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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, 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_3F), 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 (ε_{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.:

$$4\ HCOOH \rightarrow CH_4 + 3\ CO_2 + 2\ H_2O \quad (1)$$

$$4\ HCOOH \rightarrow CH_3COOH + 2\ CO_2 + 2\ H_2O \quad (2)$$

$$4\ HCOOH + SO_4^{2-} + H^+ \rightarrow HS^- + 4\ CO_2 + 4\ H_2O \quad (3)$$

Formate may also be a substrate for syntrophic bacteria, which live from the little Gibbs free energy ($\Delta G^{0'} = -3.4$ 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.

$$HCOOH \rightarrow CO_2 + H_2 \quad (4)$$

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):

$$HCOOH \leftrightarrow CO_2 + H_2 \quad (5)$$

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 (ε) of the major metabolic processes is also important. The ε 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 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 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, 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 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, 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 1987 and finally in 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 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, those with sediments of Lake Fuchskuhle 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 $CaSO_4.2H_2O$, 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 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; (iv) 5 mL 200 mM sodium formate + 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 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 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 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; (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 $CaSO_4.2H_2O$ (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 N_2. The bottles were amended as above, but in addition 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 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 -20ºC 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 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‰, 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: 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_{form} associated with formate consumption was calculated from the temporal change of $\delta^{13}C$ of formate 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 (formate) at the beginning, and δ_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 ε_{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 ε 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 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 CH_3F (Fig. 1a). Similar results were obtained with IRRI soil (Fig. S1). 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 (Fig. 1c; S1c). It is noteworthy that CH_3F inhibited the production of CH_4 (Fig. 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 two times larger under sulfidogenic 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 only for <30%, in the presence of CH_3F even less (Fig. 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 (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 preincubtion 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, CH_3F only inhibited the production of CH_4 but not that of acetate or CO_2 (Fig. 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

205 CH_4 production in the water control (not amended with formate) was negligible (Fig. 3c;
206 S3c). Production of CO_2 started without lag phase but accelerated together with formate
207 consumption (Fig. 3d; S3d). In the lake sediments, CH_4 accounted only for <10% of formate
208 consumption, while acetate was the main product when sulfate was absent (Fig. 4a, S4a). In
209 contrast to the paddy soils, formate consumption in both lake sediments was much slower
210 under sulfidogenic than under methanogenic conditions (Fig. 3a; S3a). In the sediment from
211 SW basin, formate consumption was very slow so that less than half of the formate was
212 consumed during 80 days of incubation and consumption was not completed until the end of
213 the experiment (Fig. S3a). Very little acetate was produced and no CH_4 was formed from
214 formate in both lake sediments, when sulfate was present (Fig. 4b, S4b).
215
216 *3.2 Isotope fractionation during formate consumption*
217 In the rice paddy soils, $\delta^{13}C$-values of formate increased when formate was consumed
218 indicating discrimination against the heavy carbon isotope. This process was not affected by
219 CH_3F and was similar without and with sulfate (Fig. 1e; S1e). The same was the case with the
220 sediment from the NE lake basin, but only in the absence of sulfate (Fig. 3e). With sulfate,
221 the $\delta^{13}C$ of formate slowly decreased with time (Fig. 3e). In the sediment from the SW basin,
222 $\delta^{13}C$ of formate slowly decreased (without sulfate) or stayed constant with time (with sulfate)
223 (Fig. S3e). Note that formate was not completely consumed in the SW sediment when sulfate
224 was present (Fig. S3a).
225 Mariotti plots of $\delta^{13}C$ of formate as function of f_{form} resulted in negative slopes (Fig. 4;
226 S5). Hence, the enrichment factors (ε_{form}) for the paddy soils, both without and with sulfate,
227 and for the sediments from the NE basin of Lake Fuchskuhle without sulfate showed that the
228 light isotope of formate carbon was preferred. Values of ε_{form} were in the range of -8.5 to -
229 2.5‰ (Fig. 6). Under sulfidogenic conditions, however, the Mariotti plots of the sediments
230 from the NE basin (Fig. 5) did not show a negative slope and ε_{form} could not be determined.
231 The same was the case for the sediments from the SW basin (Fig. 6).
232 The negative ε_{form} indicates that products of formate should be depleted in ^{13}C. Indeed the
233 $\delta^{13}C$ of acetate and CH_4 were generally more negative than the $\delta^{13}C$ of formate. This was the
234 case in the paddy soils from Vercelli (Fig. 1f) and the IRRI (Fig. S1f) as well as in the
235 sediments from the NE basin (Fig. 3f) and the SW basin (Fig. S3f) of Lake Fuchskuhle. In the
236 sediment of the NE basin, the $\delta^{13}C$ of acetate increased from very low -95‰ to finally about -
237 57‰ in parallel with formate consumption (Fig. 3f). CO_2 was also produced during formate
238 degradation to various extent (equ.1, 2 and 3). Since the pH was in a range of pH 7 to pH 8,
239 CO_2 was also converted to bicarbonate. The $\delta^{13}C$ of bicarbonate is generally by about 10‰
240 more positive than the $\delta^{13}C$ of CO_2 (Stumm and Morgan, 1996). The $\delta^{13}C$ of the gaseous CO_2
241 was always close to the $\delta^{13}C$ of formate or was more positive. In the paddy soils and the NE
242 basin of Lake Fuchskuhle, the $\delta^{13}C$ of CO_2 increased in parallel with the increasing $\delta^{13}C$ of

