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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 few experiments on carbon isotope fractionation and the magnitude of the isotopic enrichment factors (ε) involved, we measured propionate conversion to acetate, CH_4 and CO_2 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 phosphate buffer (pH 7.0), both in the absence and presence of sulfate (gypsum), and of methyl fluoride (CH_3F), an inhibitor of aceticlastic methanogenesis. Under methanogenic conditions, propionate was eventually degraded to CH_4 with acetate being a transient intermediate. Butyrate was also a minor intermediate. Methane was mainly produced by aceticlastic methanogenesis. Propionate consumption was inhibited by CH_3F. Whereas butyrate and CH_4 were ^{13}C-depleted relative to propionate, acetate and CO_2 were ^{13}C-enriched. The isotopic enrichment factors (ε_{prop}) 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 CH_4 production was negligible. Application of CH_3F hardly affected propionate degradation and acetate accumulation. The initially produced CO_2 was ^{13}C-depleted, whereas the acetate was ^{13}C-enriched. The values of ε_{prop} were -3.5‰. It is concluded that degradation of organic carbon via propionate to acetate and CO_2 involves only 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 of the microbial community composition published previously for the same soils.

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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 milimolar 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; DeBok et al., 2005; Imachi et al., 2002; Plugge 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 (DeBok et al., 2001). 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:

$$4\ \text{propionate} + 8\ H_2O \rightarrow 4\ \text{acetate} + 4\ CO_2 + 12\ H_2 \quad (1)$$

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)):

$$2\ \text{butyrate} + 4\ H_2O \rightarrow 4\ \text{acetate} + 4\ H_2 \quad (3)$$

The *Smithella* pathway in total:

$$4\ \text{propionate} + 4\ H_2O \rightarrow 6\ \text{acetate} + 4\ H_2 \quad (4)$$

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*:

$$4\ \text{propionate} + 2\ H_2O \rightarrow 7\ CH_4 + 5\ CO_2, \text{ or} \quad (5)$$

$$4\ \text{propionate} + 7\ \text{sulfate} + 11\ H^+ \rightarrow 7\ HS^- + 12\ CO_2 + 12\ H_2O \quad (6)$$

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 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

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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 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 (ε) of the processes involved in both production and consumption of propionate are probably small. The direct determination of ε values in microbial cultures of one propionate-producing and one propionate-consuming bacterium also showed low values (Botsch and Conrad, 2011). However, direct determination of ε 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_3F) 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 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. Sampling and soil characteristics were described before (Liu et al., 2018b).

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_2O 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_2O at a ratio of 1:1, was amended with 0.07 g $CaSO_4.2H_2O$, and 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 3 replicates) for the following incubation experiments. Two different sets of incubations were prepared. In the first set (resulting in methanogenic conditions), 5 ml soil slurry preincubated without sulfate was incubated at 25°C with 40 ml 20 mM potassium phosphate buffer (pH 7.0) in a 150-ml bottle under an atmosphere of N_2. The bottles were the amended with (i) 5 ml H_2O; (ii) 5 ml H_2O + 4.5 ml CH_3F; (iii) 5 ml 50 mM sodium propionate; (iv) 5 ml 50 mM sodium acetate + 4.5 ml CH_3F. In the second set (resulting in sulfidogenic conditions), 5 ml soil slurry preincubated with sulfate was incubated at 25°C with 40 ml 20 mM potassium phosphate buffer (pH 7.0) in a 150-ml bottle under an atmosphere of N_2. The amendments were the same as above, but with the addition of 200 µl of a $CaSO_4$ suspension corresponding to a concentration of 2.5 M (giving a final concentration of 10 mM sulfate).

