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# Fractionation of stable carbon isotopes during formate consumption in anoxic rice paddy soils and lake sediments

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**Abstract.** Formate is energetically equivalent to hydrogen and thus is an important intermediate during the breakdown of organic matter in anoxic rice paddy soils and lake sediments. Formate is a common substrate for methanogenesis, homoacetogenesis and sulfate reduction. However, how much these processes contribute to formate degradation and fractionate carbon stable isotopes is largely unknown. Therefore, we measured the conversion of formate to acetate, $CH_4$ and $CO_2$ and the $\delta^{13}C$ of these compounds in samples of paddy soils from Vercelli, Italy, and the International Rice Research Institute (IRRI) in the Philippines and of sediments from the NE and SW basins of Lake Fuchskuhle, Germany. The samples were suspended in a phosphate buffer (pH 7.0) in both the absence and presence of sulfate (gypsum) and of methyl fluoride ($CH_3F$), an inhibitor of aceticlastic methanogenesis. In the paddy soils, formate was mainly converted to acetate under both methanogenic and sulfidogenic conditions. Methane was only a minor product and was mainly formed from the acetate. In the lake sediments, the product spectrum was similar but only under methanogenic conditions. In the presence of sulfate, however, acetate and $CH_4$ were only minor products. The isotopic enrichment factors ($\varepsilon_{form}$) of formate consumption, determined by Mariotti plots, were in the low range of $-8\,\permil$ to $-2.5\,\permil$ when sulfate was absent, and formate was mainly converted to acetate and $CH_4$. However, no enrichment factor was detectable when formate was degraded with sulfate to mainly $CO_2$. The $\delta^{13}C$ of acetate was by about $25\,\permil$–$50\,\permil$ more negative than that of formate, indicating acetate production by chemolithotrophic homoacetogenesis. Hence, formate seems to be an excellent substrate for homoacetogenesis in anoxic soils and sediments, so that this process is competing well with methanogenesis and sulfate reduction.

## 1 Introduction

Formate is energetically almost equivalent to $H_2$ (Schink et al., 2017) and thus is an important intermediate in the anaerobic degradation of organic matter. Formate is a product of microbial fermentation, where it is produced in pyruvate cleavage by pyruvate formate lyase (Thauer et al., 1977) or by reduction in $CO_2$ (Schuchmann and Müller, 2013). Formate can also be produced in secondary fermentation, such as oxidation of butyrate or propionate (Dong et al., 1994; Sieber et al., 2014). In fact, formate and $H_2$ may be used equivalently as electron shuttles between secondary fermenting bacteria and methanogens (Montag and Schink, 2018; Schink et al., 2017).

Formate can serve alternatively to $H_2$ as a substrate for methanogenesis (Zinder, 1993) (homo)acetogenesis (Drake, 1994) or sulfate reduction (Widdel, 1988), i.e.,

$$4HCOOH \rightarrow CH_4 + 3CO_2 + 2H_2O, \tag{R1}$$

$$4HCOOH \rightarrow CH_3COOH + 2CO_2 + 2H_2O, \tag{R2}$$

$$4HCOOH + SO_4^{2-} + H^+ \rightarrow HS^- + 4CO_2 + 4H_2O. \tag{R3}$$
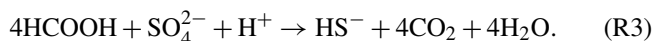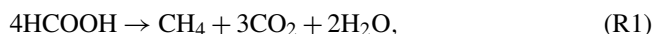
Formate may also be a substrate for syntrophic bacteria, which live from the little Gibbs free energy ($\Delta G^{0\prime} = -3.4\,kJ\,mol^{-1}$) that is generated by the conversion of formate to $H_2$ plus $CO_2$ (Dolfing et al., 2008; Kim et al., 2010; Martins et al., 2015), i.e.,

$$HCOOH \rightarrow CO_2 + H_2. \tag{R4}$$

Formate can also be enzymatically equilibrated with $H_2$ and $CO_2$ without energy generation. This reaction happens in any organism possessing the suitable enzymes, such as formate hydrogen lyase or hydrogen-dependent carbon dioxide reductase, and in anoxic sediments (DeGraaf and Cappenberg,

1996; Peters et al., 1999; Schuchmann et al., 2018):

$$HCOOH \leftrightarrow CO_2 + H_2. \tag{R5}$$

Formate has been identified as an important substrate for methanogenesis, homoacetogenesis or sulfate reduction in lake sediments (DeGraaf and Cappenberg, 1996; Lovley and Klug, 1982; Phelps and Zeikus, 1985), soils (Kotsyurbenko et al., 1996; Küsel and Drake, 1999; Rothfuss and Conrad, 1993), mires (Hausmann et al., 2016; Hunger et al., 2011; Liebner et al., 2012; Wüst et al., 2009) and marine sediments (Glombitza et al., 2015). However, it is not very clear to which extent formate-dependent methanogenesis, homoacetogenesis and sulfate reduction are actually operative and to which extent formate affects stable carbon isotope fractionation. The $\delta^{13}C$ values of compounds involved in the degradation process of organic matter provide valuable information on the metabolic pathways involved (Conrad, 2005; Elsner et al., 2005; Hayes, 1993). However, for correct interpretation, the knowledge of the enrichment factors ($\varepsilon$) of the major metabolic processes is also important. The $\varepsilon$ values of methanogenesis or homoacetogenesis from $H_2$ plus $CO_2$ are large (Blaser and Conrad, 2016). However, our knowledge of carbon isotope fractionation with formate as a substrate is scarce. In cultures of homoacetogenic bacteria, the carbon in the acetate produced from formate was strongly depleted in $^{13}C$ ($\varepsilon = -56.5\permil$), almost on the same level as with $CO_2$ as carbon source (Freude and Blaser, 2016). However, it is not known which enrichment factors operate in methanogenic or sulfidogenic environmental samples.

