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

Ralf Conrad¹, Peter Claus¹

¹Max Planck Institute for Terrestrial Microbiology, Karl-von-Frisch-Str. 10, 35043 Marburg, Germany

Correspondence to: Ralf Conrad (Conrad@mpi-marburg.mpg.de)

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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, the Philippines) and of sediments from the NE and SW basins of Lake Fuchskuhle (Germany). The samples were suspended in phosphate buffer (pH 7.0) both in the absence and presence of sulfate (gypsum) and of methyl fluoride (CH$_3$F), an inhibitor of aceticlastic methanogenesis. In the paddy soils, formate was mainly converted to acetate both under 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 ($\varepsilon_{\text{form}}$) of formate consumption, determined by Mariotti plots, were in the low range of -8‰ to -2.5‰ 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 25-50‰ 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 for example produced in pyruvate cleavage by pyruvate formate lyase (Thauer et al., 1977) or by reduction of 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 equivalently be used 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.: 

\begin{align*}
4 \text{HCOOH} &\rightarrow \text{CH}_4 + 3 \text{CO}_2 + 2 \text{H}_2\text{O} \quad (1) \\
4 \text{HCOOH} &\rightarrow \text{CH}_3\text{COOH} + 2 \text{CO}_2 + 2 \text{H}_2\text{O} \quad (2)
\end{align*}
Formate may also be a substrate for syntrophic bacteria, which live from the little Gibbs free energy ($\Delta G^0_r = -3.4 \text{ kJ mol}^{-1}$) that is generated by the conversion of formate to $\text{H}_2$ plus $\text{CO}_2$ (Dolfing et al., 2008; Kim et al., 2010; Martins et al., 2015), i.e.

$$4 \text{HCOOH} + \text{SO}_4^{2-} + \text{H}^+ \rightarrow \text{HS}^- + 4 \text{CO}_2 + 4 \text{H}_2\text{O} \quad (3)$$

Formate can also be enzymatically equilibrated with $\text{H}_2$ and $\text{CO}_2$ 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):

$$\text{HCOOH} \leftrightarrow \text{CO}_2 + \text{H}_2 \quad (4)$$

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; Küssel 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 $\text{H}_2$ plus $\text{CO}_2$ are large (Blaser and Conrad, 2016). However, our knowledge of carbon isotope fractionation with formate as substrate is scarce. In cultures of homoacetogenic bacteria the carbon in the acetate produced from formate was strongly depleted in $^{13}$C ($\varepsilon = -56.5\%$) almost similarly as with $\text{CO}_2$ 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 lake sediments amended with formate. We recorded the consumption of formate along with the production of acetate, $\text{CH}_4$ and $\text{CO}_2$ and measured the $\delta^{13}$C of these compounds. We also used the treatment with methyl fluoride ($\text{CH}_3\text{F}$) to inhibit the consumption of acetate by methanogenic archaea (Janssen and Frenzel, 1997). We used the same environmental samples as 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, 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 described before (Liu et al., 2018). The lake sediments (top 10 cm layer) were from the NE and SW basins of Lake Fuchskuhle (Casper et al., 2003). They were sampled in July 2016 using a gravity core sampler as described before (Kanaparthi et al., 2013).

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 H₂O (prepared under N₂) at a ratio of 1:1 and incubated under N₂ at 25°C for 4 weeks. In a second incubation, for sulfidogenic conditions, paddy soil was mixed with autoclaved anoxic H₂O at a ratio of 1:1, was amended with 0.07 g CaSO₄.2H₂O, and then incubated under N₂ 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 of 20 mM potassium phosphate buffer (pH 7.0) in a 150-mL bottle under an atmosphere of N₂. The bottles were amended with (i) 5 mL H₂O; (ii) 5 mL H₂O + 4.5 mL CH₃F; (iii) 5 mL 200 mM sodium formate; (iv) 5 mL 200 mM sodium formate + 4.5 mL CH₃F. In the second set (resulting in sulfidogenic conditions), 5 mL 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).