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2.2 Chemical and isotopic analyses

Chemical and isotopic analyses were performed as described in detail previously (Goevert and Conrad, 2009). Methane was analyzed by gas chromatography (GC) with flame ionization detector. Carbon dioxide was analyzed after conversion to CH_4 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 having a $^{13}C/^{12}C$ ratio ($R_{standard}$) of 0.01118: $\delta^{13}C = 10^3 (R_{sample}/R_{standard} - 1)$. The precision of the GC-C-IRMS was ± 0.2‰, that of the HPLC-IRMS was ± 0.3‰.

2.3 Calculations

Milimolar concentrations of CH_4 were calculated from the mixing ratios (1 ppmv = 10^{-6} bar) measured in the gas phase of the incubation bottles: 1000 ppmv CH_4 correspond to 0.09 µmol per ml of liquid. Note, that this is the total amount of CH_4 in the gas phase relative to the liquid phase.

Fractionation factors for reaction A → B are defined after Hayes (Hayes, 1993) as:

$$\alpha_{A/B} = (\delta_A + 1000)/ (\delta_B + 1000) \quad (7)$$

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

$$\delta_r = \delta_{ri} + \varepsilon [\ln(1-f)] \quad (8)$$

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

The fraction (f_{H2}) of CH_4 derived from hydrogenotrophic methanogenesis was determined as described before (Conrad et al., 2010) using

$$f_{H2} = (\delta^{13}C_{CH4} - \delta^{13}C_{CH4\text{-}ma})/(\delta^{13}C_{CH4\text{-}mc} - \delta^{13}C_{CH4\text{-}ma}) \quad (9)$$

with $\delta^{13}C_{CH4}$ = $\delta^{13}C$ of total CH_4 produced, $\delta^{13}C_{CH4\text{-}mc}$ = $\delta^{13}C$ of CH_4 produced from hydrogenotrophic methanogenesis, which is equivalent to the CH_4 produced in the presence of CH_3F, and $\delta^{13}C_{CH4\text{-}ma}$ = $\delta^{13}C$ of CH_4 produced from aceticlastic methanogenesis. The $\delta^{13}C_{CH4\text{-}ma}$ was approximated from the $\delta^{13}C$ of acetate in the presence of CH_3F assuming that the methyl group of acetate was depleted in ^{13}C by 8‰ (Conrad et al., 2014) and that the enrichment factor ($\varepsilon_{CH4,ac\text{-}methyl}$) for CH_4 being produced from acetate-methyl was between 0 and -20‰.

