Variation of carbon isotope fractionation in hydrogenotrophic methanogenic microbial cultures and environmental samples at different energy status

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Abstract

Methane is a major product of anaerobic degradation of organic matter and an important greenhouse gas. Its stable carbon isotope composition can be used to reveal active methanogenic pathways, if associated isotope fractionation factors are known. To clarify the causes that lead to the wide variation of fractionation factors of methanogenesis from H_2 plus CO_2 ($\alpha_{CO_2-CH_4}$), pure cultures and various cocultures were grown under different thermodynamic conditions. In syntrophic and obligate syntrophic cocultures thriving on different carbohydrate substrates, fermentative bacteria were coupled to three different species of hydrogenotrophic methanogens of the families Methanobacteriaceae and Methanomicrobiaceae. We found that C-isotope fractionation was correlated to the Gibbs free energy change (ΔG) of CH_4 formation from H_2 plus CO_2 and that the relation can be described by a semi-Gauss curve. The derived relationship was used to quantify the average ΔG that is available to hydrogenotrophic methanogenic archaea in their habitat, thus avoiding the problems encountered with measurement of low H_2 concentrations on a microscale. Boreal peat, rice field soil, and rumen fluid, which represent major sources of atmospheric CH_4, exhibited increasingly smaller $\alpha_{CO_2-CH_4}$, indicating that thermodynamic conditions for hydrogenotrophic methanogens became increasingly more favourable. Vice versa, we hypothesize that environments with similar energetic conditions will also exhibit similar isotope fractionation. Our results, thus, provide a mechanistic constraint for modelling the ^{13}C flux from microbial sources of atmospheric CH_4.

Keywords: coculture, fractionation, Gibbs free energy, hydrogen, methanogenesis, stable carbon isotopes, thermodynamic limit

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Introduction

Anoxic environments such as natural wetlands and flooded rice fields contribute approximately a third to the CH_4 budget of the atmosphere (Cicerone & Oremland, 1988). In these systems microbes almost exclusively produce CH_4 from acetate and H_2/CO_2. Hydrogenotrophic methanogenesis ($4H_2 + CO_2 \rightarrow CH_4 + 2H_2O$; $\Delta G° = -130.7 \, kJ \, mol^{-1}$) strongly prefers the isotopically lighter carbon, whereas the isotope effect is less expressed in acetoclastic methanogenesis. This difference in isotope fractionation can be used for modelling of C-flux in methanogenic environments (Conrad, 2005). In addition, measurements of carbon stable isotope ratios of CH_4 have been proven to be useful for constraining individual atmospheric CH_4 sources and sinks, and in interpreting the atmospheric CH_4 budget (Lowe et al., 1994; Gupta et al., 1996; Bräunlich et al., 2001; Fletcher et al., 2004). Although the ^{13}C/^{12}C-isotopic composition of CH_4 produced from CO_2 reduction has a characteristic signature, the magnitude of fractionation is usually considerably different between many natural systems and methanogenic microbial cultures (Fey et al., 2004; Valentine et al., 2004; Conrad, 2005). The wide variation of mag-

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nitude of fractionation (i.e. fractionation factor α) is a problem for modelling of C-fluxes (Conrad, 2005). Several authors observed this discrepancy, and assumed it was because of microbial culturing conditions, which often strongly differ from those in the natural environment (Burke, 1993; Sugimoto & Wada, 1993; Whiticar, 1999). However, the factors determining the wide range of α-values are basically unknown. Recently, Valentine *et al.* (2004) found that the fractionation of C-isotopes was affected by the supply of H_2 to a CO_2-reducing culture of *Methanothermobacter marburgensis*, and explained the observation by the differential reversibility hypothesis. They proposed that isotope fractionation in multistep enzymatic processes depends on enzymatic reversibility, which in turn depends on the Gibbs free energy of catabolism. So far, this is the only study measuring both H_2 partial pressures (p_{H_2}) and C-isotope fractionation explicitly, and only two data points exist (Valentine *et al.*, 2004). To control p_{H_2} and rigorously study its effect on C-isotope fractionation, we grew cocultures of different H_2-producing fermenting bacteria and H_2-consuming methanogenic archaea covering a broad range of p_{H_2}. This experimental approach was used to test whether a general function of methanogenic carbon isotope fractionation vs. p_{H_2} exists. We found that such a relationship indeed existed, such that α changed with the Gibbs free energy (ΔG) available to hydrogenotrophic methanogens, and that this relationship was generally valid for the different methanogenic microbial species studied.

Furthermore, we tested whether it is possible to predict α from p_{H_2} measured in samples from natural methanogenic environments. Our results indicate that this is not a reliable option, as the measured values of p_{H_2} hardly represent the Gibbs free energy (ΔG) available to hydrogenotrophic methanogens *in situ*. Such a determination has been problematic, as methanogens typically live within micro-aggregates involving steep gradients of p_{H_2} (Conrad *et al.*, 1985; Krylova & Conrad, 1998; Hoehler *et al.*, 2001), preventing the exact analysis of energetically relevant H_2 concentrations *in situ*. Instead, we propose that measurement of α gives a more reliable value of the *in situ* ΔG than the measurement of p_{H_2}, and show that more free energy is available to the hydrogenotrophic methanogens than suggested by measurement of p_{H_2}.

Material and methods

Archaeal and bacterial strains

Methanobacterium bryantii (DSM 863), *Methanobacterium formicicum* (DSM 1535), *Methanospirillum hungatei* (DSM 864), *M. marburgensis* (DSM 2133), *Acetobacterium woodii*

(DSM 1030), and *Clostridium papyrosolvens* (DSM 2782) were obtained from Deutsche Sammlung für Mikroorganismen und Zellkulturen. *Syntrophobacter fumaroxidans* (DSM 10017) was from our own culture collection (Laboratory of Microbiology, Wageningen University, The Netherlands).

