Fractionation of stable carbon isotopes during microbial propionate consumption in anoxic rice paddy soils

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Abstract. Propionate is an important intermediate during the breakdown of organic matter in anoxic flooded paddy soils. Since there are only a few experiments on carbon isotope fractionation and the magnitude of the isotopic enrichment factors (ε) involved, we measured propionate conversion to acetate, CH₄ and CO₂ in anoxic paddy soils. Propionate consumption was measured using samples of paddy soil from Vercelli (Italy) and the International Rice Research Institute (IRRI, the Philippines) suspended in a phosphate buffer (pH 7.0) both in the absence and presence of sulfate (gypsum) and of methyl fluoride (CH₃F), an inhibitor of aceticlastic methanogenesis. Under methanogenic conditions, propionate was eventually degraded to CH₄, with acetate being a transient intermediate. Butyrate was also a minor intermediate. Methane was mainly produced by aceticlastic methanogenesis. Propionate consumption was inhibited by CH₃F. Butyrate and CH₄ were ¹³C-depleted relative to propionate, whereas acetate and CO₂ were ¹³C-enriched. The isotopic enrichment factors (εprop) of propionate consumption, determined by Mariotti plots, were in a range of −8 ‰ to −3.5 ‰. Under sulfidogenic conditions, acetate was also transiently accumulated, but CH₄ production was negligible. Application of CH₃F hardly affected propionate degradation and acetate accumulation. The initially produced CO₂ was ¹³C-depleted, whereas the acetate was ¹³C-enriched. The values of εprop were −3.5 ‰. It is concluded that the degradation of organic carbon via propionate to acetate and CO₂ involves only a little isotope fractionation. The results further indicate a major contribution of Syntrophobacter-type propionate fermentation under sulfidogenic conditions and Smithella-type propionate fermentation under methanogenic conditions. This interpretation is consistent with data regarding the microbial community composition published previously for the same soils.

1 Introduction

Propionate is a common intermediate of organic matter degradation in anoxic paddy soils. In the absence of sulfate reduction or methanogenesis, propionate may accumulate to millimolar concentrations (Conrad et al., 2014; Glissmann and Conrad, 2000; Nozoe, 1997). Under methanogenic conditions, propionate is degraded by fermentation. Several different biochemical pathways are conceivable for propionate fermentation (Textor et al., 1997). The major fermentation pathways are those by Syntrophobacter (Boone and Bryant, 1980) and Smithella (Liu et al., 1999), both members of Deltaproteobacteria. Syntrophobacter operates the methylmalonyl-CoA pathway, which results in randomization of the carbon positions of propionate (Houwen et al., 1991). This pathway can also be found in Desulfotomaculum sp. and Pelotomaculum sp. (Chen et al., 2005; Imachi et al., 1996; DeBok et al., 2005; Imachi et al., 2002; Plugee et al., 2002) and apparently exists in many anoxic environments (Imachi et al., 2006; Krylova et al., 1997; Schink, 1985). Smithella, on the other hand, operates a dismutation pathway, which does not result in randomization (Krylov et al., 1997). This pathway has also been found in many anoxic environments (Gan et al., 2012; Lueders et al., 2004; Xia et al., 2019).

Propionate degradation by randomizing Syntrophobacter proceeds via succinate in the following way:

\[ 4 \text{ propionate} + 8\text{H}_2\text{O} \rightarrow 4 \text{ acetate} + 4\text{CO}_2 + 12\text{H}_2. \]  (1)
Propionate degradation by non-randomizing Smithella proceeds by dismutation of propionate:

$$4 \text{ propionate} \rightarrow 2 \text{ butyrate} + 2 \text{ acetate.} \quad (2)$$

Butyrate is then syntrophically converted (e.g., by Syntrophomonas; McInerney et al., 1981):

$$2 \text{ butyrate} + 4\text{H}_2\text{O} \rightarrow 4 \text{ acetate} + 4\text{H}_2. \quad (3)$$

The Smithella pathway, in total, is then

$$4 \text{ propionate} + 4\text{H}_2\text{O} \rightarrow 6 \text{ acetate} + 4\text{H}_2. \quad (4)$$

Propionate fermentation is thermodynamically endergonic under standard conditions and therefore requires syntrophic microbial partners that further convert the fermentation products. Under methanogenic conditions, the syntrophic partners are methanogenic archaea, which consume the products acetate and H$_2$. Under sulfidogenic conditions, sulfate-reducing bacteria replace the methanogens. Propionate can also be directly oxidized to CO$_2$ by propionate-degrading sulfate reducers. The overall reaction stoichiometry is the same for Syntrophobacter and Smithella:

$$4 \text{ propionate} + 2\text{H}_2\text{O} \rightarrow 7\text{CH}_4 + 5\text{CO}_2, \text{ or} \quad (5)$$

$$4 \text{ propionate} + 7 \text{ sulfate} + 11\text{H}^+ \rightarrow 7\text{HS}^- + 12\text{CO}_2 + 12\text{H}_2\text{O}. \quad (6)$$