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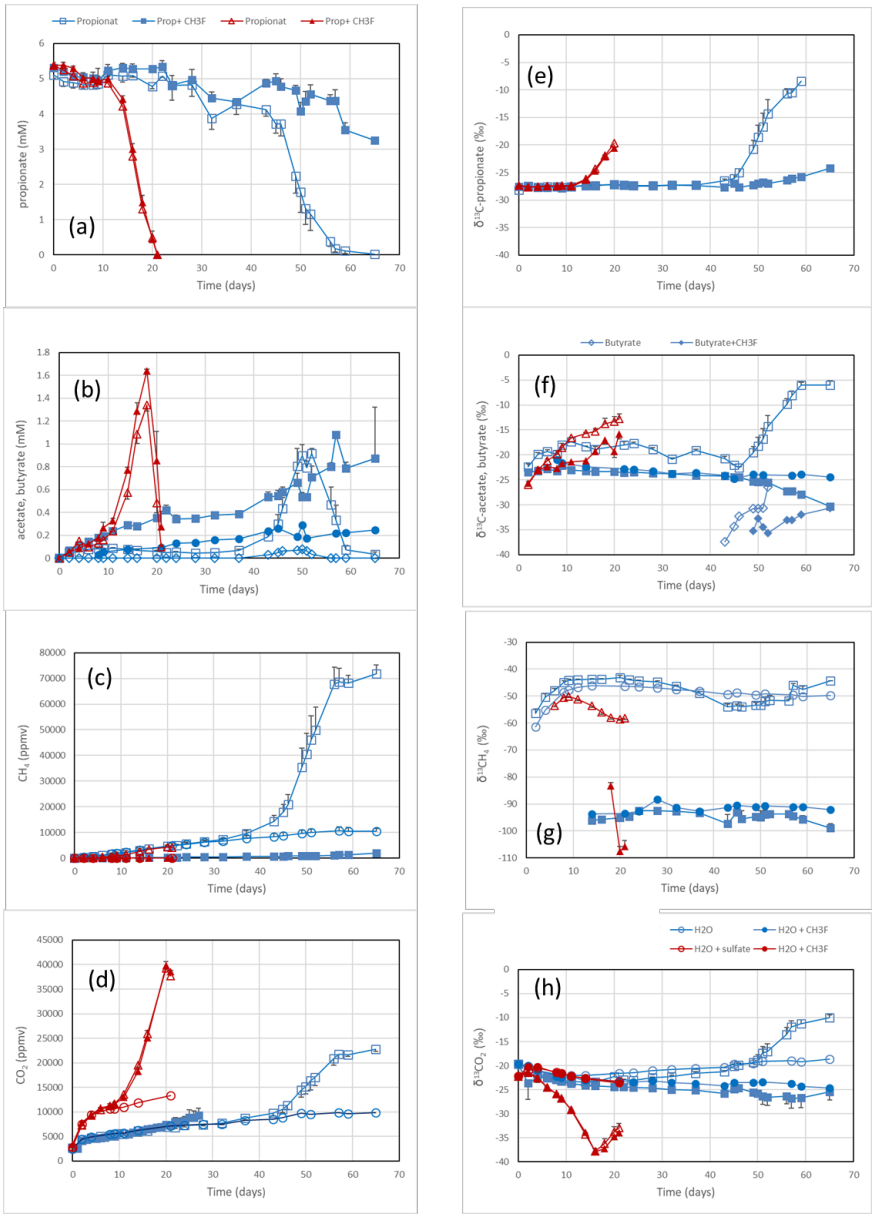

Figure 1: Propionate conversion to acetate, butyrate, CH_4 and CO_2 in suspensions of paddy soil from Vercelli (Italy) after addition of propionate without sulfate (blue squares) or propionate plus sulfate (gypsum) (red triangles) without CH_3F (open symbols) or with CH_3F (closed symbols). Controls with addition of only water (blue or red circles) are only shown occasionally. The panels show the temporal change of (a) concentrations of propionate, (b) concentrations of acetate and butyrate (blue diamonds), (c) mixing ratios of CH_4 (1 ppmv = 10^{-6} bar), (d) mixing ratios of CO_2, (e) $\delta^{13}C$ of propionate, (f) $\delta^{13}C$ of acetate and butyrate, (g) $\delta^{13}C$ of CH_4, and (h) $\delta^{13}C$ of CO_2. Means ± SE.

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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) resulted in similar patterns of propionate degradation to acetate, CH_4 and CO_2. 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), CH_4 (Fig. 1c) and CO_2 (Fig. 1d). The formation of acetate, CH_4 and CO_2 in the absence of propionate was only very small. The accumulation of acetate was only transient, except when aceticlastic methanogenesis was inhibited by CH_3F (Fig. 1b). Similar observations were made in IRRI soil (Fig. S1a-d). The production of CH_4 was roughly equimolar to the consumption of propionate, but was nearly zero when aceticlastic methanogenesis was inhibited by CH_3F (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). Butyrate was also a transient intermediate of propionate degradation and was produced and consumed simultaneously with acetate (Fig. 1b, S1b). However, the accumulated concentrations were small (<0.1 mM).

In the presence of sulfate, propionate degradation started after a lag phase of only about 10 days (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). Addition of CH_3F had no effect. Butyrate was not detected. The accumulated acetate was subsequently degraded resulting in further production of CO_2 (Fig. 1b,d).