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

The $\delta^{13}C$ values of the initial formate were about -24‰ (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‰ to -49‰ and -70‰ to -63‰, 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‰ to -52‰ and -78‰ to -72‰, 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‰ to -54‰ and -60‰ to -54‰, in the absence and presence of sulfate, respectively (Fig. 7). The $\delta^{13}C$ 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).

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 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 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_3F and the concomitant decrease of $\delta^{13}C$ of CH_4, which is characteristic for hydrogenotrophic methanogesis that is not inhibited by CH_3F (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/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 ATP 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 formate utilizers.

Formate consumption was recorded upon 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 micromolar (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 (ε_{form} of -8‰ to -2.5‰) determined from the change of $\delta^{13}C$ 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 2 CO_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 ε_{form} observed. (2) Formate first is completely converted to CO_2 plus H_2 (equ.5) or other electron equivalents. This reaction displays the ε_{form} determined by the Mariotti plots. Acetate is then produced via the WLP by the chemolithotrophic reduction of 2 CO_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‰ (Fig. 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 $\varepsilon_{ac\text{-}CH4}$ = -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 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_3F 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 ε_{form} of -8‰ to -3.5‰ (Fig. 5) and displaying a strong isotope effect with the formation of acetate ($\delta^{13}C$ = -57--52‰) and CH_4 ($\delta^{13}C$ = -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