Note that the relative production of acetate and H$_2$ is different for Syntrophobacter and Smithella fermentation, being 1 : 3 and 3 : 2, respectively. Therefore, aceticlastic methanogenesis contributes relatively more than hydrogenotrophic methanogenesis when propionate is fermented by Smithella rather than Syntrophobacter. Under methanogenic conditions, propionate degradation in anoxic paddy soils operates close to the thermodynamic limits (Krylova and Conrad, 1998; Yao and Conrad, 2001). These restrictions are more severe for Syntrophobacter than for Smithella (Dolfing, 2013).

Using paddy soil from Italy and the Philippines, Liu and their coworkers (Liu et al., 2018a; Liu and Conrad, 2017) have recently shown that propionate consumption under sulfidogenic conditions is mainly achieved by Syntrophobacter species or other Syntrophobacteraceae, which first oxidize propionate to acetate and CO$_2$ and subsequently oxidize the accumulated acetate to CO$_2$. They also showed that Smithella was probably involved in methanogenic propionate degradation. The involvement of Smithella has also been shown for other paddy soils and sediments (Gan et al., 2012; Lueders et al., 2004; Xia et al., 2019). Since we used in the present study the same soils as Liu and their coworkers (Liu et al., 2018a; Liu and Conrad, 2017), we assumed that propionate degradation was achieved by the same microorganisms.

Knowledge of carbon isotope fractionation is important for the assessment of the pathways involved in anaerobic degradation of organic matter (Conrad, 2005; Elsner et al., 2005). The $\delta^{13}$C values of organic carbon, acetate and propionate in various soils and sediments were found to be similar (Conrad et al., 2014). The similarity indicates that the enrichment factors ($\varepsilon$) of the processes involved in both the production and consumption of propionate are probably small. The direct determination of $\varepsilon$ values in microbial cultures of one propionate-producing bacterium and one propionate-consuming bacterium also showed low values (Botsch and Conrad, 2011). However, direct determination of $\varepsilon$ values in environmental samples is missing. Therefore, we decided to measure isotope fractionation in methanogenic and sulfidogenic paddy soil amended with propionate along with the recording of the production of acetate, CH$_4$ and CO$_2$. We also used the treatment with methyl fluoride (CH$_3$F) to inhibit the consumption of acetate by methanogenic archaea (Janssen and Frenzel, 1997). Recently, we determined the microbial communities in methanogenic and sulfidogenic rice field soils, which were used for the assessment of $^{13}$C isotope fractionation during acetate consumption (Conrad et al., 2021). Here, we present analogous data from the same soil suspensions prepared for the propionate degradation experiments.

2 Materials and methods

2.1 Paddy soils and incubation conditions

The soil samples were from the research stations in Vercelli, Italy, and the International Rice research Institute (IRRI) in the Philippines. The sampling and soil characteristics have been described before (Liu et al., 2018b). The main soil characteristics will be given. The Italian soil is a sandy loam with a pH of 5.75, total C of 1.1 % and total N of 0.08 %. The Philippine soil is a silt loam with a pH of 6.3, total C of 1.9 % and total N of 0.2 %.

The experimental setup was exactly the same as during a previous study on acetate consumption (Conrad et al., 2021). Paddy soil was mixed with autoclaved anoxic H$_2$O at a ratio of 1 : 1 and incubated under N$_2$ at 25°C for 4 weeks. In a second incubation, paddy soil was mixed with autoclaved anoxic H$_2$O (prepared under N$_2$) at a ratio of 1 : 1, was amended with 0.07 g CaSO$_4$ · 2H$_2$O and was then incubated under N$_2$ at 25°C for 4 weeks. These two preincubated soil slurries were sampled and stored at –20°C for later molecular analysis (see data in Conrad et al., 2021). The preincubated soil slurries were also used (in three replicates) for the following incubation experiments. Two different sets of incubations were prepared. In the first set (resulting in methanogenic conditions), 5 mL of soil slurry preincubated without sulfate was incubated at 25°C with 40 mL of 20 mM potassium phosphate buffer (pH 7.0) in a 150 mL bottle under an atmosphere of N$_2$. The bottles were then amended with (i) 5 mL H$_2$O, (ii) 5 mL H$_2$O + 4.5 mL CH$_3$F, (iii) 5 mL 50 mM sodium propionate,
and (iv) 5 mL 50 mM sodium propionate + 4.5 mL CH₃F. In the second set (resulting in sulfidogenic conditions), 5 mL of soil slurry preincubated with sulfate was incubated at 25 °C with 40 mL of 20 mM potassium phosphate buffer (pH 7.0) in a 150 mL bottle under an atmosphere of N₂. The amendments were the same as above but with the addition of 200 µL of a CaSO₄ suspension corresponding to a concentration of 2.5 M (giving a final concentration of 10 mM sulfate).