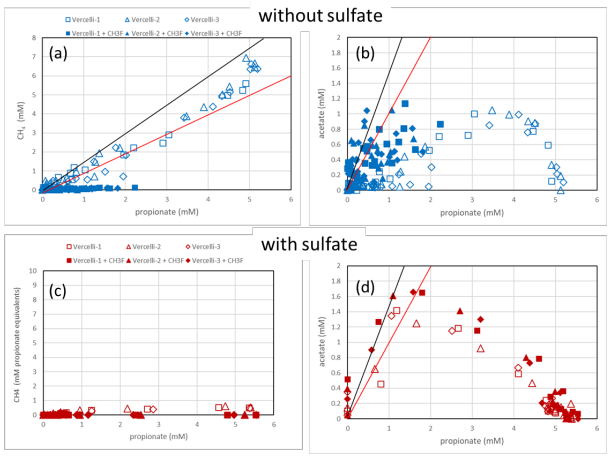

Figure 2: Balance of (a, c) produced CH_4 and (b, d) produced acetate against the consumed propionate under (a, b) methanogenic and (c, d) sulfidogenic conditions in paddy soil from Vercelli (Italy). The open and closed symbols denote conditions in the absence and the presence of CH_3F, respectively. The black and red lines in panel (a) indicate aceticlastic methanogenesis by *Smithella* and *Syntrophobacter*, respectively. The black and red lines in panel (b and d) indicate transient acetate production by *Smithella* and *Syntrophobacter*, respectively. The different symbols indicate three different replicates.

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3.2 Isotope fractionation during propionate degradation

After 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_3F, the $\delta^{13}C$ values of these compounds increased only slightly or decreased (Fig. 1e,f,h). However, the $\delta^{13}C$ of CH_4 was much more negative (30-50‰) in the presence than in the absence of CH_3F (Fig. 1g). The $\delta^{13}C$ values of CH_4 in unamended soil (H_2O control) were similar to those in propionate amended soil (Fig. 1g). To visualize the change of the metabolic ^{13}C content of the metabolic products relative to the substrates, the $\delta^{13}C$ values were plotted against the increasing fractions (f_{prop}) of propionate consumed both in soil from Vercelli (Fig.3a) and the IRRI (Fig.3b). The patterns of $\delta^{13}C$ values against the f_{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,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,b).

Under sulfidogenic conditions, only very little CH_4 was produced. Similarly as under methanogenic conditions, the $\delta^{13}C$ of propionate (Fig. 1e) and of acetate (Fig. 1f) increased after 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 days when acetate was accumulated (Fig. 1h, S1h). Inhibition of aceticlastic methanogenesis by CH_3F 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_3F (Fig. 1f). Also, $\delta^{13}C$ of CH_4 was lower in the presence than in the absence of CH_3F (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_{prop} (Fig. 3c,d). The $\delta^{13}C$ of acetate was generally by about 5-10‰ higher than the $\delta^{13}C$ of propionate but also increased with f_{prop} indicating kinetic isotope fractionation. However, the $\delta^{13}C$ of CO_2 did not increase, but instead decreased after onset of propionate degradation reaching about -35‰ when 50% of the propionate had been consumed and acetate accumulation had reached a maximum (Fig. 3c,d). Thereafter, $\delta^{13}C$ of CO_2 increased or became constant.

Mariotti plots of the ^{13}C of propionate as function of f_{prop} could be created for methanogenic and sulfidogenic incubation conditions, the latter both in the absence and the presence of CH_3F (Fig. 4). The lines were straight even when more than 70% of the propionate was consumed. Nevertheless, enrichment factors (ε) were determined only for $f_{prop} < 0.7$ and for regressions giving $r^2 > 0.9$. The ε_{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 ε_{prop} values under methanogenic conditions were about -8‰ for Vercelli and about -3.5‰ for IRRI soil. The average ε_{prop} values under sulfidogenic conditions were around -3.5‰ in both soils and irrespectively whether CH_3F was present or not.