Cultivation

Cocultures of the fermenting organisms *C. papyrosolvens* and *A. woodii* were each grown with methanogenic partner organisms (*M. bryantii* or *M. hungatei*) in glass bottles (500 mL; Müller Krempel, Bülach, Switzerland) with 250 mL as culture volume on phosphate-buffered mineral medium with defined bicarbonate concentrations (0.16–2.8 mM) under N_2. The composition was (in $g\,L^{-1}$ unless otherwise indicated as follows): KH_2PO_4, 1.9; $Na_2HPO_4 \cdot 2H_2O$, 6.4; NH_4Cl, 0.3; $MgCl_2 \cdot 6H_2O$, 0.1; NaCl, 0.3; KCl, 0.15; $CaCl_2 \cdot 2H_2O$, 0.055; $Na_2S \cdot 9H_2O$, 0.24; trace element solution, 2 mL (Chin *et al.*, 1998); alkaline trace element solution 1 mL (Stams *et al.*, 1993); vitamin solution, 1 mL (Wolin *et al.*, 1963); resazurine at 0.5% (wt/vol), 1 mL; pH adjusted to 7.2. Concentrations of glucose, cellobiose, and cellulose (expressed as anhydroglucose; $M_w = 162\,g\,mol^{-1}$) were 4.44, 2.34, and 4.94 mM, respectively. Cocultures of *S. fumaroxidans* and *M. hungatei* or *M. formicicum* were grown in glass bottles (1000 mL; Müller Krempel) under N_2 on the medium described by Stams *et al.* (1993) containing 38 mM bicarbonate and 30 mM propionate. *M. marburgensis* was grown in a flow-through batch reactor on mineral medium with $H_2/CO_2/N_2$ gas mixtures of 80/20/0% and 5/20/75%, respectively (Schönheit *et al.*, 1980). Rapid gas flow guaranteed that $\leq 3\%$ of the CO_2 supplied was converted to CH_4. This assured that inflow and outflow of CO_2 had nearly the same isotope composition and proper calculation of $\alpha_{CO_2-CH_4}$ was possible. Cultures were grown at 30 °C, except for those with *S. fumaroxidans* and *M. marburgensis* grown at 37 °C and 65 °C, respectively.

Environmental samples

Rice field soil samples were collected from rice fields of the Italian Rice Research Institute in Vercelli, Italy. Soil slurries, which represent the water-saturated conditions after flooding in rice fields, were prepared at a weight ratio of 1 : 1 with the addition of 4 mg rice straw g^{-1} soil and anoxically incubated at 30 °C. The peat was sampled at the Lakkasuo mire complex in central Finland (61°48′N, 24°19′E, ca. 150 m altitude), from a mesotrophic fen (MES), an oligotrophic fen (OLI), and an ombrotrophic bog (OMB) and anoxically incubated at 10 °C (Galand *et al.*, 2005). Methanogenesis from

acetate was inhibited with CH_3F (Janssen *et al.*, 1997). The concentration of CH_3F was optimized as described (Conrad & Klose, 1999), applying 1% in rice field soil and 2% in peat soil. Selective inhibition by CH_3F was earlier confirmed by stoichiometrical correlation of accumulated acetate with the deficit in CH_4 production (Frenzel & Bosse, 1996; Conrad & Klose, 1999). At higher CH_3F concentration hydrogenotrophic methanogenesis is partially inhibited (Conrad & Klose, 1999), but does not change its carbon isotope fractionation (Conrad, 2005). Rumen fluid was sampled at the slaughterhouse of Marburg and immediately afterwards incubated under N_2 at 30 °C or H_2/CO_2 (4 : 1) at 25 °C.

Analytical methods

CH_4 and CO_2 were analysed by GC-FID (Shimadzu, Kyoto, Japan). CO_2 was detected upon conversion to CH_4 with a methanizer (Ni-catalyst at 350 °C, Chrompack, Middelburg, Netherlands). H_2 was analysed by GC-TCD (Shimadzu) and a HgO-to-Hg conversion detector (RGD2; Trace Analytical, Menlo Park, CA, USA) (Seitz *et al.*, 1990). Stable isotope analysis of $^{13}C/^{12}C$ in gas samples was performed using a gas chromatograph combustion isotope ratio mass spectrometer (GC-C-IRMS) system (Thermoquest, Bremen, Germany). For principle operation see Brand (1996). Briefly, after conversion of CH_4 to CO_2 in the Finnigan Standard GC Combustion Interface III, isotope ratios were detected with the IRMS (Finnigan MAT model delta plus, Thermoquest, Bremen, Germany) (Fey *et al.*, 2004). Reference gas was CO_2 (99.998% purity; Messer-Griessheim, Düsseldorf, Germany), calibrated with the working standard methylstearate (Merck, Darmstadt, Germany). The latter was intercalibrated at the Max-Planck-Institut für Biogeochemie, Jena, Germany (courtesy of Dr W. A. Brand) against NBS22 and USGS 24 and reported in the delta notation vs. V-PDB:

$$\delta^{13}C = 10^3(R_{sa}/R_{st} - 1)$$

with R

$= ^{13}C/^{12}C$ of sample (sa) and standard (st), respectively.

The precision of repeated analysis was $\pm 0.2‰$ when 1.3 nmol CH_4 was injected.

Calculations

Fractionation factors for methanogenesis from H_2/CO_2 are defined according to Hayes (1993):

$$\alpha_{CO_2-CH_4} = (\delta^{13}C_{CO_2} + 1000)/(\delta^{13}C_{CH_4} + 1000). \quad (1)$$

The isotopic signature for a newly formed CH_4 (δ_n) was calculated from the isotopic signatures at two time points $t = 1$ (δ_1) and $t = 2$ (δ_2) by the following mass balance equation:

$$\delta_2 = f_n\delta_n + (1 - f_n)\delta_1 \quad (2)$$

with f_n being the fraction of the newly formed CH_4 relative to the total at $t = 2$.

Gibbs free energies for methanogenesis in the methanogenic system were calculated from the standard Gibbs free energies of formation (Thauer *et al.*, 1977) and the Nernst equation:

$$\Delta G = \Delta G° + RT \ln \frac{p_{CH_4}}{p_{H_2}^4 p_{CO_2}}. \quad (3)$$

$\Delta G°$ was corrected for a temperature of 25 °C using the Van't Hoff equation. α was corrected following the algorithm given by Whiticar *et al.* (1986), which is based on carbon isotope exchange equilibria (Richet *et al.*, 1977):

$$10^2 \ln \alpha_T = 2.92(10^3/T) - 2.96, \quad (4)$$

where T is the formation temperature in K. Correction was then made by the difference between the α_T calculated for 25 °C and the growth temperatures of the cultures (adjustment of $\alpha_{CO_2-CH_4}$ was 0.0017 for 30 °C, 0.0041 for 37 °C, and 0.0123 for 65 °C).