2.2 Chemical and isotopic analyses

Gas samples for analysis of partial pressures of CH₄ and CO₂ were taken from the headspace of the incubation bottles after vigorous manual shaking for about 30 s using a gas-tight pressure-lock syringe, which had been flushed with N₂ before each sampling. Soil slurries were sampled, centrifuged and filtered through a 0.2 µm cellulose membrane filter and were stored frozen at −20 °C for later fatty acid analysis. Chemical and isotopic analyses were performed as described in detail previously (Goewert and Conrad, 2009). Methane was analyzed by gas chromatography (GC) with a flame ionization detector. Carbon dioxide was analyzed after conversion to CH₄ with a Ni catalyst. Stable isotope analyses of $^{13}$C/$^{12}$C in gas samples were performed using GC-combustion isotope ratio mass spectrometry (GC-C-IRMS). Propionate, butyrate and acetate were measured using high-performance liquid chromatography (HPLC) linked via a Finnigan LC IsoLink to an IRMS. The isotopic values are reported in the delta notation ($\delta^{13}$C) relative to the Vienna PeeDee Belemnite standard with a $^{13}$C/$^{12}$C ratio (Rstandard) of 0.01118; $\delta^{13}$C = 103($R_{\text{sample}}$/$R_{\text{standard}}$ − 1). The precision of the GC-C-IRMS was ±0.2 ‰, and that of the HPLC-IRMS was ±0.3 ‰.

2.3 Calculations

Millimolar concentrations of CH₄ were calculated from the mixing ratios (1 ppmv ± 10⁻⁶ bar) measured in the gas phase of the incubation bottles: 1000 ppmv CH₄ correspond to 0.09 µmol mL⁻¹ of liquid. Note that this is the total amount of CH₄ in the gas phase relative to the liquid phase.

Fractionation factors for reaction A $\rightarrow$ B are defined after Hayes (Hayes, 1993) as follows:

$$\alpha_{A/B} = (\delta_A + 1000) / (\delta_B + 1000),$$

also expressed as $\epsilon \equiv 1000 (1 - \alpha)$ (in ‰). The carbon isotope enrichment factor $\epsilon_{\text{prop}}$ associated with propionate consumption was calculated from the temporal change in $\delta^{13}$C of propionate as described by Mariotti et al. (Mariotti et al., 1981) from the residual reactant

$$\delta_f = \delta_i + \epsilon [\ln (1 - f)],$$

where $\delta_i$ is the isotopic composition of the reactant (propionate) at the beginning, and $\delta_f$ is the isotopic composition of the residual propionate; both of these are indicated at the instant when $f$ is determined. $f_{\text{prop}}$ is the fractional yield of the products based on the consumption of propionate (0 < $f_{\text{prop}}$ < 1). Linear regression of $\delta^{13}$C of propionate against ln(1 − $f$) yields $\epsilon_{\text{prop}}$ as the slope of the best-fit lines. The regressions of $\delta^{13}$C of propionate were done for data in the range of $f_{\text{prop}}$ < 0.7. The linear regressions were done individually for each experimental replicate ($n = 3$) and were only accepted if $r^2 > 0.9$. The $\epsilon$ values resulting from the replicate experiments were then averaged ($\pm$ SE).

The fraction ($f_{\text{H}_2}$) of CH₄ derived from hydrogenotrophic methanogenesis was determined as described before (Conrad et al., 2010) using

$$f_{\text{H}_2} = (\delta^{13}\text{C}_{\text{CH}_4} - \delta^{13}\text{C}_{\text{CH}_4-\text{ma}}) / (\delta^{13}\text{C}_{\text{CH}_4-\text{mc}} - \delta^{13}\text{C}_{\text{CH}_4-\text{ma}}),$$

where $\delta^{13}\text{C}_{\text{CH}_4}$ = $\delta^{13}$C of the total CH₄ produced; $\delta^{13}\text{C}_{\text{CH}_4-\text{mc}}$ = $\delta^{13}$C of CH₄ produced from hydrogenotrophic methanogenesis, which is equivalent to the CH₄ produced in the presence of CH₃F; and $\delta^{13}\text{C}_{\text{CH}_4-\text{ma}}$ = $\delta^{13}$C of CH₄ produced from aceticlastic methanogenesis. The $\delta^{13}$C of CH₄ was approximated from the $\delta^{13}$C of acetate in the presence of CH₃F assuming that the methyl group of acetate was depleted in $^{13}$C by 8 ‰ (Conrad et al., 2014) and that the enrichment factor ($\epsilon_{\text{CH}_4-\text{ac}-\text{methyl}}$) for CH₄ being produced from acetae–methyl was between 0 and −20 ‰.