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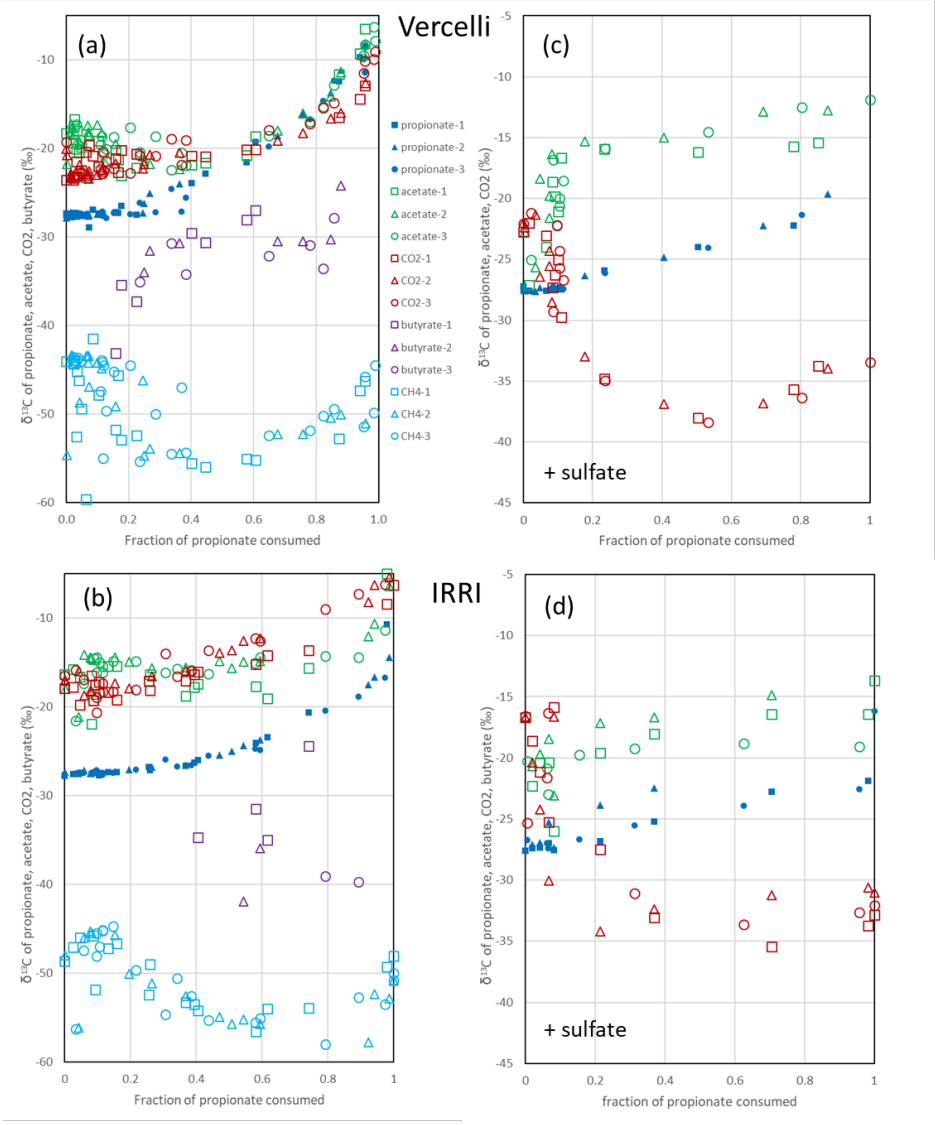

Figure 3: Change of $\delta^{13}C$ of propionate, acetate, butyrate, CO_2 and CH_4 relative to the fraction of propionate consumed (f_{prop}) under (a, b) methanogenic an (c, d) sulfidogenic conditions in paddy soil from (a, c) Vercelli (Italy) and (b, d) the IRRI (the Philippines). The different symbols indicate three different replicates.