For lake sediments under methanogenic conditions, 5 ml sediment was incubated in 3 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 N₂. The bottles were amended with (i) 5 ml H₂O; (ii) 5 ml H₂O + 4.5 ml CH₃F; (iii) 5 ml 200 mM sodium formate; (iv) 5 ml 200 mM sodium formate + 4.5 ml CH₃F. For sulfidogenic conditions, lake sediments were preincubated with sulfate by adding 0.1 g CaSO₄.2H₂O (gypsum) to 50 ml sediment and incubating at 10°C for 4 weeks. For sulfidogenic conditions, 5 ml of the preincubated sediment was incubated in 3 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 N₂. The bottles were amended as above, but in addition also with 200 µl of a CaSO₄ 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 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 stored frozen at -20°C for later fatty acid analysis. Chemical and isotopic analyses were performed as described in detail previously (Goëvert and Conrad, 2009). Methane was analyzed by gas chromatography (GC) with flame ionization detector. Carbon dioxide was analyzed after conversion to CH₄ with a Ni catalyst. Stable isotope analyses of ¹³C/¹²C 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 (δ¹³C) relative to the Vienna PeeDee Belemnite standard having a ¹³C/¹²C ratio (Rstandard) of 0.01118: δ¹³C = 10³ (Rsample/Rstandard – 1). The precision of the GC-C-IRMS was ± 0.2‰, 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 per mL 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 → B are defined after Hayes (Hayes, 1993) as:

\[ a_{A:B} = (\delta_A + 1000)/(\delta_B + 1000) \]  

also expressed as \( \varepsilon = 1000 (1 - \alpha) \) in permil. The carbon isotope enrichment factor \( \varepsilon_{form} \) associated with formate consumption was calculated from the temporal change of δ¹³C of formate as described by Mariotti et al. (Mariotti et al., 1981) from the residual reactant

\[ \delta_r = \delta_{ri} + \varepsilon \left[ \ln(1-f) \right] \]

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 δ¹³C of formate against ln(1 – f) yields \( \varepsilon_{form} \) as the slope of best fit lines. The regressions of δ¹³C 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).
Figure 1. Formate conversion to acetate, CH₄ and CO₂ in suspensions of paddy soil from Vercelli (Italy) after addition of formate without sulfate (blue squares) or formate plus sulfate (gypsum) (red triangles) without CH₃F (open symbols) or with CH₃F (closed symbols). Controls with addition of only water (blue or red X crosses) are only shown occasionally. The panels show the temporal change of (a) concentrations of formate, (b) concentrations of acetate, (c) mixing ratios of CH₄ (1 ppmv = 10⁻⁶ bar), (d) mixing ratios of CO₂, (e) δ¹³C of formate, (f) δ¹³C of acetate, (g) δ¹³C of CH₄, and (h) δ¹³C of CO₂. Means ± 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 buffer at pH 7 and amended with formate. In the Vercelli soil, formate was consumed after a lag phase of 4 days under methanogenic and 5 days 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 CH₃F (Fig. 1a). Similar results were obtained with IRRI soil (Fig. S1). Acetate was produced concomitantly with formate consumption, again without effect by CH₃F (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 (Fig. 1c; S1c). It is noteworthy that CH₃F inhibited the production of CH₄ (Fig. 1c; S1c). Finally, CO₂ was produced under all conditions without lag phase and without effect by CH₃F (Fig. 1c). In Vercelli soil, CO₂ production was about twice under sulfidogenic than under methanogenic conditions (Fig. 1c). In IRRI soil, it was only slightly larger (Fig. S1c). The accumulation of acetate plus CH₄ was equimolar to the consumption of formate in terms of electron equivalents, while the accumulation of CH₄ alone accounted only for <30%, in the presence of CH₃F even less (Fig. 2a; S2a). Hence, acetate was the more important product of formate consumption. Under sulfidogenic conditions, accumulation of acetate plus CH₄ was less than equimolar, especially in Vercelli soil (Fig. 2b), probably since formate was instead converted to CO₂. However, acetate formation was still substantial accounting for 60-80% of formate consumption (Fig. 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 preincubation with sulfate (gypsum). Substantial formate depletion did not start before about 20 days of incubation both in sediments from the NE basin (Fig. 3) and the SW basin (Fig. S3). Again, CH₃F only inhibited the production of CH₄ but not that of acetate or CO₂ (Fig. 3; S3). The main difference to the paddy soils was that CH₄ was not produced concomitantly with formate consumption, but started right from the beginning. However, the amounts of CH₄ 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 CH₄ production in the water control (not amended with formate) was negligible (Fig. 3c; S3c). Production of CO₂ started without lag phase but accelerated together with formate consumption (Fig. 3d; S3d). In the lake sediments, CH₄ accounted only for <10% of formate consumption, while acetate was the main product when sulfate was absent (Fig. 4a, S4a). In contrast to the paddy soils, formate consumption in both lake sediments was much slower under sulfidogenic than under methanogenic conditions (Fig. 3a; S3a). In the sediment from
SW basin, formate consumption was very slow so that less than half of the formate was consumed during 80 days of incubation and consumption was not completed until the end of the experiment (Fig. S3a). Very little acetate was produced and no CH₄ was formed from formate in both lake sediments, when sulfate was present (Fig. 4b, S4b).

