Carbon and hydrogen isotope fractionation during nitrite-dependent anaerobic methane oxidation by *Methyloirabilis oxyfera*

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ABSTRACT

Anaerobic oxidation of methane coupled to nitrite reduction is a recently discovered methane sink of as yet unknown global significance. The bacteria that have been identified to carry out this process, *Candidatus Methyloirabilis oxyfera*, oxidize methane via the known aerobic pathway involving the monooxygenase reaction. In contrast to aerobic methanotrophs, oxygen is produced intracellularly and used for the activation of methane by a phylogenetically distinct particulate methane monooxygenase (pMMO). Here we report the fractionation factors for carbon and hydrogen during methane oxidation by an enrichment culture of *M. oxyfera* bacteria. In two separate batch incubation experiments with different absolute biomass and methane contents, the specific methanotrophic activity was similar and the progressive isotope enrichment identical. Headspace methane was consumed up to 98% with rates showing typical first order reaction kinetics. The enrichment factors determined by Rayleigh equations were $-29.2 \pm 2.6\text{‰}$ for $\delta^{13}\text{C}$ (ϵ_{C}) and $-227.6 \pm 13.5\text{‰}$ for $\delta^2\text{H}$ (ϵ_{H}), respectively. These enrichment factors were in the upper range of values reported so far for aerobic methanotrophs. In addition, two-dimensional specific isotope analysis ($\Lambda = (\alpha_{\text{H}}^{-1}-1)/(\alpha_{\text{C}}^{-1}-1)$) was performed and also the determined Λ

25 value of 9.8 was within the range determined for other aerobic and anaerobic methanotrophs. The
26 results showed that in contrast to abiotic processes biological methane oxidation exhibits a narrow
27 range of fractionation factors for carbon and hydrogen irrespective of the underlying biochemical
28 mechanisms. This work will therefore facilitate the correct interpretation of isotopic composition of
29 atmospheric methane with implications for modeling of global carbon fluxes.

30

31 1. INTRODUCTION

32

33 Methane is the most abundant hydrocarbon in the earth's atmosphere and is a potent greenhouse gas
34 with an approximately 25 times higher global warming potential than carbon dioxide (IPCC, 2007).
35 About 69% of all methane is produced by the catabolic activity of methanogenic archaea (Conrad,
36 2009), thriving in anoxic environments (e.g. rice paddy fields, swamps, continental margins) rich in
37 organic carbon and limited in oxidants stronger than carbon dioxide.

38 Most of the produced methane is oxidized back to carbon dioxide by two major sinks – abiotic
39 oxidation by hydroxyl radicals in the upper atmosphere, and microbial oxidation under both oxic and
40 anoxic conditions (Conrad, 2009; Montzka et al., 2011; Wuebbles and Hayhoe, 2002). During
41 biological methane oxidation, the initial activation to methanol is mechanistically the most difficult
42 step due to the exceptional thermodynamic stability of methane with an activation barrier of
43 +439 kJ/mol (Thauer and Shima, 2008). Owing to its strong oxidizing capacity, oxygen was believed to
44 be the only possible electron acceptor for methane oxidation (Strous and Jetten, 2004). Aerobic
45 methane oxidation is exclusively performed by *Bacteria*, belonging to *alpha*- or *gamma*-Proteobacteria
46 and recently discovered *Verrucomicrobia* (Op den Camp et al., 2009; Semrau et al., 2008). Beginning
47 in the 1970s, anaerobic oxidation of methane with sulfate as terminal electron acceptor in consortia of
48 anaerobic methanotrophic archaea (ANME) and sulfate-reducing bacteria was shown to exist and to be
49 the dominant methane-oxidizing process in marine sediments (Knittel and Boetius, 2009). In terms of

thermodynamics, all electron acceptors in the range between sulfate and oxygen (~ -220 mV – +818 mV) could potentially be used for the oxidation of methane. In 2006, it was for the first time described that oxidized nitrogen species (i.e., nitrate, nitrite) could be used for the anaerobic oxidation of methane (AOM) (Raghoebarsing et al., 2006). The process of nitrite-dependent anaerobic methane oxidation (N-DAMO) is performed by *Candidatus* 'Methyloirabilis oxyfera' bacteria which belong to the newly described NC10 phylum (Ettwig et al., 2010; Ettwig et al., 2008; Ettwig et al., 2009). Though living anaerobically, *M. oxyfera* activates methane via the known pathway of aerobic methanotrophs, involving the monooxygenase reaction as the initial step of the process (Ettwig et al., 2010; Wu et al., 2011b). Notably, the molecular oxygen used for methane oxidation is generated intracellularly by the reduction of nitrite to nitric oxide and probably dismutation of the latter to molecular nitrogen and oxygen (Ettwig et al., 2010). Methane monooxygenase is the key enzyme of oxygen-dependent methane oxidation and occurs in nature in two different forms: membrane-bound particulate (pMMO) and cytoplasmic soluble methane monooxygenase (sMMO) (Hakemian and Rosenzweig, 2007). Both enzyme forms differ in structure, active site composition and catalytic mechanism, with sMMO being expressed under copper-limited conditions and exhibiting a broader substrate range than pMMO (Elliott et al., 1997; Hakemian and Rosenzweig, 2007; Murrell et al., 2000). Most known methanotrophs preferentially express pMMO instead of sMMO with only one genus (*Methylocella*) exclusively using the latter, and only a small number can express both simultaneously (Hakemian and Rosenzweig, 2007; Murrell et al., 2000). In its genome, *M. oxyfera* possesses a single copy gene encoding particulate methane monooxygenase, whose full length amino acid sequence of the alpha-subunit (*pmoB*) shares at most only 41% identity to the *pmoB* sequence of *Methylococcus capsulatus*.

So far, little is known about the occurrence and significance of *M. oxyfera* in the environment. Apart from an eutrophic freshwater canal in the Netherlands (Raghoebarsing et al., 2006), these bacteria have also been enriched from other freshwater sediments and a waste water treatment plant (Ettwig et al.,

2009; Hu et al., 2009; Luesken et al., 2011a). The experimental evidence for active N-DAMO *in situ* is still scarce. So far, the concomitant disappearance of methane and nitrate has only been shown to occur in a sewage-contaminated aquifer and the sediment of an oligotrophic lake (Deutzmann and Schink, 2011; Smith et al., 1991). However, 16S rRNA sequences of NC10 phylum bacteria were retrieved from several ecosystems worldwide, including contaminated aquifers, soils, lake and river sediments (Ettwig et al., 2009), thereby providing evidence for their ubiquity. Moreover, the development of primer sets specifically targeting the *pmoA* sequence of *M. oxyfera* bacteria enabled their detection on the functional gene level. *pmoA* sequences related to *M. oxyfera* were detected in several anoxic aquifers, anaerobic waste water treatment plants, peat lands and an oligotrophic lake (Deutzmann and Schink, 2011; Luesken et al., 2011a; Luesken et al., 2011b), showing the potential for nitrite-dependent anaerobic methane oxidation in these ecosystems.