Results

Pure culture experiments

Pure cultures of *M. marburgensis* were grown in a flow-through batch bioreactor on different $H_2/CO_2/N_2$ gas mixtures (80/20/0% and 5/20/75%; Fig. 1). Almost parallel increase of optical density and CH_4 concentration indicated nonlimited growth conditions in the high-H_2 reactor (Fig. 1a), whereas in the low-H_2 reactor (Fig. 1b) two phases of exponential growth were displayed. In the first 30 h the CH_4 production rate and OD_{600} increased exponentially (nonlimited growth). After that, however, the growth rate decreased, and the increase in CH_4 production rate declined and stabilized at 5 mL min^{-1} (litre of $H_2/CO_2/N_2$)$^{-1}$. Such behavior is typical for hydrogenotrophic growth of methanogens, which uncouple CH_4 production from biomass production when the supply of H_2 is not limiting (Schönheit *et al.*, 1980). During transition of the CH_4 production rate to a stable value the H_2 partial pressures (p_{H_2}) measured in the gas exhaust decreased and stabilized after 30 h (data not shown), demonstrating that H_2 limited growth conditions established. Values of $\alpha_{CO_2-CH_4}$ ranged between 1.034 and 1.039 under nonlimited conditions (Fig. 1a, b), and increased in the reactor with low H_2 concentration (Fig. 1b) to constant values between 1.062 and 1.064, when H_2 became limiting.
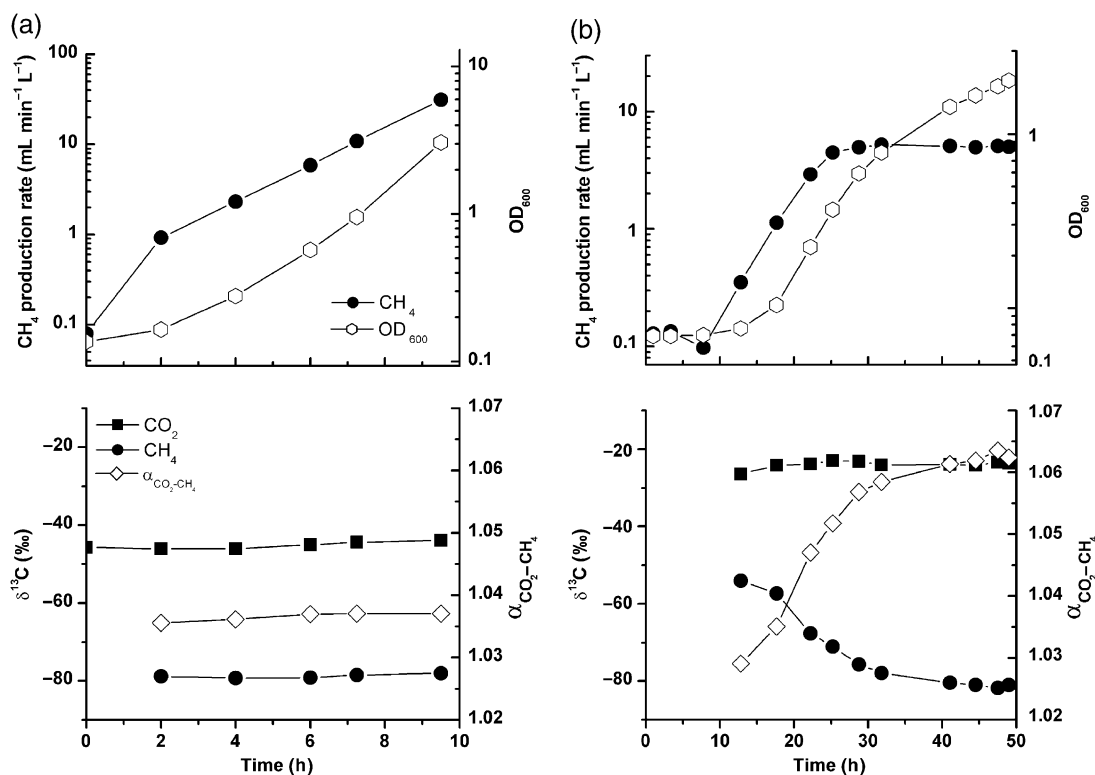
Fig. 1 Growth curve and CH_4 production of *Methanothermobacter marburgensis* in flow-through bioreactor experiments with H_2/CO_2/N_2 gas mixtures of (a) 80/20/0% and (b) 5/20/75% (upper diagrams). Time course of $\delta^{13}CH_4$, $\delta^{13}CO_2$, and α is shown in the lower diagrams of (a) and (b). Rapid gas flow guaranteed that $\leq 3\%$ of the CO_2 supplied was converted to CH_4. Unlimited availability of H_2 (a) resulted in low and stable α-values, whereas limitation of H_2 (b), as indicated by slow growth and stagnant CH_4 production rate during the late growth phase, resulted in high α-values.

Coculture experiments

Influence of p_{H_2} on carbon isotope fractionation was then tested in a closed system, where equilibrium of gases between headspace and liquid phase establishes. To create different p_{H_2} growth conditions for hydrogenotrophic methanogens, syntrophic (wide range of p_{H_2}), and obligate syntrophic cocultures (narrow p_{H_2} range) were chosen for our experiments. In syntrophic cocultures of *C. papyrosolvens* and *M. bryantii* glucose and cellobiose were completely fermented by *C. papyrosolvens* within 200 h to acetate, lactate, ethanol, formate, H_2, and CO_2. Only H_2 and CO_2 were further converted to CH_4 by the methanogenic archaeon *M. bryantii* and accumulated exponentially in the system (Fig. 2). Partial pressures of the intermediate H_2 increased transiently to more than 2.5 kPa (Fig. 2). Cellulose, by contrast, allowed only a relatively low fermentation rate limited by the hydrolysis of cellulose. In these cultures, hydrogenotrophic CH_4 production kept the p_{H_2} low (p_{H_2} <0.1 kPa) and CH_4 was linearly produced. Carbon isotope composition of CO_2 and newly formed CH_4, (Eqn (2)), from which $\alpha_{CO_2-CH_4}$ was calculated, (Eqn

(1)), is shown in Fig 2. Calculation of $\delta^{13}C$ of CH_4 was made evaluating each data point separately, as the determination is very sensitive, if changes of the measured $\delta^{13}C$ of CH_4 (accumulated) and of the CH_4 partial pressure are small. Changes of isotope ratio and concentration that were close to the analytical error of GC-C-IRMS or GC-FID measurement were therefore not included in the data evaluation. In the cocultures growing on glucose or cellobiose, CH_4 formed by *M. bryantii* at high p_{H_2} exhibited low carbon isotope fractionation ($\alpha_{CO_2-CH_4} < 1.04$), whereas at low p_{H_2} α-values increased. In contrast, on cellulose, α-values were relatively high from the beginning on ($\alpha_{CO_2-CH_4} = 1.044$) and stabilized after 500 h at $\alpha_{CO_2-CH_4} = 1.076$ with $p_{H_2} = 10$ Pa (Fig. 2).