Therefore, we measured isotope fractionation in methanogenic and sulfidogenic rice paddy soils and in lake sediments amended with formate. We recorded the consumption of formate along with the production of acetate, $CH_4$ and $CO_2$ and measured the $\delta^{13}C$ of these compounds. We also used the treatment with methyl fluoride ($CH_3F$) to inhibit the consumption of acetate by methanogenic archaea (Janssen and Frenzel, 1997). We used the same environmental samples as we did for the study of carbon isotope fractionation during consumption of acetate (Conrad et al., 2021) and propionate (Conrad and Claus, 2023), i.e., rice paddy soils from Vercelli, Italy, and the International Rice Research Institute (IRRI) in the Philippines and sediments from the NE and SW basins of Lake Fuchskuhle, Germany. The molecular data characterizing the microbial community compositions in these samples are found in Conrad et al. (2021).

## 2 Materials and methods

### 2.1 Environmental samples 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 de-

scribed before (Liu et al., 2018). The lake sediments (top 10 cm layer) were from the NE and SW basins of Lake Fuchskuhle, an acidic bog (pH 4.2–4.6) lake in northeastern Germany (Casper et al., 2003). The lake was artificially divided into four compartments in an ongoing process from 1987 to 1991, resulting in four nearly equal-sized compartments, each with a different catchment area. The NE basin is characterized by higher biomass and activity throughout all trophic levels in the water column than those of the SW basin. The lake sediments were sampled in July 2016 using a gravity core sampler as described before (Kanaparthi et al., 2013). The experiments with rice field soil were carried out in 2016, while the experiments with sediments of Lake Fuchskuhle were carried out in 2017.

The experimental setup was exactly the same as during previous studies of acetate consumption (Conrad et al., 2021) and propionate consumption (Conrad and Claus, 2023). For methanogenic conditions, paddy soil was mixed with autoclaved anoxic $H_2O$ (prepared under $N_2$) at a ratio of 1 : 1 and incubated under $N_2$ at 25 °C for 4 weeks. In a second incubation, for sulfidogenic conditions, paddy soil was mixed with autoclaved anoxic $H_2O$ at a ratio of 1 : 1, was amended with 0.07 g $CaSO_4 \cdot 2H_2O$, 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 amended with (i) 5 mL $H_2O$, (ii) 5 mL $H_2O$ + 4.5 mL $CH_3F$, (iii) 5 mL 200 mM sodium formate, and (iv) 5 mL 200 mM sodium formate + 4.5 mL $CH_3F$. 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 $N_2$. 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).

For lake sediments under methanogenic conditions, 5 mL sediment was incubated in three replicates at 10 °C (which is close to the in situ temperature) 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 amended with (i) 5 mL $H_2O$, (ii) 5 mL $H_2O$ + 4.5 mL $CH_3F$, (iii) 5 mL 200 mM sodium formate, and (iv) 5 mL 200 mM sodium formate + 4.5 mL $CH_3F$. For sulfidogenic conditions, lake sediments were preincubated with sulfate by adding 0.1 g $CaSO_4 \cdot 2H_2O$ (gypsum) to 50 mL of sediment and incubating at 10 °C for 4 weeks. For sulfidogenic conditions, 5 mL of the preincubated sediment was incubated in three

replicates at 10 °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 amended as above but also with 200 μL of a $CaSO_4$ suspension, giving a final concentration of 10 mM sulfate. Samples for later molecular analysis were taken from the original lake sediment and from the lake sediment preincubated with sulfate. The samples were stored at −20 °C (see data in Conrad et al., 2021).

## 2.2 Chemical and isotopic analyses

Gas samples for the analysis of partial pressures of $CH_4$ and $CO_2$ 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 $N_2$ before each sampling. Soil slurries were sampled, centrifuged and filtered through a 0.2 μm cellulose membrane filter and stored frozen at −20 °C for later fatty acid analysis. Chemical and isotopic analyses were performed as described in detail previously (Goevert and Conrad, 2009). Methane was analyzed by gas chromatography (GC) with a 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). Formate 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‰, and that of the HPLC-IRMS was ±0.3‰.

## 2.3 Calculations

Millimolar concentrations of $CH_4$ were calculated from the mixing ratios (1 ppmv $= 10^{-6}$ bar) measured in the gas phase of the incubation bottles, with 1000 ppmv $CH_4$ corresponding to 0.09 μmol per milliliter 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 $A \rightarrow B$ are defined after Hayes (1993) as

$$\alpha_{A/B} = (\delta_A + 1000)/(\delta_B + 1000), \tag{1}$$

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

$$\delta_r = \delta_{ri} + \varepsilon[\ln(1 - f)], \tag{2}$$

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

# 3 Results

## 3.1 Conversion of formate under methanogenic and sulfidogenic conditions

The rice paddy soils were submerged and preincubated to create methanogenic or sulfidogenic conditions. Samples of these soils were suspended in a buffer at pH 7 and amended with formate. In the Vercelli soil, formate was consumed after a lag phase of 4 d under methanogenic and 5 d under sulfidogenic conditions (Fig. 1a). During this time the pH increased from pH 7 up to pH 8 despite buffering. Formate consumption was not inhibited by $CH_3F$ (Fig. 1a). Similar results were obtained with IRRI soil (Fig. S1 in the Supplement). Acetate was produced concomitantly with formate consumption, again without effect by $CH_3F$ (Fig. 1b). The production of acetate under sulfidogenic conditions was smaller than under methanogenic conditions. Methane was also produced under both methanogenic and sulfidogenic conditions concomitantly with formate consumption (Figs. 1c; S1c). It is noteworthy that $CH_3F$ inhibited the production of $CH_4$ (Figs. 1c; S1c). Finally, $CO_2$ was produced under all conditions without lag phase and without effect by $CH_3F$ (Fig. 1d). In Vercelli soil, $CO_2$ production was about 2 times larger under sulfidogenic conditions than under methanogenic conditions (Fig. 1d). In IRRI soil, it was only slightly larger (Fig. S1d). The accumulation of acetate plus $CH_4$ was equimolar to the consumption of formate in terms of electron equivalents, while the accumulation of $CH_4$ alone accounted for only < 30 % and in the presence of $CH_3F$ even less (Figs. 2a; S2a). Hence, acetate was the more important product of formate consumption. Under sulfidogenic conditions, accumulation of acetate plus $CH_4$ was less than equimolar, especially in Vercelli soil (Fig. 2b), probably since formate was instead converted to $CO_2$. However, acetate formation was still substantial, accounting for 60 %–80 % of formate consumption (Figs. 2b; S2b).

