The stable isotopic signature of biologically produced molecular hydrogen (H₂)

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Abstract. Biologically produced molecular hydrogen (H₂) is characterised by a very strong depletion in deuterium. Although the biological source to the atmosphere is small compared to photochemical or combustion sources, it makes an important contribution to the global isotope budget of H₂. Large uncertainties exist in the quantification of the individual production and degradation processes that contribute to the atmospheric budget, and isotope measurements are a tool to distinguish the contributions from the different sources. Measurements of δD from the various H₂ sources are scarce and for biologically produced H₂ only very few measurements exist.

Here the first systematic study of the isotopic composition of biologically produced H₂ is presented. In a first set of experiments, we investigated δD of H₂ produced in a biogas plant, covering different treatments of biogas production. In a second set of experiments, we investigated pure cultures of several H₂ producing microorganisms such as bacteria or green algae. A Keeling plot analysis provides a robust overall source signature of δD = −712 ‰ (±13 ‰) for the samples from the biogas reactor (at 38°C, δD_H₂O = +73.4 ‰), with a fractionation constant ε_H₂-H₂O of −689 ‰ (±20 ‰) between H₂ and the water. The five experiments using pure culture samples from different microorganisms give a mean source signature of δD = −728 ‰ (±28 ‰), and a fractionation constant ε_H₂-H₂O of −711 ‰ (±34 ‰) between H₂ and the water. The results confirm the massive deuterium depletion of biologically produced H₂ as was predicted by the calculation of the thermodynamic fractionation factors for hydrogen exchange between H₂ and water vapour. Systematic errors in the isotope scale are difficult to assess in the absence of international standards for δD of H₂.

As expected for a thermodynamic equilibrium, the fractionation factor is temperature dependent, but largely independent of the substrates used and the H₂ production conditions. The equilibrium fractionation coefficient is positively correlated with temperature and we measured a rate of change of 2.3 ‰ /°C between 45°C and 60°C, which is in general agreement with the theoretical prediction of 1.4 ‰ /°C.

Our best experimental estimate for ε_H₂-H₂O at a temperature of 20°C is −731 ‰ (±20 ‰) for biologically produced H₂. This value is close to the predicted value of −722 ‰, and we suggest using these values in future global H₂ isotope budget calculations and models with adjusting to regional temperatures for calculating δD values.

1 Introduction

Molecular hydrogen (H₂) is the second most abundant reduced compound in the atmosphere, after methane (CH₄). With a global average mixing ratio of ~530 ppb (parts-per-billion, nmole/mole) and an atmospheric lifetime of ~2 yr, it is responsible for a large fraction of the chemical turnover of hydrogen atoms in the atmosphere and...
contributes significantly to atmospheric chemistry (Novelli et al., 1999; Hauglustaine and Ehhalt, 2002; Rahn et al., 2003; Ehhalt and Rohrer, 2009). By reaction with the hydroxyl radical (•OH), hydrogen indirectly increases the atmospheric lifetimes of other trace gases that also react with •OH, for example, CH4 and carbon monoxide (CO) (Prather, 2003; Schultz et al., 2003; Jacobson et al., 2005; Jacobson, 2008) and, therefore, acts as an ‘indirect’ greenhouse gas. In the stratosphere, oxidation of H2 is a source of water vapour, which is important for the radiative properties of the stratosphere and also forms the substrate for polar stratospheric clouds, which are key ingredients in the formation of the polar ozone holes (Tromp et al., 2003; Warwick et al., 2004; Feck et al., 2008; Jacobson, 2008).

H2 is considered as a promising future energy carrier. It can be produced chemically, physically and biologically. The shortage, increase in cost and climate impact of fossil fuels leads to increased interest in sustainable and clean production of H2. One possible source to accommodate the expected energy demand might be biologically produced H2, e.g., via fermentation or photosynthesis.

Numerous studies in the past have addressed the global atmospheric budget of H2, but still none of the individual source or sink strengths is constrained to better than ±25% (Ehhalt and Rohrer, 2009). Additional information is expected to come from the analysis of the H2 isotopic composition (δD), because the different sources of H2 have a very different deuterium content. δD is defined as the relative deviation of the D/H ratio in a sample from the same ratio in the international reference material Vienna Standard Mean Ocean Water (VSMOW). Also, the kinetic fractionation in the two main removal processes, soil deposition and reaction with OH, is different.