As methane is the second most important anthropogenic greenhouse gas in the atmosphere, the quantification and evaluation of its fluxes on a global scale has been the subject of extensive research during the last decades (Dlugokencky et al., 2009; Ettwig et al., 2009; Montzka et al., 2011; Petit et al., 1999; Robertson et al., 2000; Wuebbles and Hayhoe, 2002). The unusually strong depletion in the heavy ^{13}C isotope of biogenic methane makes it possible to distinguish between thermogenic and biotic sources (Whiticar, 1999), but the picture is often complicated by the effect of biological oxidation. In this respect, determination of the global C1 budget based on mixing ratios and isotopic composition of methane has become an essential tool in biogeochemical studies (Dlugokencky et al., 2009; Kai et al., 2011; Whiticar, 1999).

Usually, (bio)chemical bond cleavages are associated with isotope discrimination of the substrate. The atomic mass differences between isotopes lead to different bond strengths inside the molecules, with heavier nuclei possessing lower zero-point energies and thus stronger bonds (Urey, 1947). The molecules with weaker bonds react faster and the remaining substrate pool becomes enriched with the heavier isotope. However, the preference of different processes, enzymes or even different enzyme

100 isomers for lighter substrates is not uniform. Thereby, different pathways of substrate conversion can
101 often be distinguished by the specific isotopic signature of the remaining pool or the product formed
102 (Fischer et al., 2008; Mahieu et al., 2006; Meckenstock et al., 2004; Vogt et al., 2008).

103 The weighted average $\delta^{13}\text{C}$ of all biotic and abiotic methane sources is about $-54 \pm 5\%$, but the average
104 $\delta^{13}\text{C}$ of atmospheric methane is only -47% (Quay et al., 1999), indicating that methane consumption
105 processes lead to its enrichment with the heavy isotope. During aerobic methane oxidation, the
106 remaining methane pool becomes enriched with ^{13}C and ^2H under non-limiting substrate conditions,
107 which can be attributed to the kinetic effect of the initial and irreversible step of the pathway, the
108 monooxygenase reaction (Nesheim and Lipscomb, 1996; Templeton et al., 2006).

109 The isotope enrichment of the residual substrate pool is expressed in enrichment factors (ϵ). So far,
110 several enrichment factors for carbon and hydrogen during aerobic methane oxidation were determined
111 (Bergamaschi and Harris, 1995; Coleman et al., 1981; Feisthauer et al., 2011; King et al., 1989;
112 Kinnaman et al., 2007; Reeburgh et al., 1997; Snover and Quay, 2000; Tyler et al., 1994). The factors
113 showed a broad range and could not be linked to a distinct type of methane monooxygenase,
114 phylogenetic affiliation or cultivation condition. Furthermore, also enrichment cultures from marine
115 sources exhibiting sulfate-dependent anaerobic methane oxidation produced enrichment factors for
116 carbon and hydrogen similar to those of aerobic methanotrophs (Alperin et al., 1988; Holler et al.,
117 2009; Kessler et al., 2006), despite the profoundly different underlying biochemical methane activation
118 mechanisms.

119 As the observed bulk stable isotope effect can be influenced by masking effects, e. g. diffusion or
120 transport limitation, a two-dimensional analysis on the basis of hydrogen and carbon has been proposed
121 (Feisthauer et al., 2011). The masking effects are considered to influence the bulk isotope fractionation
122 of each element to the same extent, thus the ratio of both fractionation factors could potentially provide
123 a better picture about a particular biotransformation process in the environment (Feisthauer et al.,
124 2011).

125 This study aimed to investigate the isotope enrichment factors for carbon and hydrogen and to
126 determine the two-dimensional fractionation factor during nitrite-dependent anaerobic methane
127 oxidation by an *M. oxyfera* enrichment culture.

128

129 **2. MATERIALS & METHODS**

130

131 **2.1 Enrichment culture and methane oxidation**

132

133 The culture of *M. oxyfera* bacteria (corresponding to strain “Twente” in Ettwig et al. (2010)) was
134 enriched anoxically in a sequencing batch reactor under continuous supply of methane and nitrite as
135 described before (Wu et al., 2011a), and consisted of ~80% of *M. oxyfera* bacteria. The incubation for
136 isotope analyses was performed in batch incubations using 60 ml glass serum bottles with two different
137 headspace to liquid volume ratios: 0.35 (further referred to as R0.35) and 2.8 (R2.8). Biomass was
138 concentrated two times in nitrate-free mineral salt medium (Ettwig et al., 2009) buffered with 5 mM 3-
139 (N-morpholino) propanesulfonic acid (MOPS) under oxic conditions. After aerobically dispensing the
140 biomass to 12 (R0.35) and 8 (R2.8) serum bottles, these were sealed with red butyl rubber stoppers
141 (Rubber BV, Hilversum, Netherlands), crimped with aluminium caps and made anaerobic by 5 cycles
142 of successive vacuuming and gassing with helium, and a final flushing with helium for 5 min. In each
143 serum bottle, an overpressure of 0.4 bar was applied. Thereafter, methane (Air Liquide, Eindhoven,
144 Netherlands) was added to a concentration of 3-5% (v/v) of headspace gas. Culture bottles were
145 incubated horizontally on a shaker (Innova[®] 40, New Brunswick Scientific, Enfield, CT, United States)
146 at 170 rpm and 30°C. Headspace methane content of each serum bottle was monitored throughout the
147 experiment. At certain levels of oxidation (0-98% of initial concentration), bottles (one per time point)
148 were sacrificed by injection of 1 mL of 4 M sodium hydroxide and stored at 4°C until analyses. In

149 addition to biotic culture incubation, abiotic control serum bottles (without culture addition) were
150 prepared.