3.3 Hydrogenotrophic methanogenesis

The difference in the $\delta^{13}C$ of CH_4 in the presence and the absence of CH_3F 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). The percentage fractions of hydrogenotrophic methanogenesis (f_{H2}) 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 $\varepsilon_{CH4,ac\text{-}methyl}$ = -15‰,

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which is in-between the $\varepsilon_{CH4,ac\text{-}methyl}$ 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_{H2} values were 0% for Vercelli soil and 20% for IRRI soil (Fig. S3c).

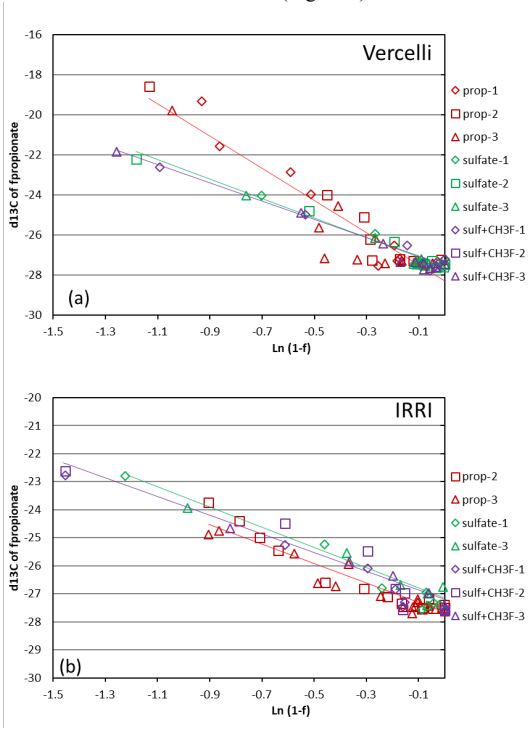

Figure 4: Mariotti plots of propionate consumption under methanogenic and sulfidogenic (± CH_3F) conditions in paddy soil from (a) Vercelli and (b) the IRRI. The different symbols indicate three different replicates; the lines give the results of linear regression averaged over the replicates.

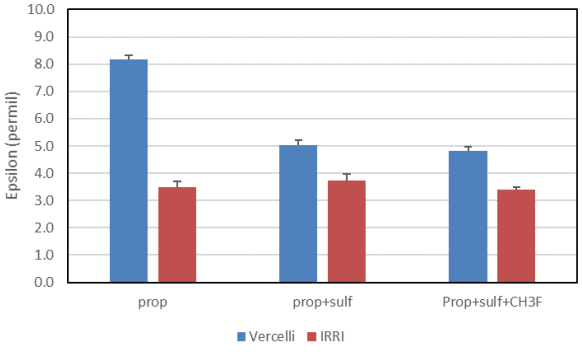

Figure 5: Isotopic enrichment factors (ε_{prop}, given as negative values) in paddy soils without and with addition of sulfate (gypsum) and CH_3F. Means ± SE.

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4 Discussion

Pathway of propionate degradation

Our results showed that propionate was degraded via acetate as main transient intermediate finally resulting in the production of CH_4 and CO_2 under methanogenic 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 *Syntrophopbacter* and *Smithella* 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 (Fig. 1, S1). In the *Smithella* pathway butyrate is further fermented to acetate and H_2. However, production of H_2 is smaller in the *Smithella* than in the *Syntrophobacter* pathway, while production of acetate is larger. Indeed, aceticlastic methanogenesis explained all the propionate-driven methanogenesis in the paddy soils (Fig. 2a, 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 than hydrogenotrophic methanogenesis to methanogenic propionate degradation supports the conclusion that *Smithella* pathway was dominating over the *Syntrophobacter* pathway. Arguments against the *Smithella* pathway are that the accumulated CH_4 amounted to less than the expected 1.75 mole per mole propionate consumed in Vercelli soil (Fig. 2a) and even less in IRRI soil (Fig. S2a). With inhibition of aceticlastic methanogenesis, acetate accumulation in Vercelli soil accounted for about 1 mole acetate per mole propionate, being 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 CH_3F. Our conclusion that propionate was degraded mainly by *Smithella* under methanogenic conditions is consistent with the microbial community structure in the paddy soils from Vercelli and IRRI, which contains 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 CO_2, or syntrophically as under methanogenic conditions, but with subsequent oxidation of H_2 and acetate by sulfate reducers. Using the same paddy soils, Liu and 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 CO_2, and subsequently oxidized the accumulated acetate to CO_2. 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