3 Results

3.1 Conversion of propionate under methanogenic and sulfidogenic conditions

Incubation of buffered suspensions of rice field soil from Vercelli (Fig. 1) and the IRRI (Fig. S1 in the Supplement) resulted in similar patterns of propionate degradation to acetate, CH₄ and CO₂. Under methanogenic conditions in the absence of sulfate, propionate degradation started after a lag phase of about 20 d (Fig. 1a), resulting in the production of acetate (Fig. 1b), CH₄ (Fig. 1c) and CO₂ (Fig. 1d). The formation of acetate, CH₄ and CO₂ in the absence of propionate was only very small. The accumulation of acetate was only transient, except when aceticlastic methanogenesis was inhibited by CH₃F (Fig. 1b). Similar observations were made in IRRI soil (Fig. S1a–d). The production of CH₄ was roughly equimolar to the consumption of propionate but was nearly zero when aceticlastic methanogenesis was inhibited by CH₃F (Fig. 2a). Under these conditions, acetate accumulated to nearly equimolar amounts with the consumed propionate (Fig. 2b), but in IRRI soil, acetate accumulation was less than equimolar (Fig. S2b in the Supplement). Butyrate was also a transient intermediate of propionate degradation and was produced and consumed simultaneously with acetate (Figs. 1b and S1b). However, the accumulated concentrations were small (< 0.1 mM).
Figure 1. Propionate conversion to acetate, butyrate, CH₄ and CO₂ in suspensions of paddy soil from Vercelli (Italy) after addition of propionate without sulfate (blue squares) or of propionate plus sulfate (gypsum) (red triangles) without CH₃F (open symbols) or with CH₃F (closed symbols). Controls with the addition of water only (blue or red circles) are only shown occasionally. The panels show the temporal change in (a) concentrations of propionate, (b) concentrations of acetate and butyrate (blue diamonds), (c) mixing ratios of CH₄ (1 ppmv = 10⁻⁶ bar), (d) mixing ratios of CO₂, (e) δ¹³C of propionate, (f) δ¹³C of acetate and butyrate, (g) δ¹³C of CH₄, and (h) δ¹³C of CO₂. Shown are the means ± SE (standard error).
Figure 2. Balance of (a, c) produced CH$_4$ and (b, d) produced acetate against the consumed propionate under (a, b) methanogenic and (c, d) sulfidogenic conditions in paddy soil from Vercelli (Italy). The open and closed symbols denote conditions in the absence and the presence of CH$_3$F, respectively. The black and red lines in (a) indicate aceticlastic methanogenesis after generation of acetate by either Smithella (Eq. 4) or Syntrophobacter (Eq. 1). The black and red lines in (b) and (d) indicate transient acetate production by Smithella and Syntrophobacter, respectively. The different symbols indicate three different replicates.

In the presence of sulfate, propionate degradation started after a lag phase of only about 10 d (Fig. 1a), resulting in the accumulation of acetate (Fig. 1b) and the production of CO$_2$ (Fig. 1d), but CH$_4$ production was close to zero (Fig. 1c). Similar results were obtained with IRRI soil (Fig. S1a–d). The accumulated acetate was equimolar (slightly less than equimolar in the IRRI soil; Fig. S2d) to the consumption of propionate (Fig. 2d), but CH$_4$ was not accumulated (Fig. 2c). The addition of CH$_3$F had no effect. Butyrate was not detected. The accumulated acetate was subsequently degraded, resulting in further production of CO$_2$ (Fig. 1b and d).

