Carbon and hydrogen isotope fractionation during nitrite-dependent anaerobic methane oxidation by *Methylomirabilis 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 *Methylomirabilis 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 ± 2.6‰ for $\delta^{13}C$ (ε_C) and -227.6 ± 13.5‰ for $\delta^{2}H$ (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 ($\Lambda = (\alpha_H^{-1}-1)/(\alpha_C^{-1}-1)$) was performed and also the determined Λ

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

1. INTRODUCTION

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

Most of the produced methane is oxidized back to carbon dioxide by two major sinks – abiotic oxidation by hydroxyl radicals in the upper atmosphere, and microbial oxidation under both oxic and anoxic conditions (Conrad, 2009; Montzka et al., 2011; Wuebbles and Hayhoe, 2002). During biological methane oxidation, the initial activation to methanol is mechanistically the most difficult step due to the exceptional thermodynamic stability of methane with an activation barrier of +439 kJ/mol (Thauer and Shima, 2008). Owing to its strong oxidizing capacity, oxygen was believed to be the only possible electron acceptor for methane oxidation (Strous and Jetten, 2004). Aerobic methane oxidation is exclusively performed by *Bacteria*, belonging to *alpha*- or *gamma*-Proteobacteria and recently discovered *Verrucomicrobia* (Op den Camp et al., 2009; Semrau et al., 2008). Beginning in the 1970s, anaerobic oxidation of methane with sulfate as terminal electron acceptor in consortia of anaerobic methanotrophic archaea (ANME) and sulfate-reducing bacteria was shown to exist and to be 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* 'Methylomirabilis 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 expresses 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 $\delta^{13}C$ of all biotic and abiotic methane sources is about -54 ± 5‰, but the average
104 $\delta^{13}C$ 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 ^{2}H 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).

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

2. MATERIALS & METHODS

2.1 Enrichment culture and methane oxidation

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

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

2.2 Analysis of nitrite, methane and protein content

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

2.3 Isotope-ratio mass spectrometry

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

2.4 Determination of isotope enrichment factors, fractionation factors and Λ

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

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

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

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

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

$$\frac{R_t}{R_0} = \frac{C_t}{C_0}^{\frac{\varepsilon}{1000}} \qquad \text{Eq. 2}$$

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

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

equation 3.

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

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

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

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

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

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

3. RESULTS & DISCUSSION

3.1 Methane oxidation kinetics

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

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

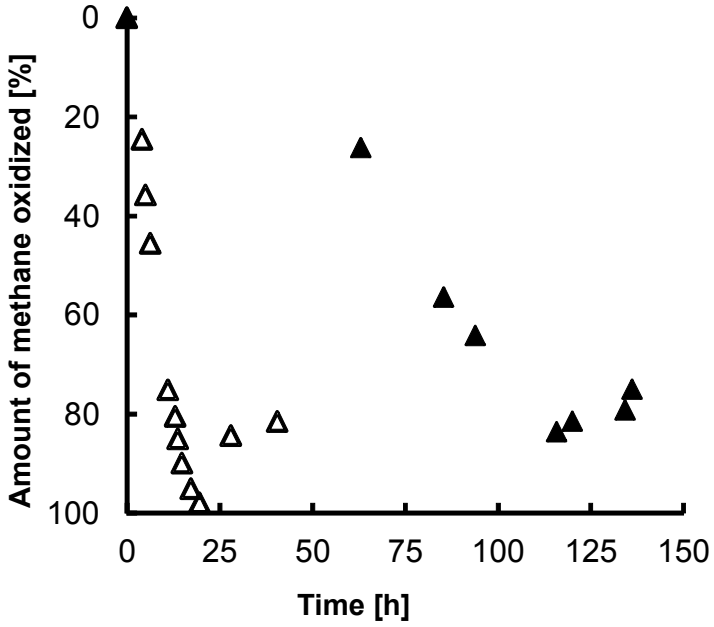

Figure 1: Methane oxidation of an *M. oxyfera* enrichment culture during both incubation experiments with different headspace to liquid ratios (R0.35 and R2.8). Headspace methane contents at the time of sacrification are plotted. R0.35 (initial concentration 4% in headspace) is shown with open triangles,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

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

233

234 **3.2 Isotope fractionation of carbon and hydrogen**

235

236 Both R0.35 and R2.8 exhibited an enrichment in heavy isotopes of carbon and hydrogen during the
237 course of incubation. The $\delta^{13}C$ of methane was -37.8 ± 0.6‰ 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 ^{13}C and increased from -130 ± 0.5‰ to +434 ± 6.4‰ after 90% of
240 headspace methane was consumed (Fig. 2B).