![Figure 2. Balance of produced acetate plus CH₄ (blue symbols) and of only CH₄ (red symbols) against the consumed formate in (a) the absence and (b) the presence of sulfate in paddy soil from Vercelli (Italy). The open and closed symbols denote conditions in the absence and the presence of CH₃F, respectively. The different symbols indicate three different replicates. The line indicate equimolarity (in terms of reducing equivalents between substrate and product.]

3.2 Isotope fractionation during formate consumption

In the rice paddy soils values of δ¹³C increased when formate was being consumed indicating discrimination against the heavy carbon isotope. This process was not affected by CH₃F and was similar without and with sulfate (Fig. 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 δ¹³C of formate slowly decreased with time (Fig. 3e). In the sediment from the SW basin, δ¹³C 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).
Figure 3. Formate conversion to acetate, CH$_4$ and CO$_2$ in suspensions of sediment from the NE basin of Lake Fuchskuhle after addition of formate without sulfate (blue squares) or formate plus sulfate (gypsum) (red triangles) without CH$_3$F (open symbols) or with CH$_3$F (closed symbols). Controls with addition of only water (blue or red X crosses) are only shown occasionally. The panels show the temporal change of (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 ± SE.
Figure 4. Balance of produced acetate plus CH₄ (blue symbols) and of only CH₄ (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. The open and closed symbols denote conditions in the absence and the presence of CH₃F, respectively. The different symbols indicate three different replicates. The line indicate equimolarity (in terms of reducing equivalents between substrate and product).

Mariotti plots of δ₁³C of formate as function of f_form resulted in negative slopes (Fig. 4; S5). Hence, the enrichment factors (ε_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 ε_form were in the range of -8.5 to -2.5‰ (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 ε_form could not be determined. The same was the case for the sediments from the SW basin (Fig. 6).

The negative ε_form indicates that products of formate should be depleted in ¹³C. Indeed the δ¹³C of acetate and CH₄ were generally more negative than the δ¹³C 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 δ¹³C of acetate increased from very low -95‰ to finally about -57‰ in parallel with formate consumption (Fig. 3f). CO₂ was also produced during formate degradation to various extent (equ. 1, 2 and 3). Since the pH was in a range of pH 7 to pH 8, CO₂ was also converted to bicarbonate. The δ¹³C of bicarbonate is generally by about 10‰
Figure 5. Mariotti plots of formate consumption in (a) paddy soil from Vercelli and (b, c) sediment from the NE basin of Lake Fuchskuhle under methanogenic (blue symbols) and sulfidogenic (red symbols) conditions both in the absence (open symbols) and in the presence (closed symbols) of CH$_3$F. The different symbols indicate three different replicates.