Additional syntrophic cocultures (*C. papyrosolvens* with *M. hungatei*, *A. woodii* with *M. bryantii*, and *A. woodii* with *M. hungatei*) were analysed the same way. Trends in these cocultures were the same as shown in Fig. 2 for *C. paprosolvens* and *M. bryantii* and are, therefore, not shown individually, but are later included in the data analysis. Isotope fractionation at very low p_{H_2} was investigated with the obligate syntrophic
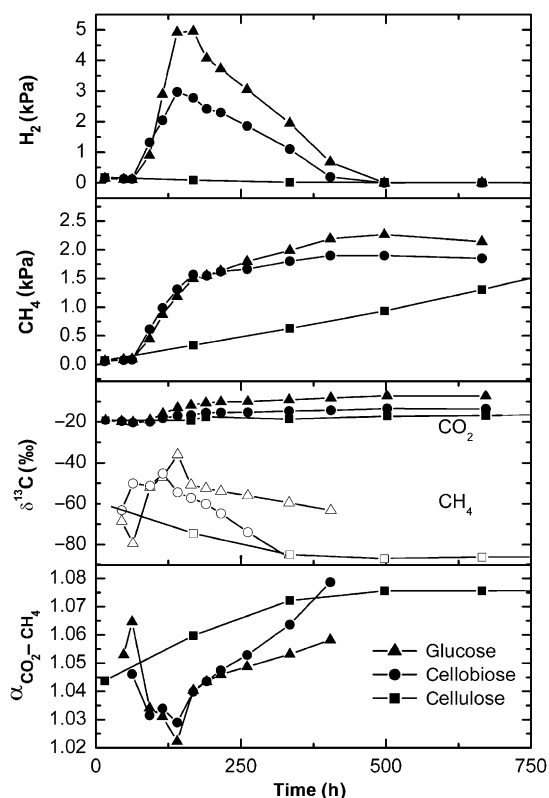
Fig. 2 Change of fractionation factor α, $\delta^{13}CH_4$, $\delta^{13}CO_2$, and partial pressures of H_2 and CH_4 in a coculture of *Clostridium papyrosolvens* and *Methanobacterium bryantii*. High p_{H_2} correspond to low α and vice versa. With cellulose, *M. bryantii* operated close to its threshold for H_2 exhibiting very high isotope fractionation.
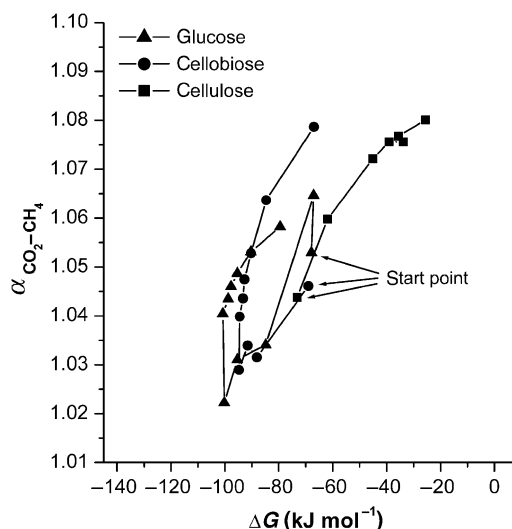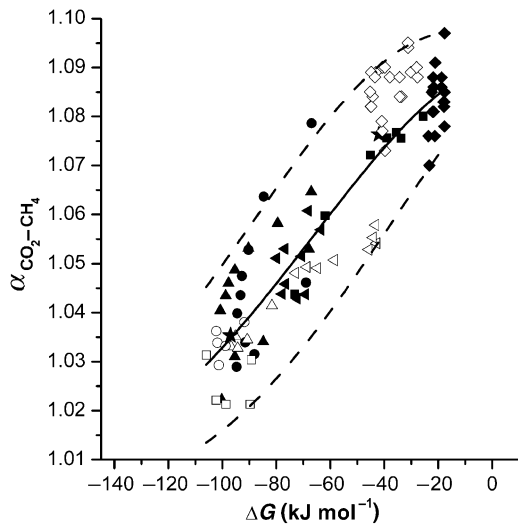

Fig. 3 α–ΔG relation in coculture of *Clostridium papyrosolvens* and *Methanobacterium bryantii* growing on different substrates, process and isotope data of which are shown in Fig. 2. ΔG and $\alpha_{CO_2-CH_4}$ well correlate and show a decrease and later increase for glucose and cellobiose, whereas for cellulose there is a steady increase.

cocultures of *S. fumaroxidans* coupled to either *M. hungatei* or *M. formicicum*. Propionate oxidation by *S. fumaroxidans* was only possible at low p_{H_2} equivalent to a ΔG of about $-15\,\mathrm{kJ\,mol^{-1}}$, similarly as observed before (Scholten & Conrad, 2000). This low p_{H_2} was maintained by the methanogen, which, in turn, was also only provided with little free energy ($\Delta G \approx -40$ to $-20\,\mathrm{kJ\,mol^{-1}}$). In contrast, the cocultures, which were not obligate syntrophic, operated at a higher p_{H_2}, and thus allowed generally more free energy ($\Delta G \approx -100$ to $-50\,\mathrm{kJ\,mol^{-1}}$) to the methanogenic partner.