Tropospheric H2 is enriched in deuterium with δD ~ +130‰ (Gerst and Quay, 2001; Rhee et al., 2006; Rice et al., 2010; Battenburg et al., 2011) compared to surface emissions from fossil fuel combustion and biomass burning (δD approximately −200‰ and −300‰, respectively) (Gerst and Quay, 2001; Rahn et al., 2002; Röckmann et al., 2010a; Vollmer et al., 2010). As originally proposed by Gerst and Quay (2001) from budget closure, the photochemical sources of H2 are also enriched in deuterium with δD between ~ +100‰ and +200‰ (Rahn et al., 2003; Röckmann et al., 2003, 2010b; Feilberg et al., 2007; Nilsson et al., 2007, 2010; Pieterse et al., 2009). Biologically produced H2 has the most exceptional isotopic composition. Biochemical reactions take place in the aqueous phase and, therefore, the isotopic composition of biologically produced H2 should reflect the thermodynamic isotope equilibrium between H2 and H2O. Bottinga (1969) calculated fractionation factors for isotope equilibrium in the system H2 – water vapour. He predicts εH2-H2O values for biologically produced H2 of −737‰ to −693‰, relative to the water, in the main biological relevant temperature range between 10°C and 40°C. Up to now only few individual measurements have been carried out to experimentally determine the isotopic composition of biologically produced H2 and confirm the extremely depleted values calculated by Bottinga (1969). Rahn et al. (2002) measured headspace samples from a jar of termites with a value of δD = −778‰ at a mixing ratio of 1.8 ppm (parts-per-million, µmole/mole), and δD = −690‰ at a mixing ratio of 4 ppm from a water headspace sample taken from an eutrophic pond. For none of these values the isotopic composition of the water was reported and it appears that the equilibrium isotope effect between H2 and H2O has never been experimentally verified. Today recent global modelling studies have incorporated biological sources with an isotopic composition of δD = −628‰ (Price et al. 2007; Pieterse et al. 2011).

Although biologically produced H2 is only responsible for approximately 10% of the annual global H2 source (Novelli et al., 1999; Hauglustaine and Ehhalt, 2002; Ehhalt and Rohrer, 2009; Pieterse et al., 2011) the extreme deuterium depletion relative to ambient atmospheric H2 makes it a quite important contributor to the isotope budget (Price et al. 2007; Pieterse et al. 2011). An increasing demand and anthropogenic biological production of H2 by e.g., industrial fermentation of biogenic waste material is associated with an expected release to the atmosphere because of leakage during production, storage, transport and use. This may increase the contribution of highly deuterium-depleted H2 to the atmosphere.

Here we present the first systematic experimental evaluation of the isotope source signature of biologically produced H2, which is then compared to the theoretical calculations of Bottinga (1969). We measured the isotopic composition of fermentative produced molecular H2 in biogas, using different production conditions and substrates. Additionally we investigated H2 produced from pure cultures of fermentative bacteria (Caldicellulosiruptor saccharolyticus, Escherichia coli, and Clostridium acetobutylicum) and of one N2-fixer (Azospirillum brasilense). We also measured photosynthetically produced H2 from the common green algae Chlamydomonas reinhardtii.

2 Experimental

2.1 Samples

2.1.1 Samples from a biogas plant

Samples were provided from a biogas plant in Freising, Germany, where also the experiments were conducted. Experiments were carried out with batch cultures (21 Merck glass bottles) and continuous cultures (301 glass container). Both were fed with different substrates from surrounding agriculture such as corn, sunflower, cellulose, grass, wheat or mixtures of these substrates. For both treatments the same inoculum was used. It was provided from a pilot-plant scaled plant.
(35001 volume). An overview about used substrates and different treatments is given in Table 1.