151

152 **2.2 Analysis of nitrite, methane and protein content**

153

154 Methane concentrations were analyzed by gas chromatography as described by Ettwig et al. (2008).
155 Each sample was measured in duplicate by manual injection of 100 μL headspace gas with a gas-tight
156 syringe (Hamilton, Bonaduz, Switzerland). Protein content from 3 representative batch incubations per
157 experiment was analyzed by bicinchoninic acid assay according to the manufacturer's instructions
158 (Ettwig et al., 2008). Nitrite content was monitored throughout the incubation with Merckoquant test
159 strips (Merck, Darmstadt, Germany) in order to prevent nitrite limitation. In experiment R2.8,
160 additional nitrite was supplied by a 100 mM anaerobic stock solution.

161

162 **2.3 Isotope-ratio mass spectrometry**

163

164 The isotopic composition of the headspace methane was analyzed with an isotope-ratio mass
165 spectrometer (Finnigan MAT 253, Thermo Finnigan Bremen, Germany) coupled to a gas
166 chromatograph (GC, HP 7890A Series, Agilent Technology, Santa Clara, CA, United States for H and
167 HP 6890 Series, Agilent Technology, Santa Clara, CA, United States for C isotopes) via a combustion
168 device. Dependent on the concentration of methane in the headspace, 50 to 1000 μL gas were injected
169 into the GC by a sample-lock syringe (Hamilton). Helium was used as a carrier gas with a constant
170 flow of 2 mL min^{-1} at 40°C . Each sample was measured at least 3 times. The standard deviation was
171 always lower than 0.6 ‰ for $\delta^{13}\text{C}$ and 7.8 ‰ for $\delta^2\text{H}$, respectively. Due to analytical limitations, $\delta^2\text{H}$ of
172 headspace methane of some serum bottles could not be determined and was abandoned from final
173 calculations.

174 2.4 Determination of isotope enrichment factors, fractionation factors and Λ

175

176 For calculations of the isotope enrichment factors, the isotopic abundance ($\delta^{13}\text{C}$, $\delta^2\text{H}$) was expressed in
177 per mill (‰) relative to Vienna PeeDee Belemnite (VPDB) and Vienna Standard Mean Ocean Water
178 (VSMOW) as international standards, respectively (Eq. 1).

179

$$180 \quad \delta^{13}\text{C or } \delta^2\text{H [‰]} = \frac{(R_{\text{sample}} - R_{\text{standard}})}{R_{\text{standard}}} \cdot 1000 \quad \text{Eq. 1}$$

181

182 in which R_{sample} and R_{standard} represent the $^{13}\text{C}/^{12}\text{C}$ and $^2\text{H}/^1\text{H}$ ratios in sample and international standard,
183 respectively.

184 Because batch incubation experiments represent closed systems, a simplified Rayleigh equation
185 approach as described by Coleman et al. (1981) can be applied for determination of enrichment factors
186 during methane oxidation. According to Coleman et al. (1981), the initial concentration of light
187 methane isotopes can be approximated by the total methane concentration as the natural abundance of
188 ^{13}C and ^2H is small (1.1% and 0.015%, respectively). Furthermore, the simplified Rayleigh equation
189 approach applies for first order reaction kinetics, where concentration of methane is the rate limiting
190 factor (Coleman et al., 1981).

191 In the current study, the isotope enrichment factors (ϵ) for carbon and hydrogen were calculated by a
192 simplified Rayleigh equation approach according to Elsner and co-workers (2005) (Eq. 2).

193

$$194 \quad \frac{R_t}{R_0} = \frac{C_t}{C_0}^{\frac{\epsilon}{1000}} \quad \text{Eq. 2}$$

195

196 in which R_t , C_t , R_0 and C_0 represent stable isotope ratios (R) and concentrations (C) of headspace
197 methane at the beginning (time point 0) and after a certain time of the experiment (time point t).

198 By combining the equations 1 and 2, the isotope enrichment factor can be expressed according to

199 equation 3.

$$\frac{\delta_t + 1000}{\delta_0 + 1000} = \frac{C_t^{\frac{\varepsilon}{1000}}}{C_0} \quad \text{Eq. 3}$$

203 The isotope enrichment factor was determined from the slope of the linear regression after plotting of
204 $\ln((\delta_t + 1000)/(\delta_0 + 1000))$ versus $\ln(C_t/C_0)$. δ_t and δ_0 represent the isotope values at the beginning and
205 after a certain time of the experiment, respectively. The standard error originating from the slope was
206 calculated with 95% confidence interval according to Elsner et al. (2007).

207 The isotope fractionation factor (α) can then be calculated according to equation 4.

$$\alpha = \frac{\varepsilon}{1000} + 1 \quad \text{Eq. 4}$$

211 In order to account for potential masking effects, a two-dimensional specific isotope analysis based on
212 isotope fractionation of two elements was performed according to Elsner et al. (2007) and is presented
213 in equation 5.

$$\Lambda = \frac{(\alpha_H^{-1} - 1)}{(\alpha_C^{-1} - 1)} \quad \text{Eq. 5}$$

217 3. RESULTS & DISCUSSION

219 3.1 Methane oxidation kinetics

221 In R0.35, methane was consumed up to 98% within 20 h of incubation, in R2.8 the oxidation did not
222 exceed 84% within 136 h (Figure 1). The different oxidation rates in R0.35 and R2.8 were consistent
223 with the absolute protein content: the specific methane oxidation activity was comparable for both

incubation experiments, 3 ± 0.3 in R0.35 and $3.2 \pm 0.2 \mu\text{mol g}^{-1} \text{protein min}^{-1}$ in R2.8, respectively. In both R0.35 and R2.8, methane oxidation followed first order reaction kinetics; most culture bottles of each experiment exhibited similar methane oxidation rates (Fig. 1).

Figure 1: Methane oxidation of an *M. oxyfera* enrichment culture during both incubation experiments with different headspace to liquid ratios (R0.35 and R2.8). Headspace methane contents at the time of sacrifice are plotted. R0.35 (initial concentration 4% in headspace) is shown with open triangles, R2.8 (initial concentration 3% in headspace) with filled triangles.

During both incubation experiments a significant increase in biomass could be excluded, as the doubling time of *M. oxyfera*-like bacteria lies in the range of one to two weeks (Ettwig et al., 2009). In a previously conducted activity test, the stoichiometry of methane to nitrite consumption was determined to be close to the theoretical ratio of 3:8, indicating that N-DAMO was the predominant denitrifying pathway.