Data analysis of cocultures

Although each individual coculture showed a good correlation between the values of p_{H_2} and $\alpha_{CO_2-CH_4}$ measured during the course of growth ($r^2 > 0.82$), there was no general relationship between p_{H_2} and $\alpha_{CO_2-CH_4}$ across all the different coculture experiments. Instead, a good correlation was found between the catabolic ΔG of the methanogenic reaction and the $\alpha_{CO_2-CH_4}$ of the reaction determined in the same incubation (Figs 3

and 4). Values of ΔG were calculated of the actual conditions using measured p_{H_2}, p_{CH_4}, and p_{CO_2}, and the Nernst (Eqn (3)). Figure 3 shows the relation for the coculture of *C. papyrosolvens* and *M. bryantii* (Fig. 2). After inoculation ΔG for the coculture was $-70\,\mathrm{kJ\,mol^{-1}}$. During the course of the experiment ΔG decreased in cocultures with glucose and cellobiose to $-100\,\mathrm{kJ\,mol^{-1}}$, concomitant with a decrease in $\alpha_{CO_2-CH_4}(1.02 - 1.03)$. With decrease in p_{H_2} and therefore increase in ΔG, $\alpha_{CO_2-CH_4}$ also increased again. In the coculture with cellulose, where ΔG steadily increased with time, $\alpha_{CO_2-CH_4}$ increased simultaneously to values up to 1.08.

Figure 4 summarizes the results of all the different syntrophic and obligate syntrophic cocultures tested. We calculated the values of $\alpha_{CO_2-CH_4}$ and ΔG separately for each replicate of the cocultures to increase precision. Syntrophic cocultures (with *C. papyrosolvens* or *A. woodii* as fermenting partner) were active within a wide range of ΔG, as well as $\alpha_{CO_2-CH_4}$, whereas obligate syntrophic cocultures (*S. fumaroxidans* as fermenting partner) could only operate within a narrow range of ΔG (-45 to $-17\,\mathrm{kJ\,mol^{-1}}$). Both, *M. bryantii* and *M. hungatei*, displayed the relation over a wide range of ΔG and $\alpha_{CO_2-CH_4}$. *M. formicicum* was only used in the obligate syntrophic cocultures and was, therefore, restricted to the high ΔG values, characteristic for these cultures. Pure culture data of *M. marburgensis* (Fig. 1) could not be included in the figure because of lack of the *in situ* p_{H_2} in the flow-through system necessary for the calcu-

Fig. 4 α–ΔG relation in cocultures of H_2-producing fermenting and H_2-consuming methanogenic microorganisms; the line is a fit (using Origin Microcal™) to the Gauss function ($y = y_0 + A \exp[-(x-x_c)^2/(2w^2)] = 1 + 0.0919 \exp[-(x-11.8376)^2/12170]$). ▲, *Clostridium papyrosolvens/Methanobacterium bryantii* (glucose); ●, *C. papyrosolvens/M. bryantii* (cellobiose); ■, *C. papyrosolvens/M. bryantii* (cellulose); ◄, *Acetobacterium woodii/M. bryantii* (glucose); △, *C. papyrosolvens/Methanospirillum hungatei* (glucose); ○, *C. papyrosolvens/M. hungatei* (cellobiose); □, *C. papyrosolvens/M. hungatei* (cellulose); ◁, *A. woodii/M. hungatei* (glucose); ◆ *Syntrophobacter fumaroxidans/Methanobacterium formicicum* (propionate); ◇, *S. fumaroxidans/M. hungatei* (propionate); ★ *M. marburgensis* (H_2/CO_2; data from Valentine *et al.* (2004))

lation of ΔG. Yet, two data points from Valentine *et al.* (2004) are included and they fit the observed α–ΔG relation well. A Gauss function, Eqn (5), was used to fit the data (justification see Discussion) and resulted in a goodness of fit of $r^2 = 0.77$:

$$y = y_0 + A \exp[-(x - x_c)^2/(2w^2)] \qquad (5)$$

with $y_0 = 1$; $A = 0.0919 \pm 0.0104$; $x_c = 11.8376 \pm 23.5222$; $w = 78.0067 \pm 14.1127$.

Because of the individual treatment of replicates no errors bars can be shown for the data points, but we calculated the 95% confidence range for the α–ΔG relation (included in Fig. 4).

Incubations of environmental samples

To test the implication of the observed α–ΔG relation for natural methanogenic systems, samples from different environments were incubated under anoxic conditions. As in the cocultures, only time points with active methanogenesis were used in the data analysis, which was performed as described for the cocultures. In the α–ΔG relation diagrams of the environmental incubations
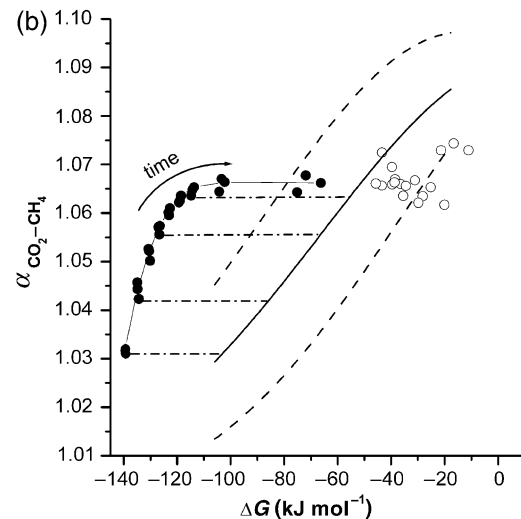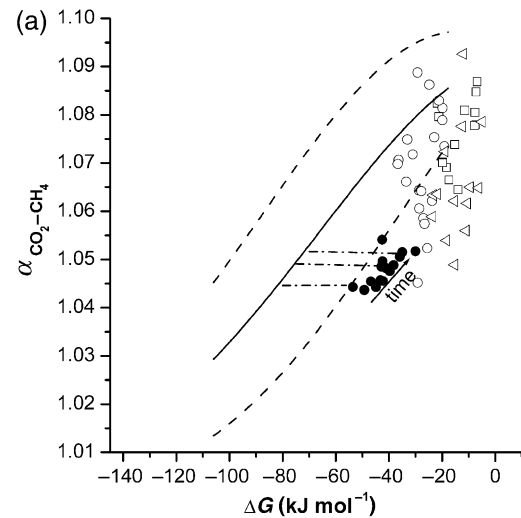
Fig. 5 α–ΔG relation in different environmental samples including regression curve and confidence lines from Fig. 4; arrows indicate the time course of incubation, and dash-dotted lines show the difference between the apparent ΔG (symbols) and the actual ΔG (regression curve) of hydrogenotrophic methanogenesis. The actual ΔG available to methanogens in their environmental habitat was up to $60 \, kJ \, mol^{-1}$ more negative than that suggested from the apparent ΔG determined from bulk measurement of p_{H_2} (difference shown by horizontal dash-dotted lines). However, in rumen fluid incubated under H_2/CO_2 it was more positive. (a) Rice field soil and peat (●, rice field soil; □, peat OLI; ○, peat MES; ◁, peat OMB). (b) Rumen fluid (○, rumen (N_2); ●, rumen (H_2/CO_2)).