The batch cultures consist of 1600 ml inoculum and were fed once with 50 g substrate (organic dry substance, oDS) and incubated at a stable temperature of 38 °C. After 35 days, headspace gas samples were taken with gas tight syringes into evacuated 12 ml glass tubes with an overpressure of approximately 1 bar.

The continuous cultures consist of 30 l inoculum and were fed daily in the morning and incubated at temperatures of 38 °C to 60 °C depending on the treatment. The treatments also differ in the amount of substrate between 2 and 3.5 kg organic dry substance/day (oDS/d). Approximately 4 h after feeding, samples were taken at a syringe port at the fermenter with gas tight syringes into evacuated 12 ml glass tubes with an overpressure of approximately 1 bar.

In total, three samples from batch cultures and 13 samples from continuous cultures were measured (see Table 1). Some samples were measured in duplicate or more. The headspace of pure inoculum was also sampled and measured.

2.1.2 Pure culture experiments

Headspace samples were taken from 5 pure cultures of known H2 producing organisms: (i) three common fermentative bacteria: Caldicellulosiruptor saccharolyticus, Escherichia coli and Clostridium acetobutylicum; (ii) one N2-fixing bacterium (Azospirillum brasilienis, strain SP7); and (iii) one limnic green algae Chlamydomonas reinhardtii (SAG strain number 11–32b).

E. coli and C. saccharolyticus were grown with 10 or 20 mM glucose and 0.2 g l−1 yeast extract in the medium as described in Stams et al. (1993). These bacteria were grown in 120 ml vials with 50 ml medium or 250 ml bottles with 100 ml medium, and a gasphase of N2/CO2 (80/20). E. coli was grown at 37 °C and C. saccharolyticus at 70 °C. C. acetobutylicum was grown at 37 °C as described by Nimcevic et al. (1998). The gas phase was N2. Gas samples were taken from the cultures at the end of growth by gastight syringes and injected in sterile vacuum vials, previously flushed with pure nitrogen.

12 ml of preincubated A. brasilienis (strain SP7) was used to inoculate 600 ml of ampicillin medium in a closed 21 borosilicate bottle. Three replicates and one control were incubated for 5 days at 30 °C. The headspace gas volume was sampled into a pre-evacuated 11 glass container (NOR-MAG, Illmenau, Germany) sealed with two polychlorotrifluoroethylen (PCTFE) valves.

The green algae C. reinhardtii was cultivated in a sulfate-limited Tris-Acetate-Phosphate (TAP) medium as part of an experiment conducted in Switzerland and described in more detail by Haus et al. (2009). For the batch used in our isotope study, a N2 headspace technique in a glass bottle was applied. After approximately 8 days of incubation, several ml of headspace gas were extracted using a gastight syringe and injected into a pre-evacuated 11 glass container of the same type as mentioned above. Synthetic air, further purified from traces of H2 using a catalyst (Sofnocaat 514, Molecular Products, Thaxted, UK) was added to 1.9 bar total pressure to dilute the sample and, thereby, making it suitable for H2 measurements. Initial H2 mixing ratio measurements were conducted at Empa before transferring the sample to IMAU for detailed H2 and δD analysis. Results for H2 mixing ratios of Empa and IMAU are in agreement within the error bars and the direct comparison is not shown here.

2.2 Determination of H2 mixing ratio and isotopic composition

The mixing ratio and isotopic composition of molecular H2 was determined by using the experimental setup developed by Rhee et al. (2004) and modified as described in Röckmann et al. (2010b). Due to a lack of international isotope standards for H2, calibration is a critical issue. Our calibration scale has been described in Batenburg et al. (2011). Samples were measured randomly and within 35 days after collection. The measurements consist of the following steps: (1) The sample is cryogenically separated at −240 °C, which means that all gaseous compounds, with the exception of H2 and some noble gases, are condensed; (2) The non-condensed fraction of the sample (including H2) is preconcentrated using a 5 Å molecular sieve at −210 °C; (3) H2 is focused on a capillary gas chromatographic column (5 Å molecular sieve) and chromatographically purified from remaining contaminants at 50 °C; (4) the D/H ratio of molecular H2 is determined by continuous flow isotope ratio mass spectrometry using a ThermoFinnigan Delta Plus XL instrument.