The sediments from Lake Fuchskuhle were methanogenic in situ so that preincubation of the samples was not required. However, sulfidogenic conditions were created analogously to the paddy soils by preincubtion with sulfate (gypsum). Substantial formate depletion did not start before about 20 d of incubation in sediments from the NE basin (Fig. 3) and the
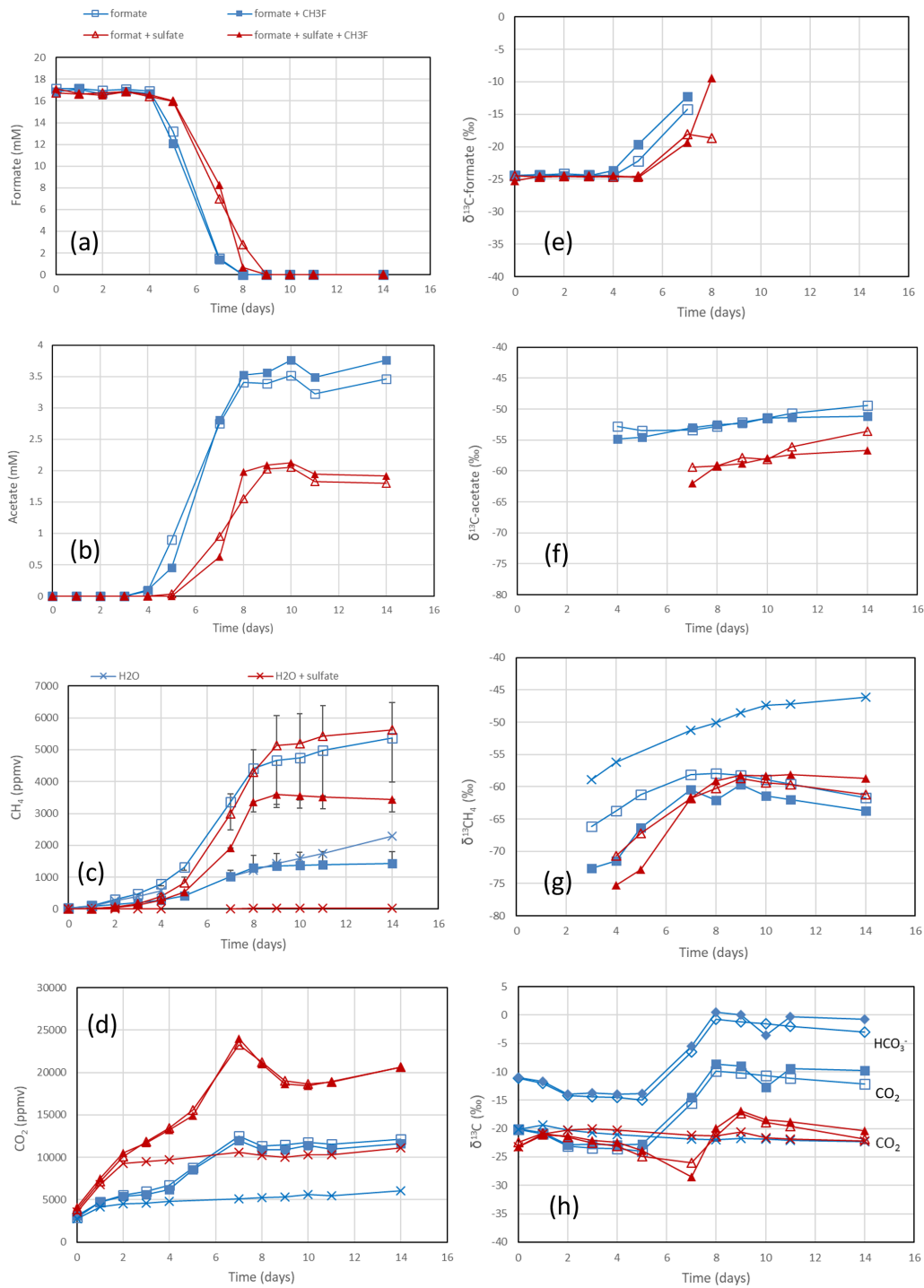
**Figure 1.** Formate conversion to acetate, $CH_4$ and $CO_2$ in suspensions of paddy soil from Vercelli, Italy, after the addition of formate without sulfate (blue squares) or formate plus sulfate (gypsum) (red triangles), without $CH_3F$ (open symbols) or with $CH_3F$ (closed symbols). Controls with the addition of only water (blue or red × crosses) are only shown occasionally. The panels show the temporal change in **(a)** concentrations of formate, **(b)** concentrations of acetate, **(c)** mixing ratios of $CH_4$ (1 ppmv $= 10^{-6}$ bar), **(d)** mixing ratios of $CO_2$, **(e)** $\delta^{13}C$ of formate, **(f)** $\delta^{13}C$ of acetate, **(g)** $\delta^{13}C$ of $CH_4$ and **(h)** $\delta^{13}C$ of $CO_2$. Means $\pm$ SE.
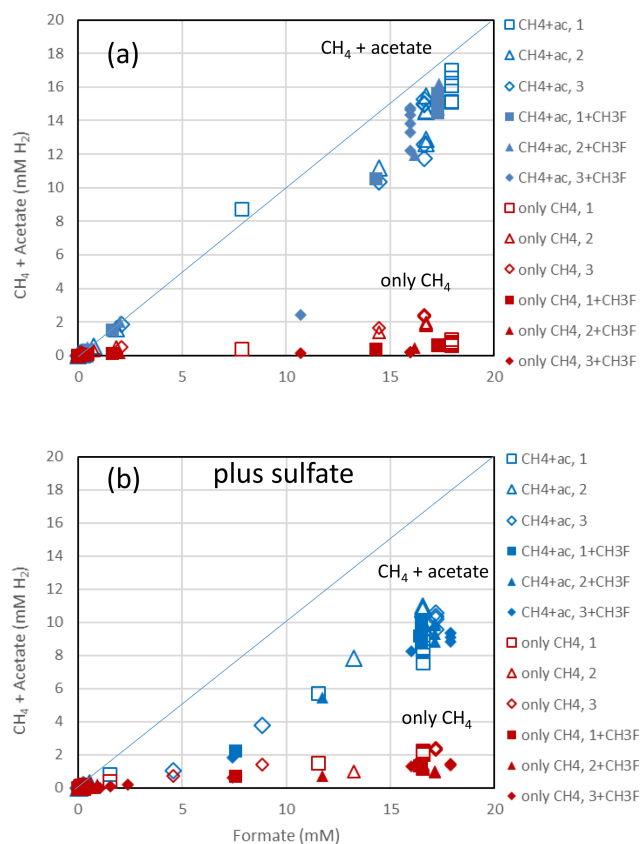
**Figure 2.** Balance of produced acetate plus $CH_4$ (blue symbols) and of only $CH_4$ (red symbols) against the consumed formate in **(a)** the absence and **(b)** the presence of sulfate in paddy soil from Vercelli, Italy. Acetate and $CH_4$ are each equivalent to $4 H_2$, while formate is equivalent to $1 H_2$. The open and closed symbols denote conditions in the absence and the presence of $CH_3F$, respectively. The different symbols indicate three different replicates. The lines indicate equimolarity (in terms of reducing equivalents between substrate and product).