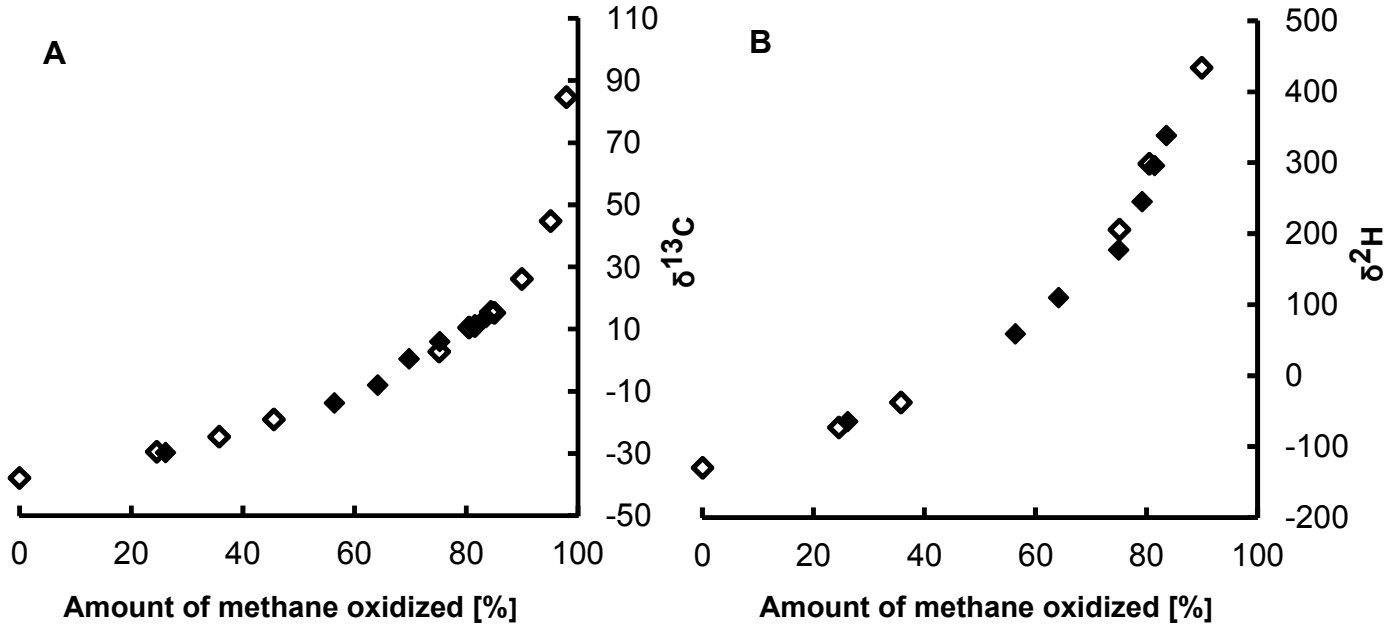

Figure 2: Enrichment in heavy isotopes (A, carbon; B, hydrogen) with progressive methane oxidation by an *M. oxyfera* enrichment culture during both incubation experiments. Values of R0.35 are shown as open rhombs, values of R2.8 as filled rhombs.

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 ± 2.6‰
247 for carbon (ε_C) and -227.6 ± 13.5‰ for hydrogen (ε_H) (Fig. 3). Both regression lines came close to a
248 correlation factor (R^2) of 1 (Figure 3).