The analytical system is designed for measurement of air samples with H2 mixing ratios in the range of typical atmospheric air samples (e.g., Röckmann et al. 2003, 2010b; Rhee et al. 2006; Batenburg et al. 2011). The samples obtained from the biogas reactor and the individual cultures have extremely high H2 molar mixing ratios between 10 ppm and 1.4 % (see Table 1), which are outside the measurement range of our instrument. To a certain degree, the analytical system has some flexibility as regards high H2 mixing ratios because simply smaller samples can be inserted into the sample volume; however, in this study the values were that high that the samples had to be diluted. Two dilution methods were adapted for samples of pure cultures and biogas samples.

Several samples from the pure cultures were expanded into 21 electropolished stainless steel canisters that are routinely used in our laboratory for airborne air sampling (Kaiser et al., 2006; Laube et al., 2008, 2010 and referenced herein). They were diluted by a factor of approximately 2000 with 4117

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Table 1. Molecular hydrogen (H$_2$) mixing ratio and δD (vs. VSMOW) from different biogas production treatments and pure cultures. Columns 4 and 5 give the raw (i.e. measured) values for mixing ratio and δD, which are used in the Keeling plot in Fig. 1. Columns 6 and 7 give the final values of the pure sample after correction for the dilution with standard gas (with known H$_2$ mixing ratio of 546.2 ppb, δD +71.4 ‰, used for biogas samples) and H$_2$ free gas (used for pure microorganism culture samples), respectively. Substrate was added in units of kg organic dry material per day [o DM/d]. Some samples are measured in duplicate or more. Uncertainties in the table are given for the reproducibility of the measurements or calculations as the average of absolute deviation of data from their mean. For single measurements we assume a mean uncertainty of 4.1 % for mixing ratios and 2.4 % for δD values.

<table>
<thead>
<tr>
<th>Culture</th>
<th>Temp. (°C)</th>
<th>Substrate (organic dry material/day)</th>
<th>Measured mixing ratio (ppb)</th>
<th>Measured δD (%)</th>
<th>Corrected mixing ratio (ppm)</th>
<th>Corrected δD (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>inoculum</td>
<td>38</td>
<td>inoculum</td>
<td>624</td>
<td>−12</td>
<td>27</td>
<td>−587</td>
</tr>
<tr>
<td>batch</td>
<td>38</td>
<td>corn</td>
<td>584</td>
<td>+12</td>
<td>13</td>
<td>−831</td>
</tr>
<tr>
<td>culture</td>
<td>38</td>
<td>corn + sunflower</td>
<td>575</td>
<td>+35</td>
<td>10</td>
<td>−636</td>
</tr>
<tr>
<td></td>
<td>38</td>
<td>cellulose</td>
<td>592</td>
<td>+21</td>
<td>16</td>
<td>−555</td>
</tr>
<tr>
<td>continuous</td>
<td>38</td>
<td>grass, 2 kg o DM/d</td>
<td>702</td>
<td>−104</td>
<td>53</td>
<td>−712</td>
</tr>
<tr>
<td>culture</td>
<td>38</td>
<td>grass, 2 kg o DM/d</td>
<td>1252</td>
<td>−370</td>
<td>240</td>
<td>−710</td>
</tr>
<tr>
<td></td>
<td>38</td>
<td>grass, 2 kg o DM/d</td>
<td>1531</td>
<td>−437</td>
<td>335</td>
<td>−718</td>
</tr>
<tr>
<td></td>
<td>38</td>
<td>corn, 2 kg o DM/d</td>
<td>675</td>
<td>−85</td>
<td>44</td>
<td>−741</td>
</tr>
<tr>
<td></td>
<td>38</td>
<td>corn, 2.5 kg o DM/d</td>
<td>587</td>
<td>+16</td>
<td>14</td>
<td>−710</td>
</tr>
<tr>
<td></td>
<td>38</td>
<td>corn, 3.5 kg o DM/d</td>
<td>689</td>
<td>−49</td>
<td>49</td>
<td>−699</td>
</tr>
<tr>
<td></td>
<td>38</td>
<td>grass, 2 kg o DM/d (n = 2)</td>
<td>1170 ± 23</td>
<td>−350 ± 6</td>
<td>212 ± 5</td>
<td>−718 ± 21</td>
</tr>
<tr>
<td></td>
<td>38</td>
<td>grass, 2 kg o DM/d (n = 3)</td>
<td>2262 ± 135</td>
<td>−520 ± 9</td>
<td>587 ± 38</td>
<td>−708 ± 20</td>
</tr>
<tr>
<td></td>
<td>38</td>
<td>grass, 2 kg o DM/d</td>
<td>1133</td>
<td>−327</td>
<td>201</td>
<td>−696</td>
</tr>
<tr>
<td></td>
<td>45</td>
<td>30 % grass + 30 % corn + 40 % cereals (n = 2)</td>
<td>946 ± 23</td>
<td>−270 ± 13</td>
<td>138 ± 7</td>
<td>−734 ± 22</td>
</tr>
<tr>
<td></td>
<td>50</td>
<td>30 % grass + 30 % corn + 40 % cereals (n = 2)</td>
<td>1371 ± 87</td>
<td>−408 ± 15</td>
<td>282 ± 15</td>
<td>−726 ± 21</td>
</tr>
<tr>
<td></td>
<td>55</td>
<td>30 % grass + 30 % corn + 40 % cereals (n = 2)</td>
<td>2273 ± 121</td>
<td>−523 ± 6</td>
<td>584 ± 32</td>
<td>−711 ± 21</td>
</tr>
<tr>
<td></td>
<td>60</td>
<td>30 % grass + 30 % corn + 40 % cereals (n = 2)</td>
<td>2510 ± 66</td>
<td>−535 ± 7</td>
<td>671 ± 26</td>
<td>−703 ± 20</td>
</tr>
</tbody>
</table>