3.2 Isotope fractionation during propionate degradation

After the onset of propionate degradation, the $\delta^{13}$C of propionate (Fig. 1e) and acetate (Fig. 1f) increased, indicating that the light isotope was preferentially consumed. The $\delta^{13}$C values of CO$_2$ also increased (Fig. 1h). The same was the case for butyrate (Fig. 1f). Similar results were obtained with IRRI soil (Fig. S1e–h). When aceticlastic methanogenesis was inhibited by CH$_3$F, the $\delta^{13}$C values of these compounds increased only slightly or decreased (Fig. 1e, f and h). However, the $\delta^{13}$C of CH$_4$ was much more negative (30–50‰) in the presence than in the absence of CH$_3$F (Fig. 1g). The $\delta^{13}$C values of CH$_4$ in unamended soil (H$_2$O control) were similar to those in propionate amended soil (Fig. 1g). To visualize the change in the $^{13}$C content of the metabolic products relative to the substrates, the $\delta^{13}$C values were plotted against the increasing fractions ($f_{\text{prop}}$) of propionate consumed in soil from both Vercelli (Fig. 3a) and the IRRI (Fig. 3b). The patterns of $\delta^{13}$C values against the $f_{\text{prop}}$ indicated kinetic isotope fractionation. Note that the $\delta^{13}$C values of acetate and CO$_2$ were higher than those of propionate, whereas the values of butyrate and CH$_4$ were lower (Fig. 3a and b). The $\delta^{13}$C of CH$_4$ decreased until about 40% of the propionate had been consumed and then increased again to its initial (low) values ($-50$‰ to $-45$‰) (Fig. 3a and b).

Under sulfidogenic conditions, only very little CH$_4$ was produced. Similarly to that under methanogenic conditions, the $\delta^{13}$C of propionate (Fig. 1e) and of acetate (Fig. 1f) increased after the onset of propionate degradation, indicating that the light isotope was preferentially consumed. However,
the $\delta^{13}C$ values of CO$_2$ decreased during the first 10–15 d when acetate was accumulated (Figs. 1h and S1h). Inhibition of acetoclastic methanogenesis by CH$_3$F had no effect on the $\delta^{13}C$ of propionate and CO$_2$, but the values of acetate increased less than in the absence of CH$_3$F (Fig. 1f). Also, $\delta^{13}C$ of CH$_4$ was lower in the presence than in the absence of CH$_3$F (Fig. 1g), but the amounts of CH$_4$ produced were only very small (Fig. 1c). The values of $\delta^{13}C$ of propionate and acetate increased with increasing $f_{\text{prop}}$ (Fig. 3c and d). The $\delta^{13}C$ of acetate was generally higher by about 5–10‰ than the $\delta^{13}C$ of propionate but also increased with $f_{\text{prop}}$, indicating kinetic isotope fractionation. However, the $\delta^{13}C$ of

Figure 3. Change in $\delta^{13}C$ of propionate, acetate, butyrate, CO$_2$ and CH$_4$ relative to the fraction of propionate consumed ($f_{\text{prop}}$) under (a, b) methanogenic and (c, d) sulfidogenic conditions in paddy soil from (a, c) Vercelli (Italy) and (b, d) the IRRI (the Philippines). The different symbols indicate three different replicates.
Mariotti plots of propionate consumption under methanogenic and sulfidogenic \( (+CH_3F) \) conditions in paddy soil from (a) Vercelli and (b) the IRRI. The different symbols indicate three different replicates; the lines give the results of linear regression averaged over the replicates.

CO\(_2\) did not increase; instead, it decreased after the onset of propionate degradation, reaching about \(-35\%\) when 50\% of the propionate had been consumed and when acetate accumulation had reached a maximum (Fig. 3c and d). Thereafter, \( \delta^{13}C \) of CO\(_2\) increased or became constant.

Mariotti plots of the \( ^{13}C \) of propionate as a function of \( f_{\text{prop}} \) could be created for methanogenic and sulfidogenic incubation conditions, the latter being the case both in the absence and the presence of CH\(_3\)F (Fig. 4). The lines were straight even when more than 70\% of the propionate was consumed. Nevertheless, enrichment factors (\( \varepsilon \)) were determined only for \( f_{\text{prop}} < 0.7 \) and for regressions giving \( r^2 > 0.9 \). The \( \varepsilon_{\text{prop}} \) values were determined for each individual incubation and then averaged over the replicates (\( n = 2-3 \)). The results for Vercelli and IRRI soils are summarized in Fig. 5. The average \( \varepsilon_{\text{prop}} \) values under methanogenic conditions were about \(-8\%\) for Vercelli and about \(-3.5\%\) for IRRI soil. The average \( \varepsilon_{\text{prop}} \) values under sulfidogenic conditions were around \(-3.5\%\) in both soils, irrespective of whether CH\(_3\)F was present or not.

Figure 4. Mariotti plots of propionate consumption under methanogenic and sulfidogenic \( (+CH_3F) \) conditions in paddy soil from (a) Vercelli and (b) the IRRI. The different symbols indicate three different replicates; the lines give the results of linear regression averaged over the replicates.

Figure 5. Isotopic enrichment factors \( (\varepsilon_{\text{prop}}, \text{given as negative values}) \) in paddy soils without and with the addition of sulfate (gypsum) and CH\(_3\)F. Shown are the means \( \pm \) SE (standard error). The differences between the incubations were examined using Tukey’s post hoc test of a one-way analysis of variance (ANOVA). Different letters on top of the bars indicate significant difference \( (P < 0.05) \) between the data.