(Fig. 5) the regression curve of the culture experiments and its 95% confidence lines are also included. In incubations of rice field soil and peat, acetoclastic methanogenesis was inhibited with methyl fluoride (Janssen & Frenzel, 1997) to avoid changes of the $\delta^{13}C$ of CH_4 by CH_4 produced from acetate (Fig. 5a). This approach has recently been proposed for the determi-

nation of $\alpha_{CO_2-CH_4}$, (Conrad, 2005) and successfully tested in eutrophic lake sediment (Chan *et al.*, 2005) and rice field soil (unpublished data). In rice field soil, ΔG (-54 to $-30 \, kJ \, mol^{-1}$) and $\alpha_{CO_2-CH_4}$, (1.043–1.053) increased simultaneously with time. The peat incubations plotted over a wide range of $\alpha_{CO_2-CH_4}$ (1.045–1.087) and at high ΔG (-37 to $-7 \, kJ \, mol^{-1}$). In the rice field soil and peat system, data followed the α–ΔG relation only for $\alpha_{CO_2-CH_4} > 1.065$, whereas for $\alpha_{CO_2-CH_4} < 1.065$, data were on the right side of the α–ΔG relation. Using the α–ΔG relation and the p_{H_2} measured in the environmental samples would thus predict (via ΔG that decreases with p_{H_2}) much higher α than actually measured. On the other hand, the α–ΔG relation and the measured α would predict much lower (more negative) values of ΔG than calculated from measured p_{H_2}.

We also used rumen fluid, as in this environment CH_4 is almost exclusively produced from H_2/CO_2 (Sharp *et al.*, 1998) and, thus, inhibition of acetoclastic methanogenesis with methyl fluoride is not necessary. Incubation of rumen fluid was conducted under N_2 and H_2/CO_2 headspace (Fig. 5b). For incubations under N_2 headspace data clustered in a relatively confined area ($\Delta G = -11$ to $-46 \, kJ \, mol^{-1}$; $\alpha_{CO_2-CH_4} = 1.061$–1.074) within the 95% confidence limit of the α–ΔG relation. In contrast, the H_2/CO_2 headspace created very low ΔG values at the beginning ($-140 \, kJ \, mol^{-1}$), which continuously decreased, when CH_4 was produced by hydrogenotrophic archaea. Values of $\alpha_{CO_2-CH_4}$ strongly increased initially (1.032–1.067) and stabilized at a similar $\alpha_{CO_2-CH_4}$ as observed in the incubation under N_2. Simultaneously, bulk p_{H_2} decreased so that the measured ΔG gradually changed from -145 to $-75 \, kJ \, mol^{-1}$, similarly as observed in rumen fluid incubated under a N_2 atmosphere. Hence, for rumen incubated under H_2/CO_2, data first plotted on the left side of the α–ΔG relation curve, but later approached and finally crossed the curve reaching the cluster of the rumen incubation under N_2. Again, the α–ΔG relation together with the measured p_{H_2} did not allow prediction of α. However, using the α–ΔG relation together with the α measured after stabilization would predict a reasonable value of ΔG available to the hydrogenotrophic methanogens.

Discussion

Pure and coculture experiments

Different hydrogenotrophic methanogenic archaea were grown under varying growth conditions to elucidate the factors causing the fractionation of carbon isotopes during CH_4 production from H_2/CO_2. In pure cultures of *M. marburgensis* (Fig. 1) we could show, that $\alpha_{CO_2-CH_4}$ depended on the p_{H_2}. Values of $\alpha_{CO_2-CH_4}$ increased with decreasing p_{H_2}, confirming the results by Valentine *et al.* (2004). To quantify the observed correlation we used cocultures in closed systems, where partial pressures of the gases involved in the methanogenic reaction could be quantified with a high precision. $\alpha_{CO_2-CH_4}$ produced from *M. bryantii* in coculture with *C. papyrosolvens* at high p_{H_2} was similar as reported for pure cultures of *Methanobacterium* spp. (Belyaev *et al.*, 1983) when grown on H_2/CO_2 at a ratio of 4:1 as required for stoichiometric conversion to CH_4. However, with decreasing p_{H_2}, as for *M. marburgensis* in the flow-through reactor, $\alpha_{CO_2-CH_4}$ strongly increased. The cocultures demonstrated, that the relation of α to p_{H_2} was not specific to pure culture data, and that the coculture experiments were not biased by the different mode of H_2/CO_2 gas transfer (i.e. between different microbes within the culture medium or between the gas phase and the culture medium). In addition, we found that the different isotopic composition of glucose and cellobiose, $\delta^{13}C = -10.85 \pm 0.05$‰ and -25.23 ± 0.47‰, respectively, resulted in a different isotopic composition of the pool of CO_2, but did not influence the value of $\alpha_{CO_2-CH_4}$ (Fig. 2). This observation shows that isotopic equilibrium was reached between the CO_2 produced by fermentation and the CO_2 in the medium.

α–ΔG relation

Although we found a good correlation of $\alpha_{CO_2-CH_4}$ with p_{H_2} for the single cocultures, the lack of a general dependency across the different coculture experiments indicated that p_{H_2} was not the only factor determining $\alpha_{CO_2-CH_4}$. The catabolic ΔG, however, did yield for all data a good correlation ($r^2 = 0.77$). Valentine *et al.* (2004) recently observed that a culture of *M. marburgensis* exhibited a larger fractionation ($\alpha_{CO_2-CH_4} = 1.064$) at low (310 Pa) and a lower fractionation ($\alpha_{CO_2-CH_4} = 1.031$) at high (80 000 Pa) p_{H_2} (data points included in Fig. 4), and proposed that this difference is because of the extent of enzymatic reversibility that is controlled by the ΔG of the methanogenic reaction. They further suggested that the differential reversibility would act in concert with the intracellular availability of CO_2. High metabolic rates would decrease the intracellular concentration of CO_2, effectively creating an irreversible flux of CO_2 to CH_4. If then CO_2 was quantitatively consumed, consequently, no fractionation would occur by methanogenesis ($\alpha_{CO_2-CH_4} = 1.0$) and only a slight fractionation by transport into the cell would be observed.