357 acetate nor CH_4 was a major product of sulfidogenic formate degradation. Hence, formate
358 was apparently degraded according to equ.3 forming CO_2 as main carbon product. This
359 formation process displayed no depletion of the heavy carbon isotope, as the Mariotti plots of
360 $\delta^{13}C$ of formate did not exhibit a negative slope. The $\delta^{13}C$ of the CO_2 slowly decreased with
361 increasing fraction of formate consumed (Fig. 3h; 5c), probably involving isotope exchange
362 between formate and CO_2 (DeGraaf and Cappenberg, 1996). The little acetate, which was
363 formed, displayed a $\delta^{13}C$ of -77‰ (Fig. 7b) indicating that it was produced by a similar
364 mechanism as in the absence of sulfate, presumably via the WLP.
365 The strong differences between rice paddy soils and lake sediments were possibly caused
366 by their different microbial communities (Conrad et al., 2021). The differences were seen in
367 the composition of the *mcrA* and *dsrB* genes coding for methyl CoM reductase and
368 dissimilatory sulfate reductase, respectively, as well as the gene coding for the bacterial 16S
369 rRNA (data are shown in Conrad et al. (2021)) . The microbial community structures based
370 on these genes were similar whether the soils and sediments were amended with sulfate or
371 not. However, they were strongly different between soils and sediments (Conrad et al., 2021).
372 Unfortunately, these data do not allow to discriminate for particular taxa of homoacetogenic
373 bacteria. Nevertheless, it is possible that formate-consuming homoacetogens were more
374 prevalent in the soils than in the sediments and accordingly competed more or less with the
375 formate-consuming sulfate reducers.
376
377 *4.3 Conclusions*
378 Formate was found to be an excellent substrate for acetate formation in the paddy soils as
379 well as in the lake sediments, confirming and extending similar observations in a fen soil
380 (Hunger et al., 2011). In the anoxic soils, acetate was the major product even in the presence
381 of sulfate, which would have allowed sulfate reduction. The acetate was strongly depleted in
382 ^{13}C relative to formate, but the consumption of formate itself displayed only a small isotopic
383 enrichment factor. Therefore, it is likely that formate was disproportionated to ^{13}C-depleted
384 acetate and ^{13}C-enriched CO_2. The $\delta^{13}C$ of CO_2 was indeed slightly higher than that of
385 formate. Acetate was most likely produced by homoacetogenesis via the WLP. The produced
386 acetate was then used by aceticlastic methanogens (probably by *Methanothrix*), but only to
387 minor extent, resulting in further depletion of ^{13}C. The homoacetogenic bacteria in the paddy
388 soils apparently competed well with both methanogenic and sulfate-reducing
389 microorganisms, when formate was the substrate. The preference of homoacetogenesis as
390 degradation pathway is unexpected, since other substrates, such as acetate and propionate, are
391 degraded in these paddy soils by methanogenesis or sulfate reduction (Conrad et al., 2021)
392 (Conrad and Claus, 2023). Only in the lake sediments, formate oxidation by sulfate reduction
393 was more prevalent than homoacetogenesis.
394

Supplement link

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

Conflicting interests: The contact author has declared that neither of the authors has any competing interests.

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555

556

Figure legends

Figure 1. Formate conversion to acetate, CH_4 and CO_2 in suspensions of paddy soil from Vercelli (Italy) after 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 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 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, formate 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 line indicate equimolarity (in terms of reducing equivalents between substrate and product.

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_3F (open symbols) or with CH_3F (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 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, formate 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 line indicate equimolarity (in terms of reducing equivalents between substrate and product.

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. Three different replicates.

Figure 6. Isotopic enrichment factors (ε_{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

592 Lake Fuchskuhle without (left panel) and with (right panel) addition of sulfate (gypsum) and
593 CH_3F. Means ± SE.

594 **Figure 7.** Average $\delta^{13}C$ of formate (at the beginning of incubation) and of CO_2, acetate and
595 CH_4 (after the depletion of formate) in paddy soils from Vercelli (blue) and the IRRI (green),
596 and in sediments from the NE basin (red) and the SW basin (yellow) of Lake Fuchskuhle in
597 the absence (filled bars) and the presence (dotted bars) of CH_3F. Means ± SE.
598