SW basin (Fig. S3). Again, $CH_3F$ only inhibited the production of $CH_4$ but not that of acetate or $CO_2$ (Figs. 3; S3). The main difference to the paddy soils was that $CH_4$ was not produced concomitantly with formate consumption but started right from the beginning. However, the amounts of $CH_4$ produced were only small and were apparently due to the little formate that was consumed in the beginning of incubation (i.e., before day 20), as seen by the fact that $CH_4$ production in the water control (not amended with formate) was negligible (Figs. 3c; S3c). Production of $CO_2$ started without lag phase but accelerated together with formate consumption (Figs. 3d; S3d). In the lake sediments, $CH_4$ accounted only for $< 10\%$ of formate consumption, while acetate was the main product when sulfate was absent (Figs. 4a, S4a). In contrast to the paddy soils, formate consumption in both lake sediments was much slower under sulfidogenic conditions than under methanogenic conditions (Figs. 3a; S3a). In

the sediment from SW basin, formate consumption was very slow; therefore, less than half of the formate was consumed during 80 d of incubation and consumption was not completed until the end of the experiment (Fig. S3a). Very little acetate was produced and no $CH_4$ was formed from formate in both lake sediments, when sulfate was present (Figs. 4b, S4b).

## 3.2 Isotope fractionation during formate consumption

In the rice paddy soils, $\delta^{13}C$ values of formate increased when formate was consumed, indicating discrimination against the heavy carbon isotope. This process was not affected by $CH_3F$ and was similar without and with sulfate (Figs. 1e; S1e). The same was the case with the sediment from the NE lake basin but only in the absence of sulfate (Fig. 3e). With sulfate, the $\delta^{13}C$ of formate slowly decreased with time (Fig. 3e). In the sediment from the SW basin, $\delta^{13}C$ of formate slowly decreased (without sulfate) or stayed constant with time (with sulfate) (Fig. S3e). Note that formate was not completely consumed in the SW sediment when sulfate was present (Fig. S3a).

Mariotti plots of $\delta^{13}C$ of formate as a function of $f_{form}$ resulted in negative slopes (Figs. 4; S5). Hence, the enrichment factors ($\varepsilon_{form}$) for the paddy soils, both without and with sulfate, and for the sediments from the NE basin of Lake Fuchskuhle without sulfate showed that the light isotope of formate carbon was preferred. Values of $\varepsilon_{form}$ were in the range of $-8.5\%o$ to $-2.5\%o$ (Fig. 6). Under sulfidogenic conditions, however, the Mariotti plots of the sediments from the NE basin (Fig. 5) did not show a negative slope and $\varepsilon_{form}$ could not be determined. The same was the case for the sediments from the SW basin (Fig. 6).