| Microorganism species | | | | | | |
|-----------------------| | | | | | |
| Pure microorganism culture | | | | | | |
| Clostridium acetobutylicum | 30 | Azospirillum brasiliensis (n = 5) | 4043 ± 1096 | −556 ± 24 | 1339 ± 420 | −664 ± 19 |

region of the extremely deuterium-depleted samples, and the isotope scale has to be extrapolated very far outside the range that was used for calibrating the reference gas (−9.5 ‰ to +205 ‰) (Batenburg et al., 2011). Therefore, for the samples from the biogas plant and the N$_2$ fixer (A. brasiliensis), a standard addition method was developed. Small amounts of a sample (usually approximately 1 ml) were added manually with a gas tight syringe to air from the laboratory reference air cylinder (H$_2$ mixing ratio = 546.2 ppb, δD = +71.4 ‰) (Batenburg et al., 2011). For the biogas samples following this procedure the measured mixing ratios after dilution were between 575 ppb and 2510 ppb, and δD values were between +35 ‰ and −535 ‰ (Table 1). This means that in the measurement procedure itself a “Keeling plot analysis” is involved, because the H$_2$ and HD measured in the isotope ratio mass spectrometer is then a mixture of the well-known reference air and the unknown sample (Fig. 1 for the biogas samples). The isotopic composition of the original sample is then inferred by extrapolation of the linear fit to the correlation between δD and inverse mixing ratio to 0 (y-axis intercept). On the one hand, this introduces an error from the extrapolation, but on the other hand the measured δD values are much closer to the range that was used for calibration of the reference gas. The manual injection of the reference gas with a syringe leads to a relatively high error for the reproducibility of mixing ratios for the original biogas samples (±4.1 ‰), whereas the reproducibility for δD is not much worse than for normal atmospheric air samples (±2.4 ‰), since an error in mixing ratio only changes the location of the mixture on the mixing line, but not the y-axis intercept (see Fig. 1). The error for measurement reproducibility is given as the average of absolute deviation of data from their mean.