Figure 6. Isotopic enrichment factors ($\varepsilon_{\text{form}}$, given as negative values) in paddy soils without and with addition of sulfate (gypsum) and CH$_3$F. Means ± SE.
more positive than the δ\(^{13}\)C of CO\(_2\) (Stumm and Morgan, 1996). The δ\(^{13}\)C of the gaseous CO\(_2\)
was always close to the δ\(^{13}\)C of formate or was more positive. In the paddy soils and the NE basin of Lake Fuchskuhle, the δ\(^{13}\)C of CO\(_2\) increased in parallel with the increasing δ\(^{13}\)C of formate (Fig. 1h, 3h; S1h). The δ\(^{13}\)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 δ\(^{13}\)C increased above these values when formate was completely consumed (Fig. 1h, 3h; S3h).

The δ\(^{13}\)C values of the initial formate were about -24‰ (Fig. 5). When formate was completely consumed, the δ\(^{13}\)C values of the products acetate and CH\(_4\) were always more negative. The average δ\(^{13}\)C values of the products after complete consumption of formate are shown in Fig. 7. In the absence of sulfate, δ\(^{13}\)C of acetate was in a range of -51‰ to -49‰ and -70‰ to -63‰, in the paddy soils and lake sediments, respectively (Fig. 7). In the presence of sulfate, δ\(^{13}\)C of acetate was in a range of -57‰ to -52‰ and -78‰ to -72‰, in the paddy soils and lake sediments (only NE basin), respectively (Fig. 7). The δ\(^{13}\)C of CH\(_4\) was in a range of -70‰ to -54‰ and -60‰ to -54‰, in the absence and presence of sulfate, respectively (Fig. 7). The δ\(^{13}\)C of gaseous CO\(_2\) (for bicarbonate plus 10‰) was in a range of -23‰ to -11‰ and -24‰ to -19‰, in the absence and presence of sulfate, respectively (Fig. 7).

**Figure 7.** Average δ\(^{13}\)C of formate (at the beginning of incubation) and of CO\(_2\), acetate and CH\(_4\) (after the depletion of formate) in soils or sediments from Vercelli (blue), the IRRI (green), the NE basin (red) and the SW basin (yellow) in the absence (filled bars) and the presence (dotted bars) of CH\(_3\)F. Means ± SE.
4 Discussion

4.1 Formate degradation under acetogenic/methanogenic conditions

In rice paddy soils formate was consumed within <10 days. The absence of sulfate did not allow sulfidogenic (equ. 3) degradation, but allowed the operation of methanogenic (equ. 1), homoacetogenic (equ. 2) or syntrophic (equ. 4) degradation. Syntrophic degradation is still disputed, since many microorganisms are able to enzymatically equilibrate \( \text{H}_2 \) and formate and thus prohibit generation of energy (Montag and Schink, 2018; Schink et al., 2017). Syntrophic formate degradation generates 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\(_3\)F 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 as well as in 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\(_3\)F and the concomitant decrease of \( \delta^{13}\text{C} \) of CH\(_4\), which is characteristic for hydrogenotrophic methanogenesis that is not inhibited by CH\(_3\)F (Conrad et al., 2010). Hence, formate was apparently primarily degraded by homoacetogenesis (equ. 1). 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\(_2/\text{CO}_2\) 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).