$$4\ \text{propionate} + 3\ \text{sulfate} + 3\ H^+ \rightarrow 3\ HS^- + 4\ \text{acetate} + 4\ CO_2 + 4\ H_2O \quad (10)$$

It was interesting, that CH_3F was not only a strong inhibitor of aceticlastic methanogenesis (which was expected), but also a relatively strong inhibitor of propionate fermentation, but only under methanogenic but 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

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conditions when acetate accumulates (Conrad et al., 2014). However, this interpretation cannot be true, since accumulation of acetate also occurred under sulfidogenic conditions, where CH_3F did not inhibit propionate degradation. In fact it is mainly the accumulation of H_2 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, CH_3F did not inhibit H_2 consumption by methanogens, as seen by the low $\delta^{13}C$ of CH_4 in the presence of CH_3F. Furthermore, the first step of the *Smithella*-type propionate fermentation does not produce any H_2 and therefore, propionate should in the presence of CH_3F at least be fermented to butyrate and acetate, which however, was not the case. Hence, the reason why CH_3F inhibited propionate fermentation under methanogenic but not under sulfidogenic conditions remains unknown. Perhaps it is *Smithella* being more sensitive to CH_3F than *Syntrophobacter.*

Fractionation during propionate degradation

The isotopic fractionation of propionate apparently followed Raleigh distillation that 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 ε_{prop} = -8‰ to -3.5‰, which is less than the enrichment factor for methanogenic acetate consumption, which has been found to be ε_{ac} = -21‰ to -17‰ (Conrad et al., 2021). The ε_{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 CO_2 and ^{13}C-depleted butyrate and CH_4. The formation of ^{13}C-depleted butyrate can be explained by 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 in various soils and sediments (Conrad et al., 2014) reporting that acetate is on the 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‰ to -17‰ and for CH_4 production from acetate in a range of -37‰ to -27‰ (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_3F, Fig. 1g), the observed difference in $\delta^{13}C$ 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

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different C positions might also affect the $\delta^{13}C$ of CH_4 and CO_2, which are 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‰.

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 ε_{prop} = -3.5‰ 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 ^{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 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 had reached a maximum (Fig. 5). Then, $\delta^{13}C$ of both acetate and CO_2 increased together with the increase of ^{13}C of propionate (Fig. 5). Increase of $\delta^{13}C$ 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, in particular during the conversion of propionate to pyruvate is unclear. We assume complications during the carboxylation and decarboxylation reactions. Unfortunately, we hardly found any literature data on the isotope fractionation of propionate fermentation. A coculture of *Syntrophobacter fumaroxidans* with *Methanobacterium formicicum* exhibited marginal propionate fractionation with ε_{prop} = 0.9‰ and the formation of acetate, that was slightly ^{13}C-enriched (about 5‰) (Botsch and Conrad, 2011), similarly as 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 (ε_{prop}) of propionate consumption in two paddy soils were generally very low (-8‰ to -3.5‰) both under 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 two times larger (Conrad et al., 2021). Hence, degradation of organic carbon via propionate to acetate and CO_2 apparently involves

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only little isotope fractionation being on the order of <10‰. 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).

Supplement link

Author contribution: RC designed the experiments, evaluated the data and wrote the manuscript, PC conducted the experiments.

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