The isotopic composition of the water used in the incubation experiments was determined by Hydroisotop GmbH, Schweitenkirchen, Germany.
The δD is defined as followed:

\[
\delta D = \left[ \frac{D/H}{\text{sample}} \right] - 1
\]

Fractionation constants \( \varepsilon_{\text{H}_2-\text{H}_2\text{O}} \) of \( \text{H}_2 \) relative to \( \text{H}_2\text{O} \) were calculated as

\[
\varepsilon_{\text{H}_2-\text{H}_2\text{O}} = \alpha_{\text{H}_2-\text{H}_2\text{O}} - 1 = \left[ \frac{D/H}{\text{H}_2\text{O}} \right] - 1
\]

\[
= \frac{\delta D_{\text{H}_2} + 1}{\delta D_{\text{H}_2\text{O}} + 1} - 1 = \frac{\delta D_{\text{H}_2} - \delta D_{\text{H}_2\text{O}}}{\delta D_{\text{H}_2\text{O}} + 1}
\]

where \( \alpha \) is the fractionation factor between the \( \text{H}_2 \) product and the \( \text{H}_2\text{O} \) reactant. All \( \delta D \) values are reported relative to Vienna Standard Mean Ocean Water (VSMOW). \( \text{H}_2 \) mixing ratios are reported as molar mixing ratios in parts per million (ppm = µmole/mole) or parts per billion (ppb = nmole/mole), or in percent (%) for high mixing ratios.

### 3 Results and discussion

Table 1 provides a summary of the results. The \( \text{H}_2 \) content of the samples differed considerably. While the samples of the batch incubations from the biogas reactor contained relatively low \( \text{H}_2 \) mixing ratios with a maximum of 16 ppm (0.0016%), the continuous incubations showed values up to 671 ppm (0.0671%). In such biogas reactors, hydrogen produced by anaerobic bacteria is metabolised by the activity of methanogenic archaea, resulting in rather low hydrogen partial pressures (Stams and Plugge, 2009). The highest \( \text{H}_2 \) mixing ratios up to 13887 ppm or 1.4% were measured in the headspace of the pure culture of \( \text{C. saccharolyticus} \).

A Keeling plot analysis provides a powerful tool to establish a robust overall source signature for the samples from the biogas reactor. The results of all measurements can fit very well \((R^2 = 0.999)\) on a straight line with a y-axis intercept (source signature) of \( \delta D = -713\%e \) (±13‰). It should be noted that this fit is not equally constrained by all samples, but more influenced by samples with high mixing ratios. To examine the effect of a possible nonlinearity in the isotope scale the analysis was repeated using only samples with \( \delta D \) values > -100‰, which results in a source signature of -718‰, which shows that nonlinearity of the isotope scale does not significantly affect our results.

Results for the individual samples are also included in Table 1. The samples from the batch incubations show individually a large degree of variability, but this is due to the low \( \text{H}_2 \) mixing ratios of the sample-reference gas mixtures measured (see Sect. 2). For individual samples, the Keeling plot analysis covers only a small range in inverse mixing ratio and the associated errors after extrapolation to 0 are large. The average of all individual source signature determinations is

\[
\delta D = -706\%e \text{ (±37%e, } n = 22) \]

This is in very good agreement with the Keeling plot approach above, which provides the more robust constraint, because the influence of the samples with low mixing ratios is reduced in the Keeling plot approach.

The \( \delta D \) source signatures of the pure cultures range between -664‰ for \( \text{A. brasilienis} \) to -758‰ for \( \text{E. coli} \) and \( \text{C. saccharolyticus} \) and give a mean source signature of \( \delta D = -728\%e \) (±28‰) (Table 2). Overall, the results from the different experimental setups confirm the very depleted source signature of biological \( \text{H}_2 \), but there are differences between the different experimental setups. For further interpretation, the \( \delta D \) values of the \( \text{H}_2 \) produced must be compared to the source water (Table 2). The isotopic composition of the waters used in the biogas plant in Freising was \( \delta D = -73.4\%e \) (±1.5‰). For the cultivation of the microorganisms the isotopic composition of the used water was -65.6‰ (±1.5‰) (\( \text{C. reinhardttii} \), Switzerland) and -52.1‰ (±1.5‰) (fermenters cultivated in Wageningen). The isotopic composition of the water used in the cultivation of \( \text{A. brasilienis} \) in Munich was not determined, but is assumed to be close to typical tap water in Freising (\( \delta D = -73.4\%e \)). With this information, the fractionation constant \( \varepsilon \) between \( \text{H}_2 \) and \( \text{H}_2\text{O} \) can be calculated Eq. (2), which eliminates the water as free parameter. It is evident from the results in Table 2 that the variability between the different experimental setups remains when corrected for the different waters.