The negative $\varepsilon_{form}$ indicates that products of formate should be depleted in $^{13}C$. Indeed, the $\delta^{13}C$ of acetate and $CH_4$ was generally more negative than the $\delta^{13}C$ of formate. This was the case in the paddy soils from Vercelli (Fig. 1f) and the IRRI (Fig. S1f), as well as in the sediments from the NE basin (Fig. 3f) and the SW basin (Fig. S3f) of Lake Fuchskuhle. In the sediment of the NE basin, the $\delta^{13}C$ of acetate increased from very low level, at $-95\%o$, to finally about $-57\%o$ in parallel with formate consumption (Fig. 3f). $CO_2$ was also produced during formate degradation to various extent (Reactions R1, R2 and R3). Since the pH was in a range of pH 7 to pH 8, $CO_2$ was also converted to bicarbonate. The $\delta^{13}C$ of bicarbonate is generally more positive than the $\delta^{13}C$ of $CO_2$ by about $10\%o$ (Stumm and Morgan, 1996). The $\delta^{13}C$ of the gaseous $CO_2$ was always close to the $\delta^{13}C$ of formate or was more positive. In the paddy soils and the NE basin of Lake Fuchskuhle, the $\delta^{13}C$ of $CO_2$ increased in parallel with the increasing $\delta^{13}C$ of formate (Figs. 1h, 3h; S1h). The $\delta^{13}C$ of the gaseous $CO_2$ produced from the formate-amended samples was initially more negative than that from the unamended samples, but eventually the $\delta^{13}C$ increased
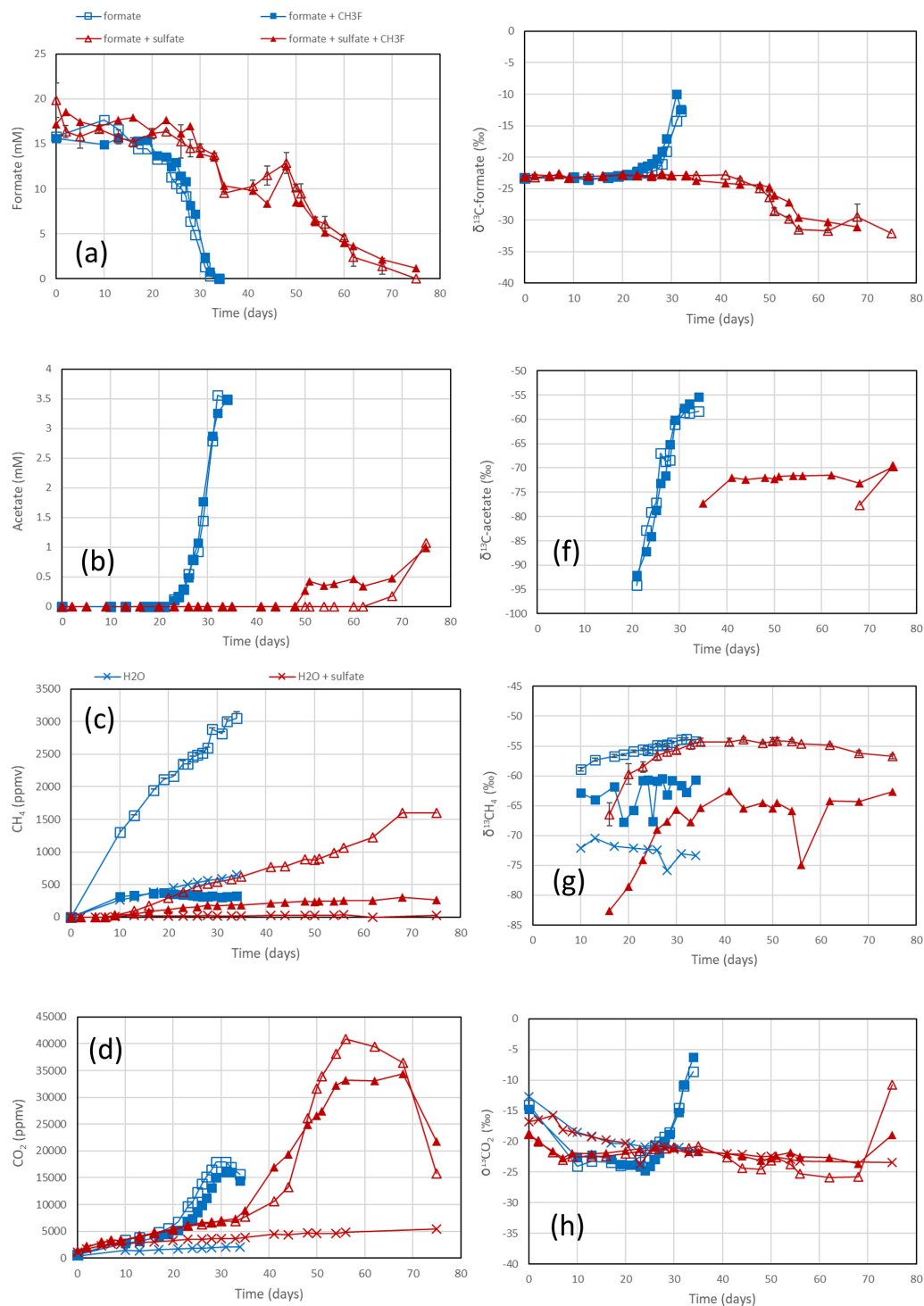
**Figure 3.** Formate conversion to acetate, $CH_4$ and $CO_2$ in suspensions of sediment from the NE basin of Lake Fuchskuhle after the addition of formate without sulfate (blue squares) or formate plus sulfate (gypsum) (red triangles), without $CH_3F$ (open symbols) or with $CH_3F$ (closed symbols). Controls with the addition of only water (blue or red $\times$ crosses) are only shown occasionally. The panels show the temporal change in **(a)** concentrations of formate, **(b)** concentrations of acetate, **(c)** mixing ratios of $CH_4$ (1 ppmv $= 10^{-6}$ bar), **(d)** mixing ratios of $CO_2$, **(e)** $\delta^{13}C$ of formate, **(f)** $\delta^{13}C$ of acetate, **(g)** $\delta^{13}C$ of $CH_4$ and **(h)** $\delta^{13}C$ of $CO_2$. Means $\pm$ SE.
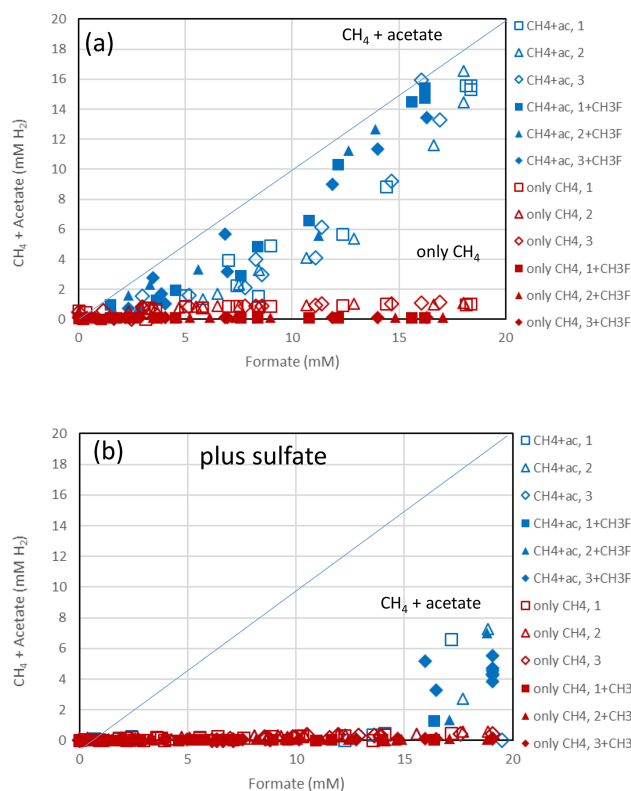
**Figure 4.** Balance of produced acetate plus $CH_4$ (blue symbols) and of only $CH_4$ (red symbols) against the consumed formate in **(a)** the absence and **(b)** the presence of sulfate in sediment from the NE basin of Lake Fuchskuhle. Acetate and $CH_4$ are each equivalent to $4 H_2$, while formate is equivalent to $1 H_2$. The open and closed symbols denote conditions in the absence and the presence of $CH_3F$, respectively. The different symbols indicate three different replicates. The lines indicate equimolarity (in terms of reducing equivalents between substrate and product).

above these values when formate was completely consumed (Figs. 1h, 3h; S3h).

