021](#)). To model the  $\text{CH}_4$  concentration profiles, two scenarios with distinct boundary conditions and input parameters are considered. In scenario (1), we assume that  $\text{CH}_4$  has significantly degassed from the cores prior to sampling. Thus, measured  $\text{CH}_4$  concentrations above saturation at ambient conditions are underestimated. The bottom  $\text{CH}_4$  bottom concentrations are assumed be at the minimum of 5 mM and 10 mM for GC01 and GC02, respectively. The vertical fluid flow velocity is taken as  $0.6 \text{ cm} \cdot \text{yr}^{-1}$ , as inferred at first order from dissolved  $\text{Cl}^-$  concentrations ([Fig. S2](#)). In scenario (2), we assume that the measured  $\text{CH}_4$  concentrations accurately capture the *in situ* concentrations (*i.e.* no degassing prior to sampling), thus requiring an substantial vertical flow velocity to fit the data (here set to  $30 \text{ cm} \cdot \text{yr}^{-1}$ ). The two scenarios have great impact on the oxidation rate profiles (panel b.) but not on the extrapolated fractionation factors (see also [Fig S4 and S5](#)). The model suggests that clumped isotope fractionation factors with  $\gamma$  significantly below unity are required to produce the extremely positive  $\Delta^{13}\text{CH}_3\text{D}$  and  $\Delta^{12}\text{CH}_2\text{D}_2$  values measured here.