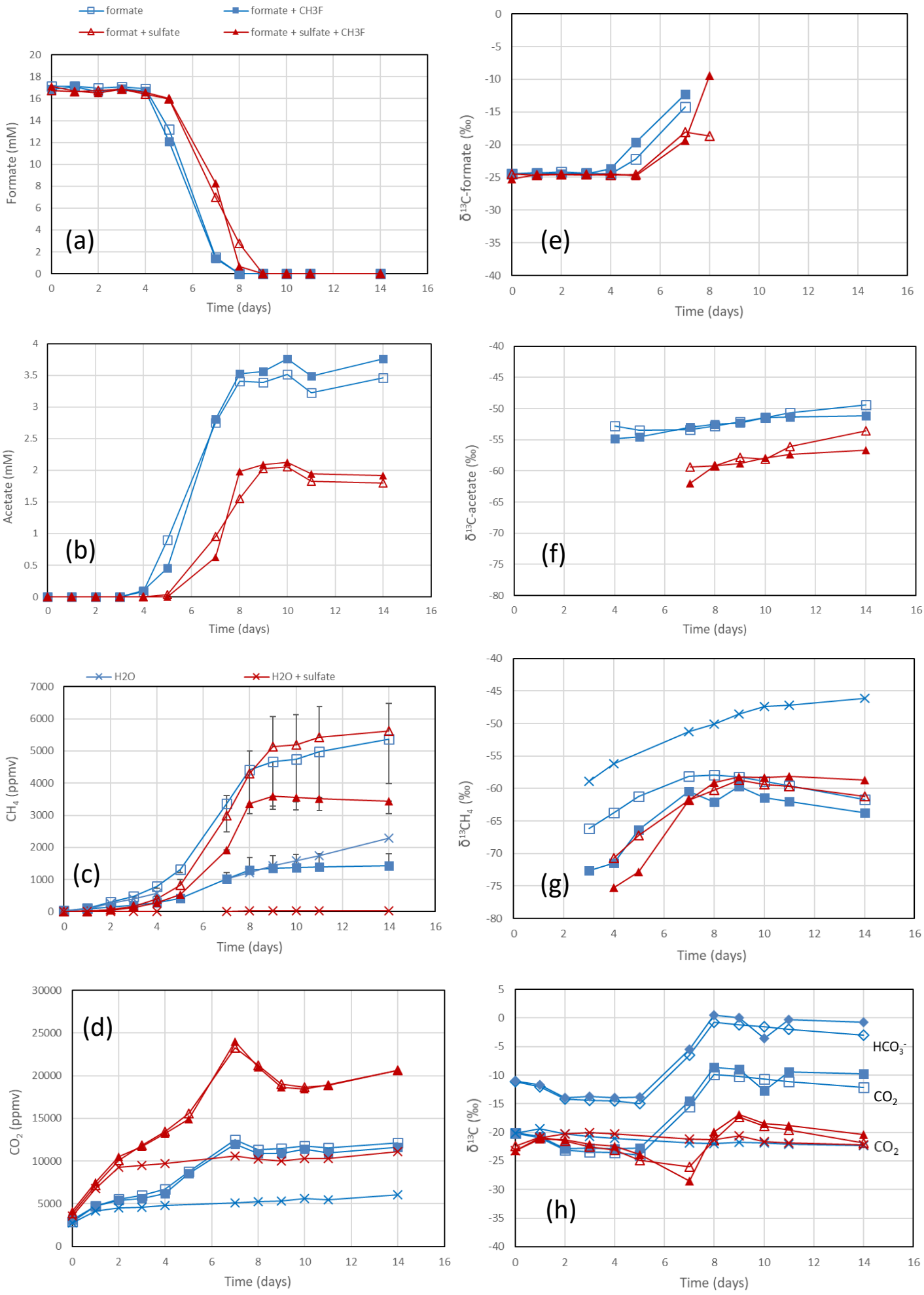

formate
formate + CH3F
format + sulfate
formate + sulfate + CH3F
Formate (mM)
Time (days)
(a)
δ13C-formate (‰)
(e)
Acetate (mM)
(b)
δ13C-acetate (‰)
(f)
H2O
H2O + sulfate
CH4 (ppmv)
(c)
δ13CH4 (‰)
(g)
CO2 (ppmv)
(d)
HCO3-
CO2
CO2
δ13C (‰)
(h)