Our results are consistent with this hypothesis demonstrating that α is indeed a function of the ΔG. With respect to energetics of methanogenesis, it is important

to note that methanogens activate CO_2 by forming formyl methanofuran. This reaction is endergonic under standard conditions and becomes exergonic only when coupled to the late reaction steps that conserve energy (e.g. methyl transferase and heterodisulfide reductase; Thauer, 1998). A low value of the ΔG (large negative values) of methanogenesis from $H_2 + CO_2$ thus facilitates activation of CO_2 by also lowering the ΔG of this single reaction, decreases reversibility, and results in low $\alpha_{CO_2-CH_4}$. A high value of the ΔG (low negative values), on the other hand, impedes activation of CO_2, increases ΔG of this reaction, and results in high $\alpha_{CO_2-CH_4}$. For activation of CO_2 a certain amount of free energy is always required, since methanogenesis can only proceed if this step is made exergonic. With increase of ΔG, less free energy will be available to drive each step of the multireaction process, further increasing the reversibility and, therefore, the fractionation of the reactions. Finally a maximum of α is reached at the thermodynamic limit of the microorganisms (Fig. 4), which in theory is equivalent to $\frac{1}{4} - \frac{1}{3}$ ATP or about -15 to $-25\,kJ\,mol^{-1}$ for mesophilic methanogens (Yao & Conrad, 1999; Hoehler *et al.*, 2001; Schink & Stams, 2003; Müller, 2004). As methanogenesis cannot proceed above $\Delta G = 0$, a semi-Gauss curve was used for correlation. The maximum fractionation is solely determined by ΔG, as isotopic equilibrium of the CO_2 pools outside and inside the cell can be assumed at the low metabolic rates associated with high ΔG. However, irreversible CO_2 flow becomes more important with decreasing ΔG and the concomitant increase of the metabolic rate, and finally governs the α–ΔG relation. At very low ΔG the hypothetical limit of zero fractionation ($\alpha_{CO_2-CH_4}$) will be reached, if all CO_2 entering the cell is quantitatively metabolized. The semi-Gauss curve accounts for this boundary condition predicted by irreversible CO_2 flow.

As explained above, the observed relation (Fig. 4) is partly explained by the reversibility hypothesis depending on the free energy of the reaction (Valentine *et al.*, 2004). For H-isotopes a similar correlation (i.e. a free-energy dependence of the kinetic isotope effect) was found, showing a bell-shaped curve with its maximum at $\Delta G = 0$ (Sühnel, 1990; Cook, 1991). There, the experimentally observed correlations in proton-transfer reactions could be theoretically explained both by the Marcus and quantum-statistical mechanical model. To our knowledge similar investigations on carbon isotopes have not been performed yet, so this interesting finding for H-isotopes should be evaluated for the element carbon. In addition, methanogenesis is a multireaction process. This further complicates the calculation of carbon isotope fractionation, as concentrations of reactants and products of each single reaction of methanogenesis have to be known to determine ΔG. There-

fore, we have to point out that the similarity between the theoretical free energy dependence of the kinetic isotope effect observed for H-isotopes and the α–ΔG relation observed for hydrogenotrophic methanogenesis in our study is probably only circumstantial. However, the observed relationship between $\alpha_{CO_2-CH_4}$ and ΔG presented here appeared to be valid for all the four different methanogenic microbial species (from three genera and two families) tested (Fig. 4), emphasizing that it is a general feature of C-isotope fractionation in hydrogenotrophic methanogenesis. Although two of the tested methanogens (*M. bryantii* and *M. hungatei*) follow the α–ΔG relation over the whole range of $\alpha_{CO_2-CH_4}$ and ΔG observed, it cannot be dismissed that specifics of enzymes – like individual binding sites and activation energies – are different in other hydrogenotrophic methanogens and might change the α–ΔG relation to a certain extent. This could be the case, if for example K- and r-strategists had developed differently fractionating sets of enzymes depending on p_{H_2}. However, regarding carbon isotope fractionation, none of this has been reported yet, and the archaea tested in this study are also typical representatives of hydrogenotrophic methanogens in natural environments (Grosskopf *et al.*, 1998; Koizumi *et al.*, 2004; Chan *et al.*, 2005).

Application of the α–ΔG relation to environmental systems

Studies determining ΔG in natural systems from measurements of p_{H_2}, p_{CH_4} and p_{CO_2} have indicated that hydrogenotrophic methanogens operate at the thermodynamic limit ($\Delta G = -15$ and $-25\,kJ\,mol^{-1}$). Although CH_4 formation may be active at $\Delta G \approx 0$ (Jackson & McInerney, 2002), such activity does not support growth, which in chemostat culture required $\Delta G \approx -36\,kJ\,mol^{-1}$ (Seitz *et al.*, 1990). The severe energetic limitation in natural systems is in contrast to observations that the composition of methanogenic communities in the environment can change with time implying growth (Peters & Conrad, 1996; Lueders & Friedrich, 2002; Krüger *et al.*, 2005). Determination of *in situ* ΔG is dependent on the measurement of realistic p_{H_2}. The actual *in situ* p_{H_2} is at the microsite where the methanogens live, which, however, may not be represented by the p_{H_2} that is typically measured in the bulk fluid or in the equilibrated gas phase of the whole environmental sample. In natural environments, methanogens are closely associated with H_2-forming bacteria so that interspecies-H_2-transfer can occur between juxtaposed partners (Conrad *et al.*, 1985; Krylova & Conrad, 1998; Hoehler *et al.*, 2001). Hence, methanogens may experience higher p_{H_2} (i.e. more negative ΔG) than

deduced from bulk measurements, which may over-estimate the effective ΔG (i.e. less negative).