3.2 Isotope fractionation of carbon and hydrogen

Both R0.35 and R2.8 exhibited an enrichment in heavy isotopes of carbon and hydrogen during the course of incubation. The $\delta^{13}\text{C}$ of methane was $-37.8 \pm 0.6\text{‰}$ at the start of incubation and increased to

238 +84.6 ± 0.4‰ after 98% of headspace methane was consumed (Fig. 2A). The enrichment in deuterium
 239 proceeded in the same manner as ¹³C and increased from -130 ± 0.5‰ to +434 ± 6.4‰ after 90% of
 240 headspace methane was consumed (Fig. 2B).

Figure 2: Enrichment in heavy isotopes (A, carbon; B, hydrogen) with progressive methane oxidation by an *M. oxyfera* enrichment culture during both incubation experiments. Values of R0.35 are shown as open rhombs, values of R2.8 as filled rhombs.

242 The trends of heavy isotope enrichment did not differ between R0.35 and R2.8, indicating that no
 243 diffusion limitation occurred in R0.35 and that slow consumption rates and *lag*-time at the beginning of
 244 the incubation in R2.8 did not affect isotope fractionation. The isotope enrichment correlated very well
 245 with the decreasing concentrations of headspace methane typical for closed incubation systems. The
 246 determined isotope enrichment factors from the slopes of simplified Rayleigh plots were $-29.2 \pm 2.6\text{‰}$
 247 for carbon (ϵ_C) and $-227.6 \pm 13.5\text{‰}$ for hydrogen (ϵ_H) (Fig. 3). Both regression lines came close to a
 248 correlation factor (R^2) of 1 (Figure 3).

Figure 3: Rayleigh plots for stable isotope fractionation (A, carbon; B, hydrogen). Data from both incubation experiments are included.

250 The results showed that *M. oxyfera*-like methanotrophs discriminate against the heavier isotopes of
 251 carbon and hydrogen with values in the upper range of what has been reported so far for other
 252 methanotrophs and methane-oxidizing environmental samples (Tab. 1). Neither the peculiar
 253 metabolism nor the distinct sequence of *M. oxyfera*-specific pMMO were reflected in its specific
 254 enrichment factors. Similar observations were made by Feisthauer et al. (2011), where type I and type
 255 II methanotrophs produced similar enrichment factors for methane regardless of the type of expressed
 256 MMO. Although Nesheim and Lipscomb (1996) determined that isotope fractionation during biological
 257 methane oxidation is primary due to catalysis by MMO, the experiments with whole cells showed that
 258 other processes might play a significant role for observed bulk isotope effect as well. Previous studies
 259 on microbial aerobic oxidation of phenol and benzoate provided evidence that growth rates and
 260 physiological features were major parameters for the variation of isotopic discrimination of carbon
 261 (Hall et al., 1999). These factors correlated, directly or indirectly, with kinetics of substrate transport
 262 into the cell and thus its availability for the activating enzyme. As concluded by Kinnaman et al.
 263 (2007), substrate limitation and transport rates during aerobic oxidation of C1-C4 alkanes were the

264 main determinants of fractionation control in methane seep enrichment cultures. As the *M. oxyfera*
265 enrichment culture primary consisted of aggregated cells, substrate diffusion limitation to inner cells
266 could be one of the parameters which would affect the extent of bulk isotope fractionation and possibly
267 mask the true fractionation in case of non-limiting substrate condition for each individual cell.
268 Substrate availability is controlled by two factors, the bulk substrate concentration on one hand, and
269 cell biomass content and its activity on the other hand. A study of Kampara et al. (2009) showed that
270 cell density had a significant impact on fractionation of carbon during aerobic toluene degradation,
271 with lower cell numbers of single-cell cultures leading to highest isotope fractionation factors. In case
272 of biofilms or cell clusters the effect could be even higher since the ratio of biovolume to surface area is
273 much higher, restricting the access of substrate even more. Templeton et al. (2006) found that methane
274 fractionation by whole cells of proteobacteria was mostly regulated by the total amount of substrate
275 oxidized per unit time, which is dependent on the cell numbers and finally the number of active
276 MMOs, and was regardless of the type of organism or type of expressed MMO. During the current
277 study, substrate limitation could be excluded as the isotope enrichment differed neither between both
278 incubation experiments nor was it affected by decreasing methane contents during oxidation within
279 each incubation experiment. One of the factors leading to the high enrichment factor of $\delta^{13}\text{C}$ could be
280 the availability of oxygen for pMMO (Templeton et al., 2006). The effect of oxygen would be inverse
281 to that of methane concentrations. Theoretically, under low oxygen concentrations the process of
282 methane oxidation would be slowed down favoring fractionation. Thus, even at low methane
283 availability, the kinetic isotope effect could be offset by low oxygen. As *M. oxyfera* produces its oxygen
284 intracellularly from nitrite via nitric oxide, this step is likely to be rate-limiting, thus restricting oxygen
285 availability for pMMO (Ettwig et al., 2010).

Table 1: Overview of known isotope enrichment factors and Λ from previous studies on enriched and pure cultures of aerobic and anaerobic methanotrophs, and methane oxidizing environmental samples. Λ average values were calculated from original publications or taken from Feisthauer et al. (2011)