3.3 Hydrogenotrophic methanogenesis

The difference between the \( \delta^{13}C \) of CH\(_4\) in the presence and in the absence of CH\(_3\)F was used together with the \( \delta^{13}C \) of acetate to roughly estimate the percentage of CH\(_4\) derived from H\(_2/CO_2\) versus acetate (Fig. S3 in the Supplement). The percentage fractions of hydrogenotrophic methanogenesis \( (f_{\text{H}}) \) in Vercelli soil reached a maximum after 40-50 d when acetate concentrations also reached a maximum (Fig. S3a) and then decreased strongly. The same was the case in IRRI soil after around 35 d (Fig. S3b). When assuming a reasonable isotopic enrichment factor of \( \varepsilon_{CH_4,ac-methyl} = -15\%\), which is in between the \( \varepsilon_{CH_4,ac-methyl} \) of aceticlastic Methanosaeta (Penning et al., 2006; Valentine et al., 2004) and Methanosarcina species (Gelwicks et al., 1994; Goevert and Conrad, 2009), the average \( f_{\text{H}} \) values were 0\% for Vercelli soil and 20\% for IRRI soil (Fig. S3c).

4 Discussion

Pathway of propionate degradation

Our results showed that propionate was degraded via acetate as the main transient intermediate, finally resulting in the production of CH\(_4\) and CO\(_2\) under methanogenic conditions and CO\(_2\) under sulfidogenic conditions. These results are consistent with previous observations by Liu and Conrad (Liu and Conrad, 2017) using the same paddy soils. Stable isotope probing and correlation network analysis of the microbial communities have shown that propionate degradation is accomplished by both Syntrophophacter and Smitheilla species (Gan et al., 2012; Liu and Conrad, 2017; Lueders et al.,...
2004). The present study showed that propionate degradation under methanogenic conditions was consistent with the major operation of the Smithella pathway. The main argument for this conclusion is the observation that butyrate was a transient intermediate of propionate degradation, albeit at low concentrations (Figs. 1 and S1). In the Smithella pathway, butyrate is further fermented to acetate and H₂. However, production of H₂ is smaller in the Smithella pathway than in the Syntrophobacter pathway, while production of acetate is larger. Indeed, aceticlastic methanogenesis explained all the propionate-driven methanogenesis in the paddy soils (Figs. 2a and S2a). The average hydrogenotrophic methanogenesis, by contrast, contributed almost zero in Vercelli soil and only about 20 % in IRRI soil (Fig. S3c). The relatively larger contribution of aceticlastic compared to hydrogenotrophic methanogenesis to methanogenic propionate degradation supports the conclusion that the Smithella pathway was dominant over the Syntrophobacter pathway. Arguments against the Smithella pathway are that the accumulated CH₄ amounted to less than the expected 1.75 mole CH₄ per mole propionate consumed in Vercelli soil (Fig. 2a) and to even less in IRRI soil (Fig. S2a). With the inhibition of aceticlastic methanogenesis, acetate accumulation in Vercelli soil accounted for about 1 mole acetate per mole propionate, which is in a range that is compatible with propionate fermentation by either Smithella or Syntrophobacter (Fig. 2b). In IRRI soil, however, acetate accumulation accounted for less than 1 mole acetate per mole propionate (Fig. S2b). Note, however, that the accumulation of acetate reflects only that part of propionate fermentation, which was not inhibited by CH₃F. Our conclusion that propionate was degraded mainly by Smithella under methanogenic conditions is consistent with the microbial community structures in the paddy soils from Vercelli and the IRRI, which contain not only Syntrophobacter species but also Smithella together with Syntrophomonas, which is able to ferment butyrate (Liu and Conrad, 2017).

Under sulfidogenic conditions, propionate can be oxidized in different ways, either directly by sulfate reducers forming acetate and CO₂ or synthrophically, as under methanogenic conditions, but with subsequent oxidation of H₂ and acetate by sulfate reducers. Using the same paddy soils, Liu and their coworkers (Liu et al., 2018a; Liu and Conrad, 2017) recently showed that, under sulfidogenic conditions, propionate consumption was mainly achieved by Syntrophobacter spp., which first oxidized propionate to acetate and CO₂ and subsequently oxidized the accumulated acetate to CO₂. These were exactly the processes observed in the present study, where propionate degradation initially resulted in almost equimolar accumulation of acetate (Fig. 2d) according to