The $\delta^{13}C$ values of the initial formate were about $-24\text{‰}$ (Fig. 5). When formate was completely consumed, the $\delta^{13}C$ values of the products acetate and $CH_4$ were always more negative. The average $\delta^{13}C$ values of the products after complete consumption of formate are shown in Fig. 7. In the absence of sulfate, $\delta^{13}C$ of acetate was in a range of $-51\text{‰}$ to $-49\text{‰}$ and $-70\text{‰}$ to $-63\text{‰}$, in the paddy soils and lake sediments, respectively (Fig. 7). In the presence of sulfate, $\delta^{13}C$ of acetate was in a range of $-57\text{‰}$ to $-52\text{‰}$ and $-78\text{‰}$ to $-72\text{‰}$, in the paddy soils and lake sediments (only NE basin), respectively (Fig. 7). The $\delta^{13}C$ of $CH_4$ was in a range of $-70\text{‰}$ to $-54\text{‰}$ and $-60\text{‰}$ to $-54\text{‰}$, in the absence and presence of sulfate, respectively (Fig. 7). The $\delta^{13}C$ of gaseous $CO_2$ (for bicarbonate plus $10\text{‰}$) was in a range of $-23\text{‰}$ to $-11\text{‰}$ and $-24\text{‰}$ to $-19\text{‰}$, in the absence and presence of sulfate, respectively (Fig. 7).
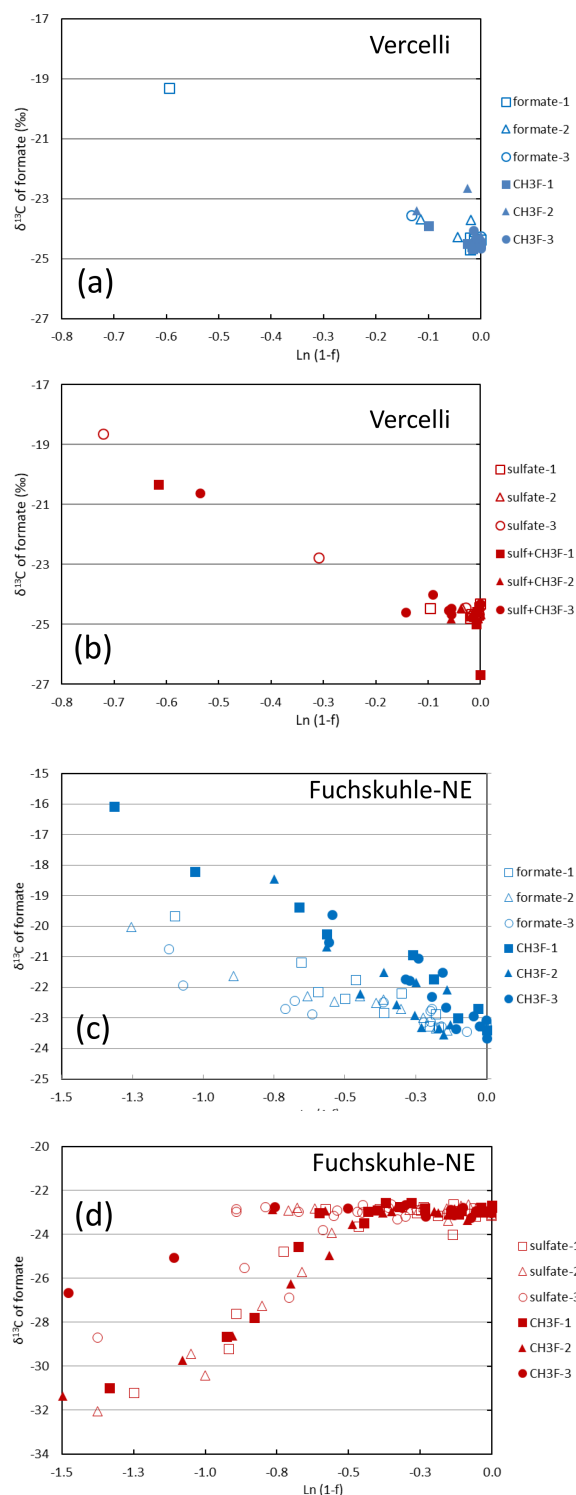
**Figure 5.** Mariotti plots of formate consumption in **(a, b)** paddy soil from Vercelli and **(c, d)** sediment from the NE basin of Lake Fuchskuhle under methanogenic (**a, c**, blue symbols) and sulfidogenic (**b, d**, red symbols) conditions, both in the absence (open symbols) and in the presence (closed symbols) of $CH_3F$.
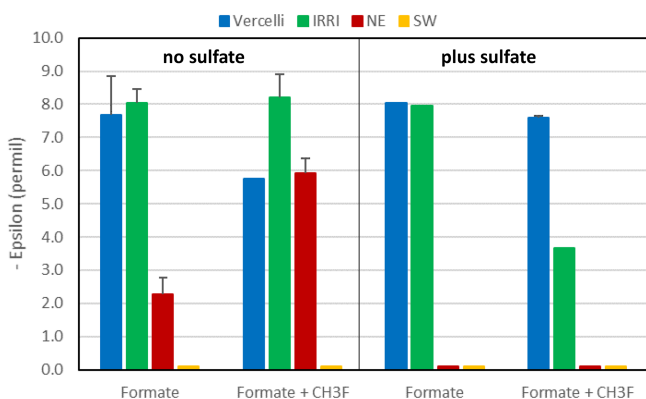
**Figure 6.** Isotopic enrichment factors ($\varepsilon_{form}$, given as negative values) in paddy soils from Vercelli and the IRRI (the Philippines) and in lake sediments from the NE and SW basins of Lake Fuchskuhle without (left panel) and with (right panel) the addition of sulfate (gypsum) and $CH_3F$. Means $\pm$ SE.
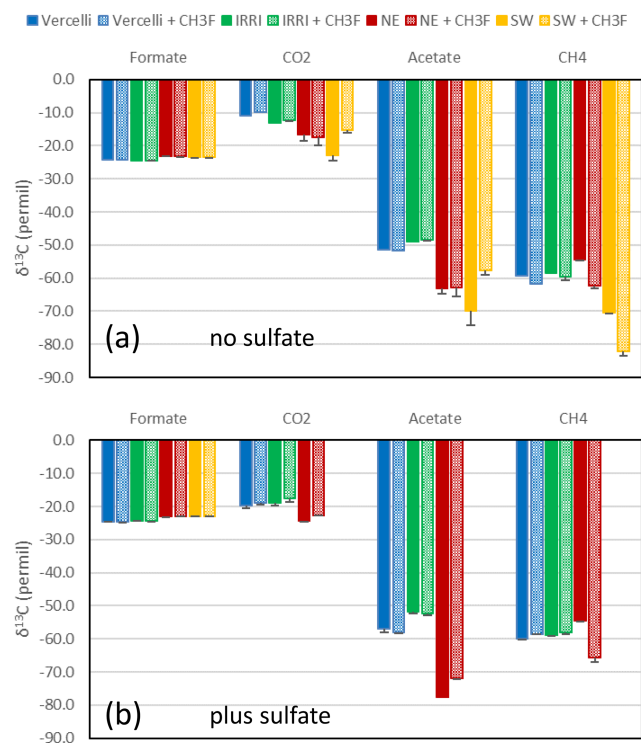