Organism/Culture/Environment	Temperature (°C)	Carbon	Hydrogen	Λ (average)	Reference
		$\epsilon_C \pm \text{Error}$	$\epsilon_H \pm \text{Error}$		
<i>Methylococcus capsulatus</i> (pure)	45	-27.9 $\pm$ 1.7 ^a	-231.5 $\pm$ 30.5	10.5	Feisthauer et al., 2011
		-22.9 $\pm$ 3.2 ^b	-192.0 $\pm$ 28.5	10.1	Feisthauer et al., 2011
<i>Methylosinus sporium</i> (pure)	30	-18.8 $\pm$ 1.4 ^a	-136.8 $\pm$ 20.1	8.3	Feisthauer et al., 2011
		-21.5 $\pm$ 2.7 ^b	-182.6 $\pm$ 23.7	10.2	Feisthauer et al., 2011
<i>Methylocystis parvus</i> (pure)	30	-19.1 $\pm$ 1.0 ^a	-168.2 $\pm$ 9.1	10.4	Feisthauer et al., 2011
<i>Methylomonas methanica</i> (pure)	30	-27.7 $\pm$ 2.3 ^a	-225.5 $\pm$ 17.8	10.2	Feisthauer et al., 2011
<i>Methylocaldum gracile</i> (pure)	30	-14.8 $\pm$ 0.9 ^a	-110.0 $\pm$ 11.5	8.2	Feisthauer et al., 2011
<i>Methylomirabilis oxyfera</i> (enrichment) ^d	30	-29.2 $\pm$ 2.6 ^a	-227.6 $\pm$ 13.5	9.8	This study
Shane Seep, CA, USA (enrichment)	15	-26.6 $\pm$ 1.6	-156.4 $\pm$ n.d.	6.8	Kinnaman et al., 2007
Brian Seep, CA, USA (enrichment)	15	-24.9 $\pm$ 1.2	-319.9 $\pm$ n.d.	18.4	Kinnaman et al., 2007
Drip tray of ice-making machine (enrichment)	26	-24.6 $\pm$ 0.7	-245.3 $\pm$ 25	12.9	Coleman et al., 1981
Water sample (enrichment)	11.5	-12.8 $\pm$ 0.2	-93.4 $\pm$ 6	7.9	Coleman et al., 1981
	26	-15.4 $\pm$ 0.3	-229.0 $\pm$ 2	19	Coleman et al., 1981
Hydrate Ridge, Pacific Ocean (enrichment) ^d	12	-11.9 $\pm$ 0	-114.7 $\pm$ 19.3	10.8	Holler et al., 2009
Amon Mud Volcano, Mediterranean Sea (enrichment) ^d	20	-20.6 $\pm$ 2	-139.4 $\pm$ 16.7	7.7	Holler et al., 2009
Black Sea microbial mat (enrichment) ^d	12	-35.7 $\pm$ 2	-229.6 $\pm$ 14.9	8.1	Holler et al., 2009
Compost and sand biofilters (6-8% CH ₄)	22	-17.7 $\pm$ 0.5	-138.5 $\pm$ 11.4	8.9	Powelson et al., 2007
Tundra soil, AK, USA	14	-26 $\pm$ n.d.	n.d.	-	King et al., 1989
	4	-16 $\pm$ n.d.	n.d.	-	King et al., 1989
Hardwood forest soil, NH, USA	8-23	-22 $\pm$ 4	n.d.	-	Tyler et al., 1994
Black Spruce soil, AK, USA	2.5	-25.3 $\pm$ n.d.	n.d.	-	Reeburgh et al., 1997
Aspen soil, AK, USA	5.5	-22.5 $\pm$ n.d.	n.d.	-	Reeburgh et al., 1997
Black Sea water column ^d	9	-20.6 $\pm$ 1	-166.7 $\pm$ 20	9.5	Kessler et al., 2006
Eckernförde Bay pore water, Baltic Sea ^d	8	-11.9 $\pm$ 1	-107.1 $\pm$ 20	10	Martens et al., 1999
Skan Bay sediment, AK, USA ^d	4	-8.7 $\pm$ 1	-135.7 $\pm$ 23	17.8	Alperin et al., 1988

	Grassland soil, WA, USA	8.3	-17 ± 1	-90.1 ± 30	5.7	Snover & Quay, 2000
	Forest soil, WA, USA	11.6	-17.8 ± 0.4	-61.9 ± 7	3.6	Snover & Quay, 2000
	Flooded Hardwood swamp, FL, USA	-	-19.6 ± n.d.	-69.8 ± n.d.	3.7	Happell et al., 1994
		-	-18.6 ± n.d.	-57.5 ± n.d.	3.2	Happell et al., 1994
	Landfill cover soil, Mainz area, Germany	-	-7.9 ± 4	-42.1 ± 20	5.5	Bergamaschi & Harris, 1995
	Landfill cover soils, Europe	-	-7.9 ± 4	-37.5 ± 26	4.9	Bergamaschi et al., 1998
	Landfill cover soils, New England, USA	-	-21.5 ± 8	n.d.	-	Liptay et al., 1998
286	^a = expressed pMMO					
287	^b = expressed sMMO					
288	n.d. = not determined					
289	^d = anoxic					

290 3.3 Two-dimensional specific isotope analysis

291

292 The two-dimensional specific isotope analysis was performed in order to compare the determined
293 isotope enrichment factors and to minimize the possible masking effects during methane oxidation by
294 *M. oxyfera*, and resulted in a lambda (Λ) value of 9.8. This approach was recently introduced in order
295 to identify specific biodegradation processes (Elsner et al., 2007; Elsner et al., 2005). However, the
296 study of Elsner et al. (2007) focused on oxidation of methyl *tert*-butyl ether, a compound more
297 complex and containing several non-reactive atom positions in contrast to methane. In a recent study,
298 Feisthauer et al. (2011) determined several Λ values for sMMO and pMMO of phylogenetically distinct
299 proteobacteria, but could not observe significantly different lambda values dependent on the type of
300 enzyme expressed. The authors compared the determination of Λ by two approaches, by plotting $\delta^{13}\text{C}$
301 versus $\delta^2\text{H}$ and calculation according to equation 5. The first approach was shown to be applicable only
302 for enrichment factors of hydrogen not exceeding -100‰, which was out of the range during the
303 current study. In general, there is a broad range of variation in Λ known from previous reports on
304 methane oxidizing environmental gas samples and enrichment cultures (Tab. 1). The values range
305 between 3.2 and 19 without an obvious correlation with phylogeny or environmental conditions. Thus,
306 neither the phylogenetic affiliation nor the specific catalytic mechanism can be inferred from the two-
307 dimensional specific isotope analysis and this tool is not valuable for discrimination of different
308 biological methane sinks.

309

310 3.4 Environmental implications

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312 It is evident from all studies on stable isotope fractionation during aerobic methane oxidation
313 conducted so far that the determined enrichment and Λ factors reveal neither the underlying enzymatic
314 pathway nor the phylogenetic affiliation of active methanotrophs. Only in environmental settings where

the electron acceptors are known, the *M. oxyfera*-specific isotope enrichment factors determined herein could be used for quantification of methane degradation. Also methanogenesis might be an important factor which must be taken into consideration when interpreting isotope data from anoxic environments. Methanogenic activity may overlap with anaerobic methanotrophy, leading to a partial recycling of produced carbon dioxide thereby complicating the interpretation of isotope data. However, under nitrate/nitrite rich conditions in the habitat of *M. oxyfera* methanogenesis might be restricted due to the high redox potential and unfavorable kinetics in comparison with denitrifiers.