$$4 \text{ propionate} + 3 \text{ sulfate} + 3H^+ \rightarrow 3\text{HS}^-$$

$$+ 4 \text{ acetic} + 4\text{CO}_2 + 4\text{H}_2\text{O}. \quad (10)$$

It was interesting that CH₃F was not only a strong inhibitor of aceticlastic methanogenesis (which was expected) but also a relatively strong inhibitor of propionate fermentation, though this was only the case under methanogenic conditions and not under sulfidogenic conditions. Inhibition of propionate fermentation under methanogenic conditions has been observed before in three different paddy soils and has been interpreted as being due to the adverse thermodynamic conditions when acetate accumulates (Conrad et al., 2014). However, this interpretation cannot be true since the accumulation of acetate also occurred under sulfidogenic conditions, where CH₃F did not inhibit propionate degradation. In fact, it is mainly the accumulation of H₂ rather than acetate to which propionate degradation is thermodynamically sensitive. This is the reason why the Smithella pathway is less sensitive to thermodynamic inhibition than the Syntrophobacter pathway (Dolfing, 2013). However, CH₃F did not inhibit H₂ consumption by methanogens, as seen by the low $\delta^{13}C$ of CH₄ in the presence of CH₃F. Furthermore, the first step of the Smithella-type propionate fermentation does not produce any H₂, and therefore, propionate in the presence of CH₃F should at least be fermented to butyrate and acetate; however, this was not the case. Hence, the reason why CH₃F inhibited propionate fermentation under methanogenic conditions but not under sulfidogenic conditions remains unknown. Perhaps it is Smithella being more sensitive to CH₃F than Syntrophobacter.

**Fractionation during propionate degradation**

The isotopic fractionation of propionate apparently followed Raleigh distillation, which is characteristic for kinetic isotope fractionation in a closed system. The isotopic enrichment factor, which was determined from Mariotti plots, was in the range of $\epsilon_{\text{prop}} = -8 \%e$ to $-3.5 \%e$, which is less than the enrichment factor for methanogenic acetate consumption, which has been found to be $\epsilon_{\text{ac}} = -21 \%e$ to $-17 \%e$ (Conrad et al., 2021). The $\epsilon_{\text{prop}}$ values are on the same order as those predicted from $\delta^{13}C$ values of propionate, acetate and organic carbon measured in various methanogenic soils and sediments (Conrad et al., 2014). Propionate degradation resulted in the formation of $^{13}C$-enriched acetate and CO₂ and $^{13}C$-depleted butyrate and CH₄. The formation of $^{13}C$-depleted butyrate can be explained by the kinetic isotope effect with the preferential utilization of $^{13}C$-depleted propionate in the initial dismutation reaction by Smithella. However, the production of $^{13}C$-enriched acetate cannot be explained by a linear kinetic isotope effect. We assume that the dismutation of propionate is a branch point (Fry, 2003; Hayes, 2001) at which the carbon flow is split into the production of $^{13}C$-enriched acetate and $^{13}C$-depleted butyrate. At the branch point, the carbon isotope flow shows a preferential flow of $^{12}C$ into the product generated by the reaction with the larger fractionation factor, which would be butyrate. The further conversion of butyrate should produce
acetate that is depleted in $^{13}$C. This acetate together with the acetate produced from propionate dismutation should result in the $\delta^{13}$C-acetate that is observed. The total acetate pool initially had a $\delta^{13}$C that was up to 10‰ heavier than the $\delta^{13}$C of propionate. In the end, the $\delta^{13}$C values were about equal. The observation that acetate was $^{13}$C-enriched relative to propionate is consistent with $\delta^{13}$C data for various soils and sediments (Conrad et al., 2014) reporting that acetate is, on average, enriched by 6‰ relative to propionate. Acetate was further converted to CH$_4$ and to CO$_2$. In Vercelli soil, the $\delta^{13}$C of CH$_4$ was about 25–35‰ lighter than the $\delta^{13}$C of acetate. In IRRI soil, $^{13}$C depletion was even larger (30–40‰). In both soils, the isotopic enrichment factors for acetate consumption were in a range of $-12\%_{\text{e}}$ to $-17\%_{\text{e}}$ and, for CH$_4$ production from acetate, they were in a range of $-37\%_{\text{e}}$ to $-27\%_{\text{e}}$ (Conrad et al., 2021). Considering that a certain percentage (albeit small) of CH$_4$ was formed from CO$_2$ reduction by hydrogenotrophic methanogenesis, which displays relatively negative enrichment factors (see the $\delta^{13}$C of CH$_4$ in the presence of CH$_3$F – Fig. 1g), the observed difference in the $\delta^{13}$C of CH$_4$ versus acetate is reasonable. In Smithella fermentation, the only CO$_2$ production occurs during the fermentation of butyrate and the aceticlastic conversion of acetate. In both cases, CO$_2$ should be $^{13}$C-depleted relative to the substrates. Note that this was not the case. Unfortunately, the $^{13}$C contents of the individual C atoms of propionate, butyrate and acetate are not known. The $^{13}$C content in the different C positions might also affect the $\delta^{13}$C of CH$_4$ and CO$_2$, which is formed. It is also possible that, besides Smithella fermentation, the Syntrophobacter fermentation contributed to propionate degradation. In summary, the detailed process of isotope fractionation during the pathway of propionate degradation is unclear. However, the magnitude of the enrichment factors involved was relatively small, being on the order of $<10\%_{\text{e}}$.