**Figure 7.** Average $\delta^{13}C$ of formate (at the beginning of incubation) and of $CO_2$, acetate and $CH_4$ (after the depletion of formate) in paddy soils from Vercelli (blue) and the IRRI (green) and in sediments from the NE basin (red) and the SW basin (yellow) of Lake Fuchskuhle in the absence (filled bars) and the presence (dotted bars) of $CH_3F$. Means $\pm$ SE.

# 4 Discussion

## 4.1 Formate degradation under acetogenic/methanogenic conditions

In rice paddy soils, formate was consumed within < 10 d. The absence of sulfate did not allow sulfidogenic (Reaction R3) degradation but allowed the operation of methanogenic (Reaction R1), homoacetogenic (Reaction R2) or syntrophic (Reaction R4) degradation. Syntrophic degradation is still disputed, since many microorganisms are able to enzymatically equilibrate $H_2$ and formate and thus prohibit exploitation of the difference in the energy content (Montag and Schink, 2018; Schink et al., 2017). Syntrophic formate degradation is exergonic by only a few kilojoules of Gibbs free energy per mole and requires the coupling with methanogenesis or other efficient hydrogen (electron) scavengers. Although formate-driven $CH_4$ production was observed in our study, the production was sensitive to inhibition by $CH_3F$, indicating that $CH_4$ was predominantly produced from acetate rather than from $H_2$. Therefore, syntrophic formate oxidation coupled to $CH_4$ production was probably not a major pathway.

Acetate was the most important product of formate degradation in the paddy soils and the lake sediments. Methane also was a product but was much less important than acetate. Furthermore, it was predominantly produced from acetate, as shown by the inhibition by $CH_3F$ and the concomitant decrease in $\delta^{13}C$ of $CH_4$, which is characteristic of hydrogenotrophic methanogesis that is not inhibited by $CH_3F$ (Conrad et al., 2010). Hence, formate was apparently primarily degraded by homoacetogenesis (Reaction R1). Only part of the produced acetate was immediately used by aceticlastic methanogenesis generating $CH_4$ as secondary product. Although formate is a perfect substrate for homoacetogenic bacteria operating the Wood–Ljungdahl pathway (WLP) (Drake, 1994), the yield of Gibbs free energy per mole formate is less for homoacetogenic than for methanogenic degradation (Dolfing et al., 2008). Thus, it is surprising that formate-driven homoacetogenesis prevailed over methanogenesis. Nevertheless, simultaneous operation of homoacetogenesis and methanogenesis from formate has been observed before in a fen soil (Hunger et al., 2011). Homoacetogenesis prevailing over methanogenesis has also frequently been observed with $H_2/CO_2$ as substrate (Conrad et al., 1989; Nozhevnikova et al., 1994), indicating that homoacetogens can take particular advantage from low temperatures (Conrad, 2023) or the availability of secondary substrates (Peters et al., 1998). It is noteworthy that homoacetogens have to invest energy for fixation of formate, while methanogens are able to bypass this step (Lemaire et al., 2020). Perhaps it is such energy investment which makes the homoacetogens to competitive with formate utilizers.

Formate consumption was recorded upon the addition of formate to initial concentrations of about 15 mM, which was

much higher than the in situ concentration being typically on the order of a few micromoles per liter (Montag and Schink, 2018). However, the increased concentration allowed stable isotope fractionation, which would not occur under formate limitation. The $\delta^{13}C$ of the produced acetate was by about 24‰–33‰ lower than that of formate. This isotopic discrimination between formate and acetate is similar to that measured in a culture of the homoacetogen *Thermoanaerobacter kivui* (Freude and Blaser, 2016). However, this discrimination is much larger than the isotopic enrichment factors ($\varepsilon_{form}$ of −8‰ to −2.5‰) determined from the change in $\delta^{13}C$ during formate consumption. There are two conceivable explanations for this observation. (1) Formate is disproportionated to $CO_2$ and acetate. In the WLP, three formate are oxidized to $CO_2$, one formate is reduced to the methyl group of acetate and one of the produced $CO_2$ is reduced to the carboxyl group of acetate. The disproportionation of formate to acetate and $2CO_2$ is possibly a branch point (Fry, 2003; Hayes, 2001), at which the carbon flow is split into the production of $^{13}C$-enriched $CO_2$ and $^{13}C$-depleted acetate, which together result in the $\varepsilon_{form}$ observed. (2) Formate first is completely converted to $CO_2$ plus $H_2$ (Reaction R5) or other electron equivalents. This reaction displays the $\varepsilon_{form}$ determined by the Mariotti plots. Acetate is then produced via the WLP by the chemolithotrophic reduction of $2CO_2$ to acetate, of which the isotopic enrichment factor is typically on the order of about −55‰ (Blaser and Conrad, 2016). In any case, it is plausible to assume that acetate was formed via the WLP. In the WLP, oxidation of formate is catalyzed by a formate dehydrogenase, which provides $CO_2$ to the carboxyl branch of the WLP. The methyl branch of the WLP normally starts with formate being converted to formyl-THF. However, it can also start with the reduction of $CO_2$ to formate with a hydrogen-dependent carbon dioxide reductase (HDCD). Homoacetogens (e.g., *Acetobacter woodii, T. kivui*) contain such a HDCD, which allows the interconversion of formate and $H_2$ plus $CO_2$ (Jain et al., 2020; Schuchmann et al., 2018). The isotope discrimination in our experiments indicates that the $CO_2$ produced from formate has been enriched in $^{13}C$ rather than depleted, thus supporting the first explanation. The $\delta^{13}C$ of $CO_2$ produced from formate was initially lower than that of the unamended soil or sediment being on the order of −20‰ to −10‰ (Figs. 1h, 3h, S1h, S3h). Eventually, however, $\delta^{13}C$ of $CO_2$ reached values of −25‰ to −10‰ (Fig. 7). The $\delta^{13}C$ of bicarbonate is 10‰ more positive than that of $CO_2$. This mixed inorganic carbon would be the $CO_2$ substrate for WLP, which together with formate generates the acetate having a $\delta^{13}C$ of about −70‰ to −50‰ (Fig. 7).