The \( \delta^{13}\text{C} \) 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 \textit{Thermoanaerobacter kivui} (Freude and Blaser, 2016). However, this discrimination is much larger than the isotopic enrichment factors (\( \epsilon_{\text{form}} \) of -8‰ to -2.5‰) determined from the change of \( \delta^{13}\text{C} \) 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\textsubscript{2} is reduced to the carboxyl group of acetate. The disproportionation of formate to acetate and 2 CO\textsubscript{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\textsubscript{2} and $^{13}$C-depleted acetate, which together result in the $\varepsilon_{\text{form}}$ observed. (2) Formate first is completely converted to CO\textsubscript{2} plus H\textsubscript{2} (equ.5) or other electron equivalents This reaction displays the $\varepsilon_{\text{form}}$ determined by the Mariotti plots. Acetate is then produced via the WLP by the chemolithotrophic reduction of 2 CO\textsubscript{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\textsubscript{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\textsubscript{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\textsubscript{2} plus CO\textsubscript{2} (Jain et al., 2020; Schuchmann et al., 2018). The isotope discrimination in our experiments indicates that the CO\textsubscript{2} produced from formate has been enriched in $^{13}$C rather than depleted, thus supporting the first explanation. The $\delta^{13}$C of CO\textsubscript{2} produced from formate was initially lower than that of the unamended soil or sediment being on the order of -20‰ to -10‰ (Fig. 1h, 3h, S1h, S3h). Eventually, however, $\delta^{13}$C of CO\textsubscript{2} reached values of -25‰ to -10‰ (Fig. 7). The $\delta^{13}$C of bicarbonate is 10‰ more positive than that of CO\textsubscript{2}. This mixed inorganic carbon would be the CO\textsubscript{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\textsubscript{4} formation was strongly inhibited by CH\textsubscript{3}F, it was most likely produced from acetate by aceticlastic methanogens. Since CH\textsubscript{4} production from the soils or sediments was much lower without formate amendment, the CH\textsubscript{4} must have primarily been produced from the acetate that was generated from formate. The $\delta^{13}$C of CH\textsubscript{4} in the soil incubations was more negative than that of acetate (Fig. 7). The difference between the $\delta^{13}$C of CH\textsubscript{4} and the $\delta^{13}$C of acetate indicated an isotopic enrichment factor of $\varepsilon_{\text{ac-CH}_4} = -10‰$ 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\textsubscript{4} and acetate were 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 ten days when sulfate was present, 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$_2$ (equ.3). 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$_3$F indicating that aceticlastic methanogenesis was the main process of CH$_4$ production. The carbon isotope fractionation of formate was similar as under non-sulfidogenic conditions, exhibiting a small $\varepsilon_{\text{form}}$ of -8‰ to -3.5‰ (Fig. 5) and displaying a strong isotope effect with the formation of acetate ($\delta^{13}\text{C} = -57--52‰$) and CH$_4$ ($\delta^{13}\text{C} = -60--58‰$). 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 days. 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 equ.3 forming CO$_2$ as main carbon product. This formation process displayed no depletion of the heavy carbon isotope, as the Mariotti plots of $\delta^{13}\text{C}$ of formate did not exhibit a negative slope. The $\delta^{13}\text{C}$ of the CO$_2$ slowly decreased with increasing fraction of formate consumed (Fig. 3h; 5c), probably involving isotope exchange between formate and CO$_2$ (DeGraaf and Cappenberg, 1996). The little acetate, which was formed, displayed a $\delta^{13}\text{C}$ of -77‰ (Fig. 7b) indicating that it was produced by a similar mechanism as 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 16S rRNA. The composition of these genes was similar whether the soils and sediments were amended with sulfate or not. However, they were strongly different between soils and sediments (Conrad et al., 2021). Unfortunately, these data do not allow to discriminate for particular taxa of homoacetogenic bacteria. Nevertheless, it is possible that formate-consuming homoacetogens were more prevalent in the soils than in the sediments and accordingly competed more or less with the formate-consuming sulfate reducers.
4.3 Conclusions

Formate was found to be an excellent substrate for acetate formation in the paddy soils as well as 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 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 degradation pathway is unexpected, since other substrates, such as acetate and propionate, are in these paddy soils degraded by methanogenesis or sulfate reduction (Conrad et al., 2021) (Conrad and Claus, 2023). Only in the lake sediments, formate oxidation by sulfate reduction was more prevalent than homoacetogenesis.

Supplement link

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

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References


Figure legends

Figure 1. Formate conversion to acetate, CH₄ and CO₂ in suspensions of paddy soil from Vercelli (Italy) after addition of formate without sulfate (blue squares) or formate plus sulfate (gypsum) (red triangles) without CH₃F (open symbols) or with CH₃F (closed symbols). Controls with addition of only water (blue or red X crosses) are only shown occasionally. The panels show the temporal change of (a) concentrations of formate, (b) concentrations of acetate, (c) mixing ratios of CH₄ (1 ppmv = 10⁻⁶ bar), (d) mixing ratios of CO₂, (e) δ¹³C of formate, (f) δ¹³C of acetate, (g) δ¹³C of CH₄, and (h) δ¹³C of CO₂. Means ± SE.