A study of Holler et al. (2009) on isotope fractionation by enrichment cultures of AOM consortia (ANME II clade) from various marine environments revealed ϵ_C values of -11.9‰ – -35.7‰ and ϵ_H of -114.7‰ – -229.6‰. Despite the profoundly different biochemical mechanism underlying anaerobic methanotrophy, its isotope enrichment factors resemble those of aerobic methanotrophs. Similar values were also reported by Kessler et al. (2006) and Martens et al. (1999) for gas samples from anoxic marine environments. These findings together indicate a narrow range of fractionation specific for biological methane oxidation. This can be valuable for the quantitative distinction between biotic and abiotic methane oxidation. The hydroxyl-driven abiotic methane oxidation in the atmosphere is responsible for almost one third of all methane removal (Thauer, 2011) and exhibits an Λ value of 75, a value of one magnitude larger than that of biologically mediated oxidation (Bergamaschi et al., 2000; Feilberg et al., 2005; Feisthauer et al., 2011; Saueressig et al., 1996; Saueressig et al., 2001), making both processes well distinguishable. With the knowledge based on these findings, different methane sinks can be more strictly confined, which is important in the context of the recent debate on the evaluation of global methane budget (Heimann, 2011).

336

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338

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343 **References**

344

345

346 Alperin M. J., Reeburgh W. S., and Whiticar M. J. (1988) Carbon and hydrogen isotope
347 fractionation resulting from anaerobic methane oxidation. *Global Biogeochem. Cycles* **2**,
348 279-288.

349 Bergamaschi P., Bräunlich M., Marik T., and Brenninkmeijer C. A. M. (2000) Measurements of the
350 carbon and hydrogen isotopes of atmospheric methane at Izaña, Tenerife: seasonal cycles
351 and synoptic-scale variations. *J. Geophys. Res.* **105**, 14531-14546.

352 Bergamaschi P., Lubina C., Königstedt R., Fischer H., Veltkamp A. C., and Zwaagstra O. (1998)
353 Stable isotopic signatures ($\delta^{13}\text{C}$, δD) of methane from European landfill sites. *J. Geophys.*
354 *Res.* **103**, 8251-8265.

355 Bergamaschi P. and Harris G. W. (1995) Measurements of stable isotope ratios ($^{13}\text{CH}_4/^{12}\text{CH}_4$;
356 $^{12}\text{CH}_3\text{D}/^{12}\text{CH}_4$) in landfill methane using a Tunable Diode Laser Absorption Spectrometer.
357 *Global Biogeochem. Cycles* **9**, 439-447.

358 Coleman D. D., Risatti J. B., and Schoell M. (1981) Fractionation of carbon and hydrogen isotopes
359 by methane-oxidizing bacteria. *Geochim. Cosmochim. Acta* **45**, 1033-1037.

360 Conrad R. (2009) The global methane cycle: recent advances in understanding the microbial
361 processes involved. *Environ. Microbiol. Rep.* **1**, 285-292.

362 Deutzmann J. S. and Schink B. (2011) Anaerobic oxidation of methane in sediments of Lake
363 Constance, an oligotrophic freshwater lake. *Appl. Environ. Microbiol.* **77**, 4429-4436.

364 Dlugokencky E. J., Bruhwiler L., White J. W. C., Emmons L. K., Novelli P. C., Montzka S. A.,
365 Masarie K. A., Lang P. M., Crotwell A. M., Miller J. B., and Gatti L. V. (2009)
366 Observational constraints on recent increases in the atmospheric CH_4 burden. *Geophys. Res.*
367 *Lett.* **36**, L18803.

368 Elliott S. J., Zhu M., Tso L., Nguyen H. H. T., Yip J. H. K., and Chan S. I. (1997) Regio- and

369 stereoselectivity of particulate methane monooxygenase from *Methylococcus capsulatus*
370 (Bath). *J. Am. Chem. Soc.* **119**, 9949-9955.

371 Elsner M., McKelvie J., Lacrampe Couloume G., and Sherwood Lollar B. (2007) Insight into
372 methyl *tert*-butyl ether (MTBE) stable isotope fractionation from abiotic reference
373 experiments. *Environ. Sci. Technol.* **41**, 5693-5700.

374 Elsner M., Zwank L., Hunkeler D., and Schwarzenbach R. P. (2005) A new concept linking
375 observable stable isotope fractionation to transformation pathways of organic pollutants.
376 *Environ. Sci. Technol.* **39**, 6896-6916.

377 Ettwig K. F., Butler M. K., Le Paslier D., Pelletier E., Mangenot S., Kuypers M. M. M., Schreiber
378 F., Dutilh B. E., Zedelius J., de Beer D., Gloerich J., Wessels H. J. C. T., van Alen T.,
379 Luesken F., Wu M. L., van de Pas-Schoonen K. T., Op den Camp H. J. M., Janssen-Megens
380 E. M., Francoijs K.-J., Stunnenberg H., Weissenbach J., Jetten M. S. M., and Strous M.
381 (2010) Nitrite-driven anaerobic methane oxidation by oxygenic bacteria. *Nature* **464**, 543-
382 548.

383 Ettwig K. F., Shima S., van de Pas-Schoonen K. T., Kahnt J., Medema M. H., Op den Camp H. J.
384 M., Jetten M. S. M., and Strous M. (2008) Denitrifying bacteria anaerobically oxidize
385 methane in the absence of Archaea. *Environ. Microbiol.* **10**, 3164-3173.

386 Ettwig K. F., van Alen T., van de Pas-Schoonen K. T., Jetten M. S. M., and Strous M. (2009)
387 Enrichment and molecular detection of denitrifying methanotrophic bacteria of the NC10
388 phylum. *Appl. Environ. Microbiol.* **75**, 3656-3662.

389 Feilberg K. L., Griffith D. W. T., Johnson M. S., and Nielsen C. J. (2005) The ¹³C and D kinetic
390 isotope effects in the reaction of CH₄ with Cl. *Int. J. Chem. Kinet.* **37**, 110-118.

391 Feisthauer S., Vogt C., Modrzynski J., Szlenkier M., Krüger M., Siegert M., and Richnow H.-H.
392 (2011) Different types of methane monooxygenases produce similar carbon and hydrogen
393 isotope fractionation patterns during methane oxidation. *Geochim. Cosmochim. Acta* **75**,
394 1173-1184.

395 Fischer A., Herklotz I., Herrmann S., Thullner M., Weelink S. A. B., Stams A. J. M., Schlömann M.,
396 Richnow H.-H., and Vogt C. (2008) Combined carbon and hydrogen isotope fractionation
397 investigations for elucidating benzene biodegradation pathways. *Environ. Sci. Technol.* **42**,
398 4356-4363.