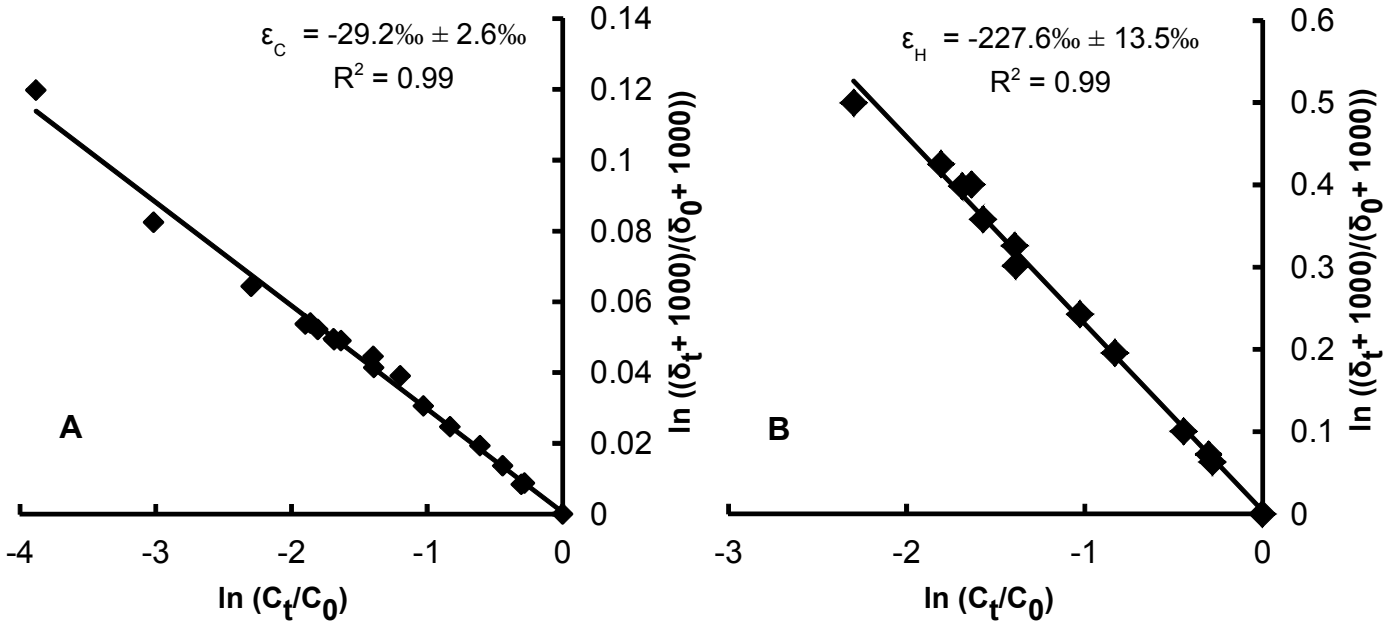

Figure 3: Rayleigh plots for stable isotope fractionation (A, carbon; B, hydrogen). Data from both incubation experiments are included.

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

main determinants of fractionation control in methane seep enrichment cultures. As the *M. oxyfera* enrichment culture primary consisted of aggregated cells, substrate diffusion limitation to inner cells could be one of the parameters which would affect the extent of bulk isotope fractionation and possibly mask the true fractionation in case of non-limiting substrate condition for each individual cell. Substrate availability is controlled by two factors, the bulk substrate concentration on one hand, and cell biomass content and its activity on the other hand. A study of Kampara et al. (2009) showed that cell density had a significant impact on fractionation of carbon during aerobic toluene degradation, with lower cell numbers of single-cell cultures leading to highest isotope fractionation factors. In case of biofilms or cell clusters the effect could be even higher since the ratio of biovolume to surface area is much higher, restricting the access of substrate even more. Templeton et al. (2006) found that methane fractionation by whole cells of proteobacteria was mostly regulated by the total amount of substrate oxidized per unit time, which is dependent on the cell numbers and finally the number of active MMOs, and was regardless of the type of organism or type of expressed MMO. During the current study, substrate limitation could be excluded as the isotope enrichment differed neither between both incubation experiments nor was it affected by decreasing methane contents during oxidation within each incubation experiment. One of the factors leading to the high enrichment factor of $\delta^{13}C$ could be the availability of oxygen for pMMO (Templeton et al., 2006). The effect of oxygen would be inverse to that of methane concentrations. Theoretically, under low oxygen concentrations the process of methane oxidation would be slowed down favoring fractionation. Thus, even at low methane availability, the kinetic isotope effect could be offset by low oxygen. As *M. oxyfera* produces its oxygen intracellularly from nitrite via nitric oxide, this step is likely to be rate-limiting, thus restricting oxygen 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
		$\varepsilon_C \pm$ Error	$\varepsilon_H \pm$ Error		
Methylococcus capsulatus (pure)	45	-27.9 ± 1.7[a]	-231.5 ± 30.5	10.5	Feisthauer et al., 2011
		-22.9 ± 3.2[b]	-192.0 ± 28.5	10.1	Feisthauer et al., 2011
Methylosinus sporium (pure)	30	-18.8 ± 1.4[a]	-136.8 ± 20.1	8.3	Feisthauer et al., 2011
		-21.5 ± 2.7[b]	-182.6 ± 23.7	10.2	Feisthauer et al., 2011
Methylocystis parvus (pure)	30	-19.1 ± 1.0[a]	-168.2 ± 9.1	10.4	Feisthauer et al., 2011
Methylomonas methanica (pure)	30	-27.7 ± 2.3[a]	-225.5 ± 17.8	10.2	Feisthauer et al., 2011
Methylocaldum gracile (pure)	30	-14.8 ± 0.9[a]	-110.0 ± 11.5	8.2	Feisthauer et al., 2011
Methylomirabilis oxyfera (enrichment)[d]	30	-29.2 ± 2.6[a]	-227.6 ± 13.5	9.8	This study
Shane Seep, CA, USA (enrichment)	15	-26.6 ± 1.6	-156.4 ± n.d.	6.8	Kinnaman et al., 2007
Brian Seep, CA, USA (enrichment)	15	-24.9 ± 1.2	-319.9 ± n.d.	18.4	Kinnaman et al., 2007
Drip tray of ice-making machine (enrichment)	26	-24.6 ± 0.7	-245.3 ± 25	12.9	Coleman et al., 1981
Water sample (enrichment)	11.5	-12.8 ± 0.2	-93.4 ± 6	7.9	Coleman et al., 1981
	26	-15.4 ± 0.3	-229.0 ± 2	19	Coleman et al., 1981
Hydrate Ridge, Pacific Ocean (enrichment)[d]	12	-11.9 ± 0	-114.7 ± 19.3	10.8	Holler et al., 2009
Amon Mud Volcano, Mediterranean Sea (enrichment)[d]	20	-20.6 ± 2	-139.4 ± 16.7	7.7	Holler et al., 2009
Black Sea microbial mat (enrichment)[d]	12	-35.7 ± 2	-229.6 ± 14.9	8.1	Holler et al., 2009
Compost and sand biofilters (6-8% CH_4)	22	-17.7 ± 0.5	-138.5 ± 11.4	8.9	Powelson et al., 2007
Tundra soil, AK, USA	14	-26 ± n.d.	n.d.	-	King et al., 1989
	4	-16 ± n.d.	n.d.	-	King et al., 1989
Hardwood forest soil, NH, USA	8-23	-22 ± 4	n.d.	-	Tyler et al., 1994
Black Spruce soil, AK, USA	2.5	-25.3 ± n.d.	n.d.	-	Reeburgh et al., 1997
Aspen soil, AK, USA	5.5	-22.5 ± n.d.	n.d.	-	Reeburgh et al., 1997
Black Sea water column[d]	9	-20.6 ± 1	-166.7 ± 20	9.5	Kessler et al., 2006
Eckernförde Bay pore water, Baltic Sea[d]	8	-11.9 ± 1	-107.1 ± 20	10	Martens et al., 1999
Skan Bay sediment, AK, USA[d]	4	-8.7 ± 1	-135.7 ± 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