Methane was a minor product of formate degradation in all soils and sediments. Since $CH_4$ formation was strongly inhibited by $CH_3F$, it was most likely produced from acetate by aceticlastic methanogens. Since $CH_4$ production from the soils or sediments was much lower without formate amendment, the $CH_4$ must have primarily been produced from the acetate that was generated from formate. The $\delta^{13}C$ of $CH_4$

in the soil incubations was more negative than that of acetate (Fig. 7). The difference between the $\delta^{13}C$ of $CH_4$ and the $\delta^{13}C$ of acetate indicated an isotopic enrichment factor of $\varepsilon_{ac\text{-}CH_4} = -10‰$ to −8‰, which is close to the enrichment factor of aceticlastic *Methanosaeta (Methanothrix) concilii* (Penning et al., 2006). In the lake sediments, the $\delta^{13}C$ of $CH_4$ and acetate was not much different, indicating that acetate was instantaneously consumed by methanogens, as it was produced by homoacetogens so that carbon isotopes were not discriminated. Both paddy soils and lake sediments contained *mcrA* genes (coding for a subunit of methyl CoM reductase) of *Methanosaetaceae (Methanotrichaceae)* (Conrad et al., 2021).

## 4.2 Formate degradation under sulfidogenic conditions

In the rice paddy soils, formate was consumed within 10 d when sulfate was present and not quite as fast as without sulfate. In the lake sediments, however, sulfidogenic formate consumption was much slower. Formate degradation by sulfate reduction normally results in complete oxidation to $CO_2$ (Reaction R3). In the lake sediments, $CO_2$ was indeed the main degradation product. However, in the paddy soils, substantial amounts of acetate and even $CH_4$ were also produced. The homoacetogenic bacteria in these soils apparently competed well with the sulfate reducing bacteria, although the soils had been adapted by preincubation in the presence of sulfate. The production of acetate and $CH_4$ was dependent on formate degradation, since no production was observed in the unamended control. Production of $CH_4$ was inhibited by $CH_3F$, indicating that aceticlastic methanogenesis was the main process of $CH_4$ production. The carbon isotope fractionation of formate was similar under non-sulfidogenic conditions, exhibiting a small $\varepsilon_{form}$ of −8‰ to −3.5‰ (Fig. 5) and displaying a strong isotope effect with the formation of acetate ($\delta^{13}C = -57‰$ to −52‰) and $CH_4$ ($\delta^{13}C = -60‰$ to −58‰). The mechanism of fractionation is probably the same (see above).

In the lake sediments, however, sulfidogenic degradation of formate was much slower than methanogenic/acetogenic degradation. In the sediment of the SW basin, formate was not even completely degraded within 80 d. In the sediments of both lake basins, neither acetate nor $CH_4$ was a major product of sulfidogenic formate degradation. Hence, formate was apparently degraded according to Reaction (R3) forming $CO_2$ as the main carbon product. This formation process displayed no depletion of the heavy carbon isotope, as the Mariotti plots of $\delta^{13}C$ of formate did not exhibit a negative slope. The $\delta^{13}C$ of the $CO_2$ slowly decreased with an increasing fraction of formate consumed (Figs. 3h; 5c), probably involving isotope exchange between formate and $CO_2$ (DeGraaf and Cappenberg, 1996). The little acetate which was formed displayed a $\delta^{13}C$ of −77‰ (Fig. 7b), indicating that it was produced by a mechanism similar to the one found in the absence of sulfate, presumably via the WLP.

The strong differences between rice paddy soils and lake sediments were possibly caused by their different microbial communities (Conrad et al., 2021). The differences were seen in the composition of the *mcrA* and *dsrB* genes coding for methyl CoM reductase and dissimilatory sulfate reductase, respectively, as well as the gene coding for the bacterial 16S rRNA (data are shown in Conrad et al., 2021). The microbial community structures based on these genes were similar whether the soils and sediments were amended with sulfate or not. However, they were strongly different between soils or sediments were used (Conrad et al., 2021). Unfortunately, these data do not allow for the discrimination of particular taxa of homoacetogenic bacteria. Nevertheless, it is possible that formate-consuming homoacetogens were more prevalent in the soils than in the sediments and competed accordingly with the formate-consuming sulfate reducers, more or less.

## 4.3 Conclusions

Formate was found to be an excellent substrate for acetate formation in the paddy soils and in the lake sediments, confirming and extending similar observations in a fen soil (Hunger et al., 2011). In the anoxic soils, acetate was the major product even in the presence of sulfate, which would have allowed sulfate reduction. The acetate was strongly depleted in $^{13}$C relative to formate, but the consumption of formate itself displayed only a small isotopic enrichment factor. Therefore, it is likely that formate was disproportionated to $^{13}$C-depleted acetate and $^{13}$C-enriched $CO_2$. The $\delta^{13}C$ of $CO_2$ was indeed slightly higher than that of formate. Acetate was most likely produced by homoacetogenesis via the WLP. The produced acetate was then used by aceticlastic methanogens (probably by *Methanothrix*) but only to a minor extent, resulting in further depletion of $^{13}$C. The homoacetogenic bacteria in the paddy soils apparently competed well with both methanogenic and sulfate-reducing microorganisms when formate was the substrate. The preference of homoacetogenesis as a degradation pathway is unexpected, since other substrates, such as acetate and propionate, are degraded in these paddy soils by methanogenesis or sulfate reduction (Conrad et al., 2021; Conrad and Claus, 2023). Only in the lake sediments was formate oxidation by sulfate reduction was more prevalent than homoacetogenesis.

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