399 Hakemian A. S. and Rosenzweig A. C. (2007) The biochemistry of methane oxidation. *Annu. Rev.*
400 *Biochem.* **76**, 223-241.

401 Hall J. A., Kalin R. M., Larkin M. J., Allen C. C. R., and Harper D. B. (1999) Variation in stable
402 carbon isotope fractionation during aerobic degradation of phenol and benzoate by
403 contaminant degrading bacteria. *Org. Geochem.* **30**, 801-811.

404 Happell J. D., Chanton J. P., and Showers W. S. (1994) The influence of methane oxidation on the
405 stable isotopic composition of methane emitted from Florida swamp forests. *Geochim.*
406 *Cosmochim. Acta* **58**, 4377-4388.

407 Heimann M. (2011) Atmospheric science: enigma of the recent methane budget. *Nature* **476**, 157-
408 158.

409 Holler T., Wegener G., Knittel K., Boetius A., Brunner B., Kuypers M. M. M., and Widdel F. (2009)
410 Substantial $^{13}\text{C}/^{12}\text{C}$ and D/H fractionation during anaerobic oxidation of methane by marine
411 consortia enriched *in vitro*. *Environ. Microbiol. Rep.* **1**, 370-376.

412 Hu S., Zeng R. J., Burow L. C., Lant P., Keller J., and Yuan Z. (2009) Enrichment of denitrifying
413 anaerobic methane oxidizing microorganisms. *Environ. Microbiol. Rep.* **1**, 377-384.

414 IPCC, Intergovernmental Panel on Climate Change (2007) Climate Change 2007: The physical
415 science basis. Contribution of working group I to the fourth assessment report of the
416 Intergovernmental Panel on Climate Change, Cambridge University Press, Cambridge.

417 Kai F. M., Tyler S. C., Randerson J. T., and Blake D. R. (2011) Reduced methane growth rate
418 explained by decreased Northern Hemisphere microbial sources. *Nature* **476**, 194-197.

419 Kampara M., Thullner M., Harms H., and Wick L. (2009) Impact of cell density on microbially
420 induced stable isotope fractionation. *Appl. Microbiol. Biotechnol.* **81**, 977-985.

421 Kessler J. D., Reeburgh W. S., and Tyler S. C. (2006) Controls on methane concentration and stable
 422 isotope ($\delta^2\text{H-CH}_4$ and $\delta^{13}\text{C-CH}_4$) distributions in the water columns of the Black Sea and
 423 Cariaco Basin. *Global Biogeochem. Cycles* **20**, GB4004.

424 King S. L., Quay P. D., and Lansdown J. M. (1989) The $^{13}\text{C}/^{12}\text{C}$ Kinetic isotope effect for soil
 425 oxidation of methane at ambient atmospheric concentrations. *J. Geophys. Res.* **94**, 18273-
 426 18277.

427 Kinnaman F. S., Valentine D. L., and Tyler S. C. (2007) Carbon and hydrogen isotope fractionation
 428 associated with the aerobic microbial oxidation of methane, ethane, propane and butane.
 429 *Geochim. Cosmochim. Acta* **71**, 271-283.

430 Knittel K. and Boetius A. (2009) Anaerobic oxidation of methane: progress with an unknown
 431 process. *Annu. Rev. Microbiol.* **63**, 311-334.

432 Liptay K., Chanton J., Czepiel P., and Mosher B. (1998) Use of stable isotopes to determine
 433 methane oxidation in landfill cover soils. *J. Geophys. Res.* **103**, 8243-8250.

434 Luesken F., van Alen T., van der Biezen E., Frijters C., Toonen G., Kampman C., Hendrickx T.,
 435 Zeeman G., Temmink H., Strous M., Op den Camp H., and Jetten M. (2011a) Diversity and
 436 enrichment of nitrite-dependent anaerobic methane oxidizing bacteria from wastewater
 437 sludge. *Appl. Microbiol. Biotechnol.* **92**, 845-854.

438 Luesken F. A., Zhu B., van Alen T. A., Butler M. K., Diaz M. R., Song B., Op den Camp H. J. M.,
 439 Jetten M. S. M., and Ettwig K. F. (2011b) *pmoA* primers for detection of anaerobic
 440 methanotrophs. *Appl. Environ. Microbiol.* **77**, 3877-3880.

441 Mahieu K., Visscher A. D., Vanrolleghem P. A., and Cleemput O. V. (2006) Carbon and hydrogen
 442 isotope fractionation by microbial methane oxidation: Improved determination. *Waste*
 443 *Manage.* **26**, 389-398.

444 Martens C. S., Albert D. B., and Alperin M. J. (1999) Stable isotope tracing of anaerobic methane
 445 oxidation in the gassy sediments of Eckernförde Bay, German Baltic Sea. *Am. J. Sci.* **299**,
 446 589-610.

447 Meckenstock R. U., Morasch B., Griebler C., and Richnow H. H. (2004) Stable isotope
 448 fractionation analysis as a tool to monitor biodegradation in contaminated aquifers. *J.*
 449 *Contam. Hydrol.* **75**, 215-255.

450 Montzka S. A., Dlugokencky E. J., and Butler J. H. (2011) Non-CO₂ greenhouse gases and climate
 451 change. *Nature* **476**, 43-50.

452 Murrell J. C., McDonald I. R., and Gilbert B. (2000) Regulation of expression of methane
 453 monooxygenases by copper ions. *Trends Microbiol.* **8**, 221-225.

454 Nesheim J. C. and Lipscomb J. D. (1996) Large kinetic isotope effects in methane oxidation
 455 catalyzed by methane monooxygenase: evidence for C–H bond cleavage in a reaction cycle
 456 intermediate. *Biochemistry* **35**, 10240-10247.

457 Op den Camp H. J. M., Islam T., Stott M. B., Harhangi H. R., Hynes A., Schouten S., Jetten M. S.
 458 M., Birkeland N.-K., Pol A., and Dunfield P. F. (2009) Environmental, genomic and
 459 taxonomic perspectives on methanotrophic Verrucomicrobia. *Environ. Microbiol. Rep.* **1**,
 460 293-306.

461 Petit J. R., Jouzel J., Raynaud D., Barkov N. I., Barnola J. M., Basile I., Bender M., Chappellaz J.,
 462 Davis M., Delaygue G., Delmotte M., Kotlyakov V. M., Legrand M., Lipenkov V. Y., Lorius
 463 C., Pepin L., Ritz C., Saltzman E., and Stievenard M. (1999) Climate and atmospheric
 464 history of the past 420,000 years from the Vostok ice core, Antarctica. *Nature* **399**, 429-436.