3.3 Two-dimensional specific isotope analysis

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

3.4 Environmental implications

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

315 the electron acceptors are known, the *M. oxyfera*-specific isotope enrichment factors determined herein
316 could be used for quantification of methane degradation. Also methanogenesis might be an important
317 factor which must be taken into consideration when interpreting isotope data from anoxic
318 environments. Methanogenic activity may overlap with anaerobic methanotrophy, leading to a partial
319 recycling of produced carbon dioxide thereby complicating the interpretation of isotope data. However,
320 under nitrate/nitrite rich conditions in the habitat of *M. oxyfera* methanogenesis might be restricted due
321 to the high redox potential and unfavorable kinetics in comparison with denitrifiers.
322 A study of Holler et al. (2009) on isotope fractionation by enrichment cultures of AOM consortia
323 (ANME II clade) from various marine environments revealed ε_C values of -11.9‰ – -35.7‰ and ε_H of
324 -114.7‰ – -229.6‰. Despite the profoundly different biochemical mechanism underlying anaerobic
325 methanotrophy, its isotope enrichment factors resemble those of aerobic methanotrophs. Similar values
326 were also reported by Kessler et al. (2006) and Martens et al. (1999) for gas samples from anoxic
327 marine environments. These findings together indicate a narrow range of fractionation specific for
328 biological methane oxidation. This can be valuable for the quantitative distinction between biotic and
329 abiotic methane oxidation. The hydroxyl-driven abiotic methane oxidation in the atmosphere is
330 responsible for almost one third of all methane removal (Thauer, 2011) and exhibits an Λ value of 75, a
331 value of one magnitude larger than that of biologically mediated oxidation (Bergamaschi et al., 2000;
332 Feilberg et al., 2005; Feisthauer et al., 2011; Saueressig et al., 1996; Saueressig et al., 2001), making
333 both processes well distinguishable. With the knowledge based on these findings, different methane
334 sinks can be more strictly confined, which is important in the context of the recent debate on the
335 evaluation of global methane budget (Heimann, 2011).
336
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338
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Figure captions

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 sacrification are plotted. R0.35 (initial concentration 4% in headspace) is shown with open triangles, R2.8 (initial concentration 3% in headspace) with filled triangles.

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.

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