The fact that the \( \text{H}_2 \) samples from the pure cultures were measured after dilution, and the biogas samples with the online mixing approach is unlikely to cause the difference, as a potential contamination during dilution of the pure culture samples should not lead to even lower \( \delta D \) values for the mixtures. It was beyond the scope of this project to further
investigate whether these differences are significant, but this would be an interesting task for the future. In the absence of further information, it may not be appropriate to simply average the results from this to some degree arbitrary selection of samples to obtain a representative mean.

The result from the biogas samples is best constrained, however, this value is determined for a temperature range of 38 °C to 60 °C. Including only biogas samples at 38 °C (inoculum and treatments at higher temperatures are excluded from the Keeling plot, Fig. 1) we end up with a δD of −712 ‰ ± 13 ‰ and a fractionation constant εH2-H2O of −689 ‰ ± 20 ‰. This value is our best estimate for a fractionation constant εH2-H2O at 38 °C. Although systematic errors in the isotopic scale cannot be excluded due to a lack of international isotope standards, the good agreement with the theoretically calculated value of δD = −695 ‰ (Bottinga, 1969) provides strong support for the validity of our results.

Bottinga (1969) also reports the temperature dependence of εH2-H2O. We determined this temperature dependence experimentally over the incubation range 45 °C–60 °C with otherwise identical conditions (same inoculum and substrate, 30 % grass, 30 % maize, 40 % cereals). As expected for an enzymatic-catalysed reaction in this temperature range, the mixing ratio of H2 is increasing with increasing temperatures (Fig. 2a). Figure 2b shows that εH2-H2O increases with increasing incubation temperature from −713 ‰ at 45 °C to −680 ‰ at 60 °C, thus, by 2.3 ‰/°C. Gray diamonds in Fig. 2b indicate the theoretically predicted temperature dependency from Bottinga (1969), which is slightly smaller with 1.4 ‰/°C over the same temperature range. The measurements show a distinct offset of 28 ‰ at 45 °C reducing to 16 ‰ at 60 °C, relative to the theoretical results over this temperature range (Fig. 2). This offset is slightly larger than our estimated experimental uncertainty and remains at present unexplained. Possible contributing factors in the measurements are the potential errors in the absolute isotope calibration (Batenburg et al. 2011) or a nonlinearity in the isotope scale at very low δD values, which is not obvious from the Keeling plot. Nevertheless, the overall temperature dependence is in good qualitative agreement with the calculations of Bottinga (1969), and we conclude that the experimental techniques are sufficiently advanced now to detect such small changes in the region of very depleted isotope values.

In order to derive a revised δD value for H2 from biological sources that can be used in global models or isotope budget calculations, we calculated εH2-H2O at a mean temperature of 20 °C using the measured value at 38 °C and our experimentally determined temperature dependence, yielding a value of εH2-H2O = −731 ‰ (±20 ‰). For calculating a global average δD value of H2 from biological sources we used a global average value of δD of precipitation of δD = −37.8 ‰ (Hoffmann et al. 1998; Bowen and Revenaugh, 2003), and then calculate δD = −741 ‰ (±20 ‰). Using the theoretical temperature dependence of Bottinga, we calculate εH2-H2O = −715 ‰ (±20 ‰) and δD = −726 ‰ (±20 ‰). Our experimental values are in a very good agreement to the predicted value by Bottinga (1969) of εH2-H2O = −722 ‰, which gives a δD = −733 ‰ (20 °C, δD of precipitation of −37.8 ‰).

### 4 Summary, conclusions and outlook

The isotopic composition of biologically produced H2 was investigated systematically and our measurements confirm the massive deuterium depletion as predicted by Bottinga (1969). Using a Keeling plot analysis, we establish an overall source signature of δD = −712 ‰ (±13 ‰) for biologically produced H2, with a fractionation constant of
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