Under sulfidogenic conditions, propionate was most probably degraded by Syntrophobacter spp., first to acetate, then finally to CO$_2$ (Liu et al., 2018a; Liu and Conrad, 2017). The carbon isotope fractionation of propionate consumption was, with an enrichment factor of $\epsilon_{\text{prop}} = -3.5\%_{\text{e}}$, comparatively small. Propionate was eventually converted to two carbon products, of which one was depleted (the CO$_2$) and the other was enriched (the acetate) in terms of $^{13}$C. In case of Syntrophobacter-type degradation, acetate and CO$_2$ are produced from the conversion of pyruvate, which is generated in the methylmalonyl-CoA pathway. In this pathway, CO$_2$ is first consumed by the conversion of propionyl-CoA to methylmalonyl-CoA and is then produced by the conversion of oxaloacetate to pyruvate. Pyruvate is finally converted to acetate and CO$_2$, which should both be $^{13}$C-depleted with respect to pyruvate (DeNiro and Epstein, 1977). However, both acetate and CO$_2$ were initially $^{13}$C-enriched relative to propionate (about 2–5‰) and then changed in opposite directions, with acetate becoming increasingly $^{13}$C-enriched and CO$_2$ becoming increasingly $^{13}$C-depleted until the time when acetate accumulation reached a maximum (Fig. 5). Then, $\delta^{13}$C of both acetate and CO$_2$ increased together with the increase in $^{13}$C of propionate (Fig. 5). The increase in $\delta^{13}$C of acetate is often explained by consumption, especially through aceticlastic methanogenesis (Heuer et al., 2010; Heuer et al., 2009). However, hardly any CH$_4$ was produced under sulfidogenic conditions, and the $^{13}$C enrichment occurred during the phase of acetate accumulation. Therefore, the enrichment likely happened during acetate production from propionate degradation. The increasing $^{13}$C depletion of CO$_2$ can also not be explained by consumption but only by the production from propionate. Hence, isotope fractionation during the conversion of propionate, particularly during the conversion of propionate to pyruvate, is unclear. We assume complications during the carboxylation and decarboxylation reactions. Unfortunately, we found hardly any literature data on the isotope fractionation of propionate fermentation. A co-culture of Syntrophobacter fumaroxidans with Methanobacterium formicicum exhibited marginal propionate fractionation with $\epsilon_{\text{prop}} = 0.9\%_{\text{e}}$ and the formation of acetate that was slightly $^{13}$C-enriched (about 5‰) (Botsch and Conrad, 2011), similarly to what is observed here. In summary, the mechanism of isotope fractionation during the conversion of propionate is not completely clear, but the magnitude of isotope fractionation is quite low.

### 5 Conclusions

Propionate degradation under sulfidogenic conditions was explained by the metabolism of Syntrophobacteraceae, which, in a first step, converted propionate to $^{13}$C-enriched acetate and $^{13}$C-depleted CO$_2$. By contrast, propionate degradation under methanogenic conditions was at least partially due to metabolism by Smithella, which, in a first step, converted propionate to $^{13}$C-enriched acetate and $^{13}$C-depleted butyrate. However, the isotopic enrichment factors ($\epsilon_{\text{prop}}$) of propionate consumption in two paddy soils were generally very low ($-8\%_{\text{e}}$ to $-3.5\%_{\text{e}}$) under both methanogenic and sulfidogenic conditions. This low range is consistent with literature values of $\delta^{13}$C collected for propionate, acetate and organic carbon in various soils and sediments (Conrad et al., 2014). Fractionation of propionate carbon actually seems to be smaller than fractionation of acetate, which is at least 2 times larger (Conrad et al., 2021). Hence, degradation of organic carbon via propionate to acetate and CO$_2$ apparently involves only a little isotope fractionation on the order of $<10\%_{\text{e}}$. By contrast, further degradation of acetate and CO$_2$ (+H$_2$) to CH$_4$ involves substantial isotope fractionation. This is also the case for chemolithotrophic acetate production (Conrad et al., 2014).

Data availability. The data are all contained in the figures and in the Supplement.

Supplement. The supplement related to this article is available online at: https://doi.org/10.5194/bg-20-3625-2023-supplement.

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