Figure 2. Balance of produced acetate plus CH₄ (blue symbols) and of only CH₄ (red symbols) against the consumed formate in (a) the absence and (b) the presence of sulfate in paddy soil from Vercelli (Italy). The open and closed symbols denote conditions in the absence and the presence of CH₃F, respectively. The different symbols indicate three different replicates. The line indicate equimolarity (in terms of reducing equivalents between substrate and product).

Figure 3. Formate conversion to acetate, CH₄ and CO₂ in suspensions of sediment from the NE basin of Lake Fuchskuhle after addition of formate without sulfate (blue squares) or formate plus sulfate (gypsum) (red triangles) without CH₃F (open symbols) or with CH₃F (closed symbols). Controls with addition of only water (blue or red X crosses) are only shown occasionally. The panels show the temporal change of (a) concentrations of formate, (b) concentrations of acetate, (c) mixing ratios of CH₄ (1 ppmv = 10⁻⁶ bar), (d) mixing ratios of CO₂, (e) δ¹³C of formate, (f) δ¹³C of acetate, (g) δ¹³C of CH₄, and (h) δ¹³C of CO₂. Means ± SE.

Figure 4. Balance of produced acetate plus CH₄ (blue symbols) and of only CH₄ (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. The open and closed symbols denote conditions in the absence and the presence of CH₃F, respectively. The different symbols indicate three different replicates. The line indicate equimolarity (in terms of reducing equivalents between substrate and product).

Figure 5. Mariotti plots of formate consumption in (a) paddy soil from Vercelli and (b, c) sediment from the NE basin of Lake Fuchskuhle under methanogenic (blue symbols) and sulfidogenic (red symbols) conditions both in the absence (open symbols) and in the presence (closed symbols) of CH₃F. The different symbols indicate three different replicates.

Figure 6. Isotopic enrichment factors (εform, given as negative values) in paddy soils without and with addition of sulfate (gypsum) and CH₃F. Means ± 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 soils or sediments from Vercelli (blue), the IRRI (green), the NE basin (red) and the SW basin (yellow) in the absence (filled bars) and the presence (dotted bars) of CH$_3$F. Means ± SE.

Legends of the supplemental figures

Fig. S1: Formate conversion to acetate, CH$_4$ and CO$_2$ in suspensions of paddy soil from the International Rice Research Institute (IRRI) after addition of formate without sulfate (blue squares) or formate plus sulfate (gypsum) (red triangles) without CH$_3$F (open symbols) or with CH$_3$F (closed symbols). Controls with addition of only water (blue or red X crosses) are only shown occasionally. The panels show the temporal change of (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 ± SE.

Fig. S2: 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 the IRRI. The open and closed symbols denote conditions in the absence and the presence of CH$_3$F, respectively. The different symbols indicate three different replicates. The line indicate equimolarity (in terms of reducing equivalents between substrate and product.

Fig. S3: Formate conversion to acetate, CH$_4$ and CO$_2$ in suspensions of sediment from the SW basin of Lake Fuchskuhle after addition of formate without sulfate (blue squares) or formate plus sulfate (gypsum) (red triangles) without CH$_3$F (open symbols) or with CH$_3$F (closed symbols). Controls with addition of only water (blue or red X crosses) are only shown occasionally. The panels show the temporal change of (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 ± SE.

Fig. S4: 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 SW basin of Lake Fuchskuhle. The open and closed symbols denote conditions in the absence and the presence of CH$_3$F, respectively. The different symbols indicate three different replicates. The line indicate equimolarity (in terms of reducing equivalents between substrate and product.
Fig. S5: Mariotti plots of formate consumption in (a) paddy soil from the IRRI and (b, c) sediment from the SW basin of Lake Fuchskuhle under methanogenic (blue symbols) and sulfidogenic (red symbols) conditions both in the absence (open symbols) and in the presence (closed symbols) of CH$_3$F. The different symbols indicate three different replicates.