Having an alternative way to determine ΔG (i.e. the α–ΔG relation), we tested the hypothesis that the ΔG effective for the methanogens in natural systems may be lower than expected from p_{H_2} measurements. During incubation of rumen fluid the p_{H_2} experienced by the methanogens is probably different than that measured in the headspace, as microbial aggregates are formed (see above) and rumen methanogens to a large part intimately associate with protozoa (Sharp *et al.*, 1998) creating p_{H_2} gradients. p_{H_2} gradients lead to a measured p_{H_2} that does not agree with the p_{H_2} experienced by the methanogenic organism. In the case of H_2/CO_2 in the headspace, a lower p_{H_2} was present in close proximity to the methanogens, which consumed H_2. In the case of a N_2 headspace, it was the opposite, since the products of fermentation, which are the substrates of methanogenesis, were produced by fermenting organisms next to the methanogenic archaea. The methanogens, therefore, had already depleted the p_{H_2} before it could be quantified in the bulk fluid or the headspace in equilibrium with the bulk liquid. An exception to this rule was some of the peat samples (Fig. 5a; $\alpha_{CO_2-CH_4} > 1.065$), in which degradable organic substrates and thus H_2 production rates were presumably low. In these incubations metabolic activity of methanogens kept the p_{H_2} close to the threshold of hydrogenotrophic methanogenesis, that the ΔG determined from bulk p_{H_2} measurements approached the α–ΔG relation close to the maximum α (i.e. a gradient between the p_{H_2} experienced by the archaea and the p_{H_2} measured in the headspace was reduced to a minimum; Fig. 5a). Collectively, these results are consistent with our hypothesis that $\alpha_{CO_2-CH_4}$ is a good predictor of the ΔG available to the methanogens *in situ*. The values of ΔG determined the conventional way, (Eqn (3)), and using the α–ΔG relation are exemplarily shown by horizontal dash-dotted lines for the incubation of rumen under H_2/CO_2 headspace and rice field soil and show a difference of up to 60 kJ mol^{-1} (Fig. 5). We propose that the more reliable ΔG values were those extrapolated from the α–ΔG relation, and that a large fraction of the methanogens actually experienced a more negative ΔG than indicated by the measurement of bulk p_{H_2}.

The α–ΔG relation found in our coculture experiments may overcome the problem of biased p_{H_2} measurements in natural methanogenic environments, as the average ΔG available to the hydrogenotrophic methanogenic community can now be constrained by the C-isotope fractionation. The resulting *in situ* ΔG values show that hydrogenotrophic methanogens often thrive at ΔG of -40 to -100 kJ mol^{-1}. These ΔG values are lower than those derived from measurements of p_{H_2} in the bulk of

environmental samples (i.e. about -20 kJ mol^{-1} in anoxic rice field soil; Yao & Conrad, 1999), and are sufficient for the synthesis of $1/2$ to 1 ATP per reaction. Thus, there is a chance to solve the dilemma of earlier observations where relatively high ΔG values, hardly sufficient for active growth of methanogenic populations, were associated with changes in community composition or even net growth of methanogenic populations (Peters & Conrad, 1996; Lueders & Friedrich, 2002; Krüger *et al.*, 2005).

Conclusions

We have shown that the fractionation of stable C-isotopes during hydrogenotrophic methanogenesis is related to the Gibbs free energy of catabolism, thus confirming the differential reversibility hypothesis of Valentine *et al.* (2004). The existence of a α–ΔG relation that is common to all species of H_2/CO_2-consuming methanogens implies that the mechanistic basis for stable C-isotope fractionation is the same. This implication is quite likely, since the enzymology of hydrogenotrophic methanogenesis is rather uniform (Thauer, 1998), but should be confirmed by more detailed research.

We have also shown that the α–ΔG relation can be used to determine the free energy available for hydrogenotrophic methanogenesis *in situ* without the need to measure the notoriously low H_2 partial pressures, which may be biased. Comparison of ΔG calculated from the α–ΔG relation and by the traditional way may be used to interpret the microenvironment of methanogenic microbial consortia with respect to the H_2 gradient experienced by the microbiota. Such a knowledge, even when approximate, helps judge the *in situ* living conditions of the methanogenic microbiota, a metabolic microbial group with high impact on global atmospheric chemistry and physics in the past (Kasting, 1993), the present (Lelieveld & Crutzen, 1992), and the future (Wang *et al.*, 2004).

Accepting a common α–ΔG relation for hydrogenotrophic methanogenesis, this would have implications for the interpretation of stable isotope signatures measured in natural environments. Values of $\delta^{13}C$ in environmental CH_4 and CO_2 are frequently interpreted with respect to the predominant path of CH_4 formation. Calculating an apparent α ($\alpha_c = (\delta^{13}CO_2 + 10^3)/(\delta^{13}CH_4 + 10^3)$) it is generally assumed that low $\alpha_c < 1.055$ and high $\alpha_c > 1.065$ are indicative of the predominant operation of acetotrophic and hydrogenotrophic methanogenesis, respectively (Whiticar *et al.*, 1986; Whiticar, 1999). However, hydrogenotrophic methanogenesis may also cause low α_c, if the methanogens operate in a microenvironment with plenty of free energy

available (e.g. eutrophic anoxic systems), in which H_2 production by fermenting bacteria is high. Hence, one should be more cautious when interpreting $\delta^{13}C$ values without determining the fractionation factor of hydrogenotrophic methanogenesis separately.

In addition, the α–ΔG relation explains the wide variation of $\alpha_{CO_2-CH_4}$ in the environment. Although we cannot use the relation to precisely quantify C-isotope fractionation, we can better predict $\alpha_{CO_2-CH_4}$ in different types of environments. We tested three environments (peat, rice field soil, and rumen), which are important for global CH_4, showing that the relatively narrow range of energy conditions in these environments determines the observed range of α-values. These findings agree with environmental studies, where low values of $\alpha_{CO_2-CH_4}$ were found in the high-p_{H_2} environment rumen ($\alpha_{CO_2-CH_4} = 1.031$; Levin et al., 1993), whereas under energy-limiting conditions as in bogs values of $\alpha_{CO_2-CH_4}$ were close to 1.07 (Lansdown et al., 1992; Avery et al., 1999). Because of the relatively favorable energy conditions for hydrogenotrophic methanogens in rice field soils, values of α there usually show intermediate isotope fractionation ($\alpha_{CO_2-CH_4} = 1.045$–$1.060$; Sugimoto & Wada, 1993; Tyler et al., 1997; Bilek et al., 1999). With the α–ΔG relation as theoretical background in combination with experimental data from methanogenic environments, we may better predict values of $\alpha_{CO_2-CH_4}$ for CH_4-producing environments for which no detailed isotope studies exist. Our results thus provide a mechanistic constraint for modelling the ^{13}C flux from microbial sources of atmospheric CH_4.

Acknowledgements

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