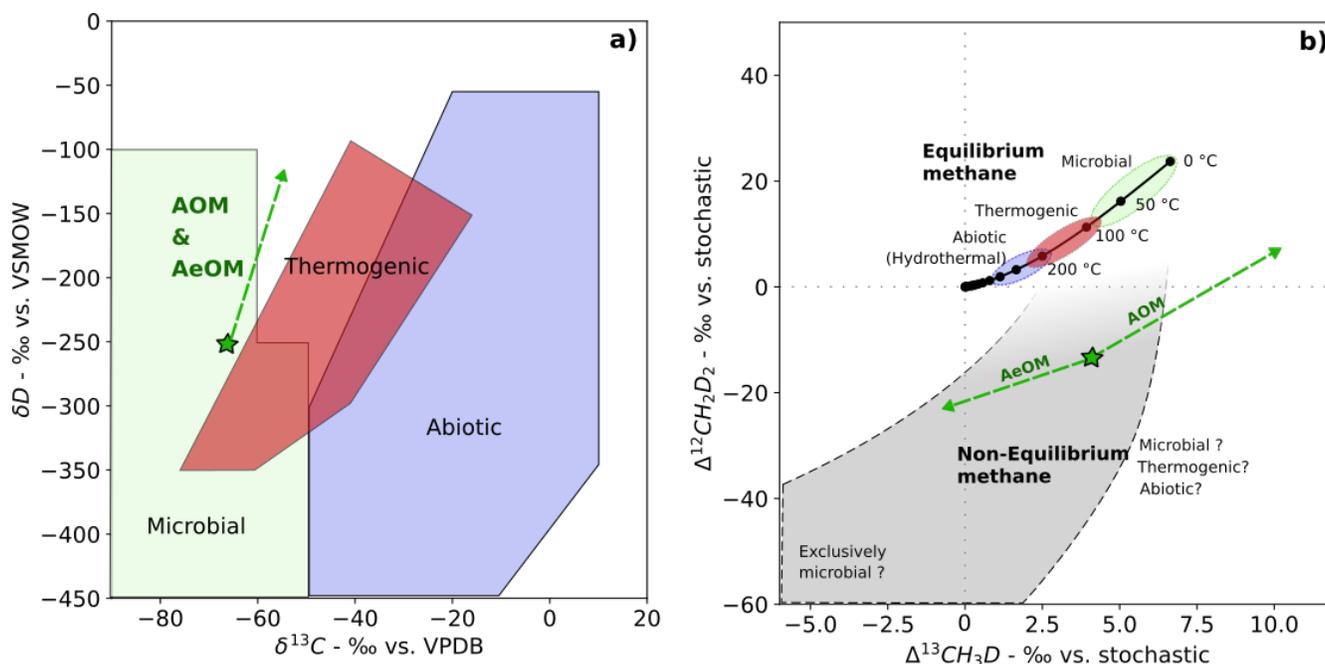


**Figure 8:** Reactive transport modeling with partially reversible AOM reaction in the core GC01. The CH<sub>4</sub> concentration profile is modeled according to Scenario 1 (see Fig. 7). In this depth interval, the pore-water is assumed to be isotopically homogeneous with  $\delta D_{\text{water}} = -15 \text{ ‰}$ . The reversibility of the AOM reaction is set here to depend on a threshold sulfate concentration of 0.55 mM (*ca.* 95 cm depth, panel b.). Below this concentration, the reversibility value is optimized to fit the data and found to be of *ca.* 0.98 (panel b.). Such a high reversibility of the AOM reaction (or at least part of its intermediate reactions) is required to explain why  $\Delta^{13}\text{CH}_3\text{D}$  and  $\Delta^{12}\text{CH}_2\text{D}_2$  are approaching equilibrium at the bottom of the cores, despite net methane degradation presumably occurring (panel a.). Meanwhile, above the threshold sulfate concentration of 0.55 mM, AOM reaction is assumed to be largely dominated by kinetics, with a minimal reversibility of 0.05 (Holler et al., 2011). The kinetic isotope fractionation factors derived here further support the idea that clumped isotopologue fractionation factors of AOM exhibit a significant departure from the product of bulk isotope fractionation factors ( $\gamma < 1$ ). Other scenarios for modeling CH<sub>4</sub> concentration profile in GC01, as well as in GC02, result in similar fractionation factors and maximum reversibility values ranging between 0.72 and 0.98 (Fig. S4 and S5). (\*)



**Figure 9:** Behaviors of (a.)  $\Delta^{13}\text{CH}_3\text{D}$  and (b.)  $\Delta^{12}\text{CH}_2\text{D}_2$  during methanotrophy in a closed or in an open system as function of remaining fraction of methane  $n/n_0$ , with  $n_0$  being the initial quantity of methane in the reservoir. Isotope fractionations factors used for these plots are from this study for AOM (GC01, Fig. 8) and AeOM (Lake Pavin water column, Fig. 5), and from Haghnegahdar et al., (2017) for  $\bullet\text{Cl}$  and  $\bullet\text{OH}$  oxidation reactions. In a closed system, the evolution isotopologues abundances is described with a simple Rayleigh formalism, and simplifies for clumped isotopologues in Equations (12) and (13). In an open system, each isotopologue satisfies the basic mass balance equation  $dn/dt = S - kn$ . The solution can be written in the form  $n(t)/n_0 = e^{-kt} + Da(1 - e^{-kt})$ , where  $Da$  is the Damköhler number establishing the relevance of the reaction over that of the source such that  $Da = \tau_k/\tau_S$ , with  $\tau_k = 1/k$  and  $\tau_S = n_0/S$ . Eventually, all open systems converge to unique steady-state signatures (dotted-lines in panels a. and b.) that can be approached with Equations (14) and (15), but are expected to mimic closed system behavior for sufficiently small  $Da$  values ( $\ll 1$ , dashed-line in panel b.).

120



125 **Figure 10:** Biological and non-biological processes in the bulk isotope space (a) and the clumped isotope space (b). Empirical  
 130 fields for microbial, thermogenic and abiotic attribution based on  $\delta^{13}C$  and  $\delta D$  are those reported in Milkov and Etiope  
 (2018). In the clumped isotope space, we may distinguish natural methane samples with equilibrium or non-equilibrium  
 signatures. To date, it was recognized that abiotic methane from marine hydrothermal vents, thermogenic methane in  
 sedimentary reservoirs or microbial methane in the deep biosphere are likely to be plotting on or closed to the equilibrium  
 curve (Young *et al.*, 2017, 2019; Giunta *et al.*, 2019; Ash *et al.*, 2019; Labidi *et al.*, 2020; Zhang *et al.*, 2021; Mangenot *et al.*,  
 135 2021). Thus, for natural methane samples with equilibrium signatures, the apparent low temperature is perhaps a simple  
 criteria to support a biological mediation of the methane. Note that there is a range of equivalent temperatures (*ca.* 80 – 120°C)  
 that would encompass both microbial and thermogenic methanogenesis temperature ranges, so that the apparent temperature  
 may not always be relevant for discriminating between these two processes. On the other hand, methane with non-equilibrium  
 signatures (gray) are thought to reflect kinetic and or statistical effects associated with the methane synthesis (e.g. Taenzer *et al.*,  
 2020). It is now established from field, experimental and computational studies that all methane production pathways,  
 whether abiotic, thermogenic or microbial, can produce disequilibrium signatures among methane isotopologue (e.g. Wang *et al.*,  
 2015; Young *et al.*, 2017; Gruen *et al.*, 2019; Cao *et al.*, 2019; Dong *et al.*, 2021). Thus, a disequilibrium signature may  
 not be sufficient to infer a biological mediation (although both negative  $\Delta^{13}CH_3D$  and  $\Delta^{12}CH_2D_2$  signatures seem to be  
 140 exclusive to microbial methanogenesis so far). Secondary processes such methanotrophic reactions will affect pristine source  
 signature of the methane and may further contribute to, or generate, disequilibrium among clumped isotopologues. Here, we  
 report in both spaces the general trajectory of methane undergoing microbial oxidation through AOM or AeOM.