465 Powelson D. K., Chanton J. P., and Abichou T. (2006) Methane oxidation in biofilters measured by
 466 mass-balance and stable isotope methods. *Environ. Sci. Technol.* **41**, 620-625.

467 Quay P., Stutsman J., Wilbur D., Snover A., Dlugokencky E., and Brown T. (1999) The isotopic
 468 composition of atmospheric methane. *Global Biogeochem. Cycles* **13**, 445-461.

469 Raghoebarsing A. A., Pol A., van de Pas-Schoonen K. T., Smolders A. J. P., Ettwig K. F., Rijpstra
 470 W. I. C., Schouten S., Damste J. S. S., Op den Camp H. J. M., Jetten M. S. M., and Strous
 471 M. (2006) A microbial consortium couples anaerobic methane oxidation to denitrification.
 472 *Nature* **440**, 918-921.

473 Reeburgh W. S., Hirsch A. I., Sansone F. J., Popp B. N., and Rust T. M. (1997) Carbon kinetic
 474 isotope effect accompanying microbial oxidation of methane in boreal forest soils. *Geochim.*
 475 *Cosmochim. Acta* **61**, 4761-4767.

476 Robertson G. P., Paul E. A., and Harwood R. R. (2000) Greenhouse gases in intensive agriculture:
 477 contributions of individual gases to the radiative forcing of the atmosphere. *Science* **289**,
 478 1922-1925.

479 Saueressig G., Bergamaschi P., Crowley J. N., Fischer H., and Harris G. W. (1996) D/H kinetic
 480 isotope effect in the reaction $\text{CH}_4 + \text{Cl}$. *Geophys. Res. Lett.* **23**, 3619-3622.

481 Saueressig G., Crowley J. N., Bergamaschi P., Brühl C., Brenninkmeijer C. A. M., and Fischer H.
 482 (2001) Carbon 13 and D kinetic isotope effects in the reactions of CH_4 with O (1 D) and
 483 OH: new laboratory measurements and their implications for the isotopic composition of
 484 stratospheric methane. *J. Geophys. Res.* **106**, 23127-23138.

485 Semrau J. D., DiSpirito A. A., and Murrell J. C. (2008) Life in the extreme: thermoacidophilic
 486 methanotrophy. *Trends Microbiol.* **16**, 190-193.

487 Smith R. L., Howes B. L., and Garabedian S. P. (1991) In situ measurement of methane oxidation in
 488 groundwater by using natural-gradient tracer tests. *Appl. Environ. Microbiol.* **57**, 1997-2004.

489 Snover A. K. and Quay P. D. (2000) Hydrogen and carbon kinetic isotope effects during soil uptake
 490 of atmospheric methane. *Global Biogeochem. Cycles* **14**, 25-39.

491 Strous M. and Jetten M. S. M. (2004) Anaerobic oxidation of methane and ammonium. *Annu. Rev.*
 492 *Microbiol.* **58**, 99-117.

493 Templeton A. S., Chu K.-H., Alvarez-Cohen L., and Conrad M. E. (2006) Variable carbon isotope
 494 fractionation expressed by aerobic CH_4 -oxidizing bacteria. *Geochim. Cosmochim. Acta* **70**,
 495 1739-1752.

496 Thauer R. K. (2011) Anaerobic oxidation of methane with sulfate: on the reversibility of the
 497 reactions that are catalyzed by enzymes also involved in methanogenesis from CO_2 . *Curr.*

498 *Opin. Microbiol.* **14**, 292-299.

499 Thauer R. K. and Shima S. (2008) Methane as fuel for anaerobic microorganisms. *Ann. N. Y. Acad.*
500 *Sci.* **1125**, 158-170.

501 Tyler S. C., Crill P. M., and Brailsford G. W. (1994) $^{13}\text{C}/^{12}\text{C}$ Fractionation of methane during
502 oxidation in a temperate forested soil. *Geochim. Cosmochim. Acta* **58**, 1625-1633.

503 Urey H. C. (1947) The thermodynamic properties of isotopic substances. *J. Chem. Soc.*, 562-581.

504 Vogt C., Cyrus E., Herklotz I., Schlosser D., Bahr A., Herrmann S., Richnow H.-H., and Fischer A.
505 (2008) Evaluation of toluene degradation pathways by two-dimensional stable isotope
506 fractionation. *Environ. Sci. Technol.* **42**, 7793-7800.

507 Whiticar M. J. (1999) Carbon and hydrogen isotope systematics of bacterial formation and
508 oxidation of methane. *Chem. Geol.* **161**, 291-314.

509 Wu M. L., de Vries S., van Alen T. A., Batler M. K., Op den Camp H. J. M., Keltjens J. T., Jetten M.
510 S. M., and Strous M. (2011a) Physiological role of the respiratory quinol oxidase in the
511 anaerobic nitrite-reducing methanotroph ‘*Candidatus Methyloirabilis oxyfera*’.
512 *Microbiology* **157**, 890-898.

513 Wu M. L., Ettwig K. F., Jetten M. S. M., Strous M., Keltjens J. T., and van Niftrik L. (2011b) A new
514 intra-aerobic metabolism in the nitrite-dependent anaerobic methane-oxidizing bacterium
515 *Candidatus ‘Methyloirabilis oxyfera’*. *Biochem. Soc. Trans.* **39**, 243–248.

516 Wuebbles D. J. and Hayhoe K. (2002) Atmospheric methane and climate change. *Earth-Sci. Rev.*
517 **57**, 177-210.

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527 **Figure captions**

528

529 **Figure 1:** Methane oxidation of an *M. oxyfera* enrichment culture during both incubation
530 experiments with different headspace to liquid ratios (R0.35 and R2.8). Headspace methane
531 contents at the time of sacrifice are plotted. R0.35 (initial concentration 4% in headspace) is
532 shown with open triangles, R2.8 (initial concentration 3% in headspace) with filled triangles.

533 **Figure 2:** Enrichment in heavy isotopes (A, carbon; B, hydrogen) with progressive methane
534 oxidation by an *M. oxyfera* enrichment culture during both incubation experiments. Values of R0.35
535 are shown as open rhombs, values of R2.8 as filled rhombs.

536 **Figure 3:** Rayleigh plots for stable isotope fractionation (A, carbon; B, hydrogen). Data from both
537 incubation experiments are included.