Fig. 1

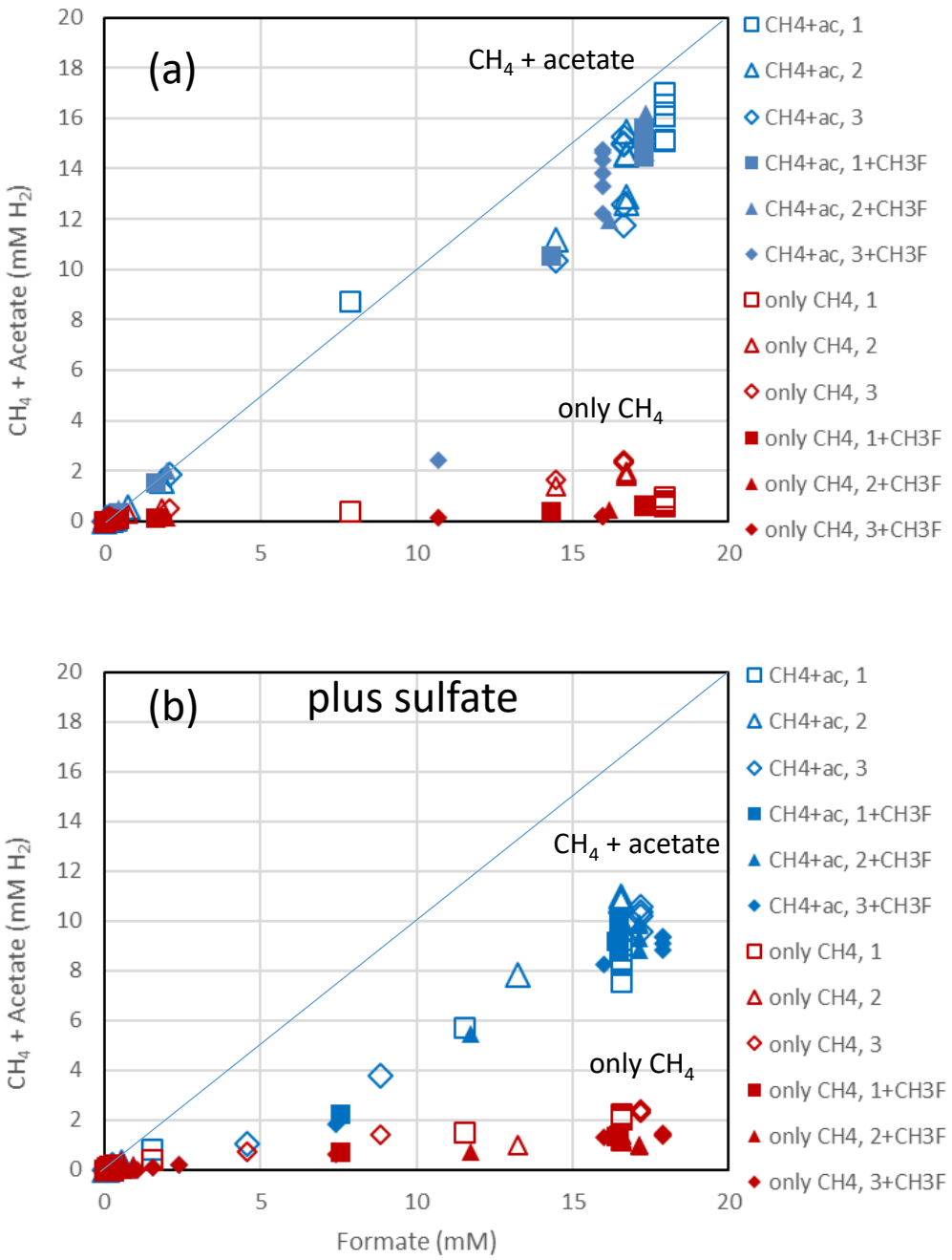

(a)
CH4 + acetate
only CH4
CH4 + Acetate (mM H2)
0
2
4
6
8
10
12
14
16
18
20
0
5
10
15
20
CH4+ac, 1
CH4+ac, 2
CH4+ac, 3
CH4+ac, 1+CH3F
CH4+ac, 2+CH3F
CH4+ac, 3+CH3F
only CH4, 1
only CH4, 2
only CH4, 3
only CH4, 1+CH3F
only CH4, 2+CH3F
only CH4, 3+CH3F
(b)
plus sulfate
CH4 + acetate
only CH4
CH4 + Acetate (mM H2)
Formate (mM)

Fig. 2

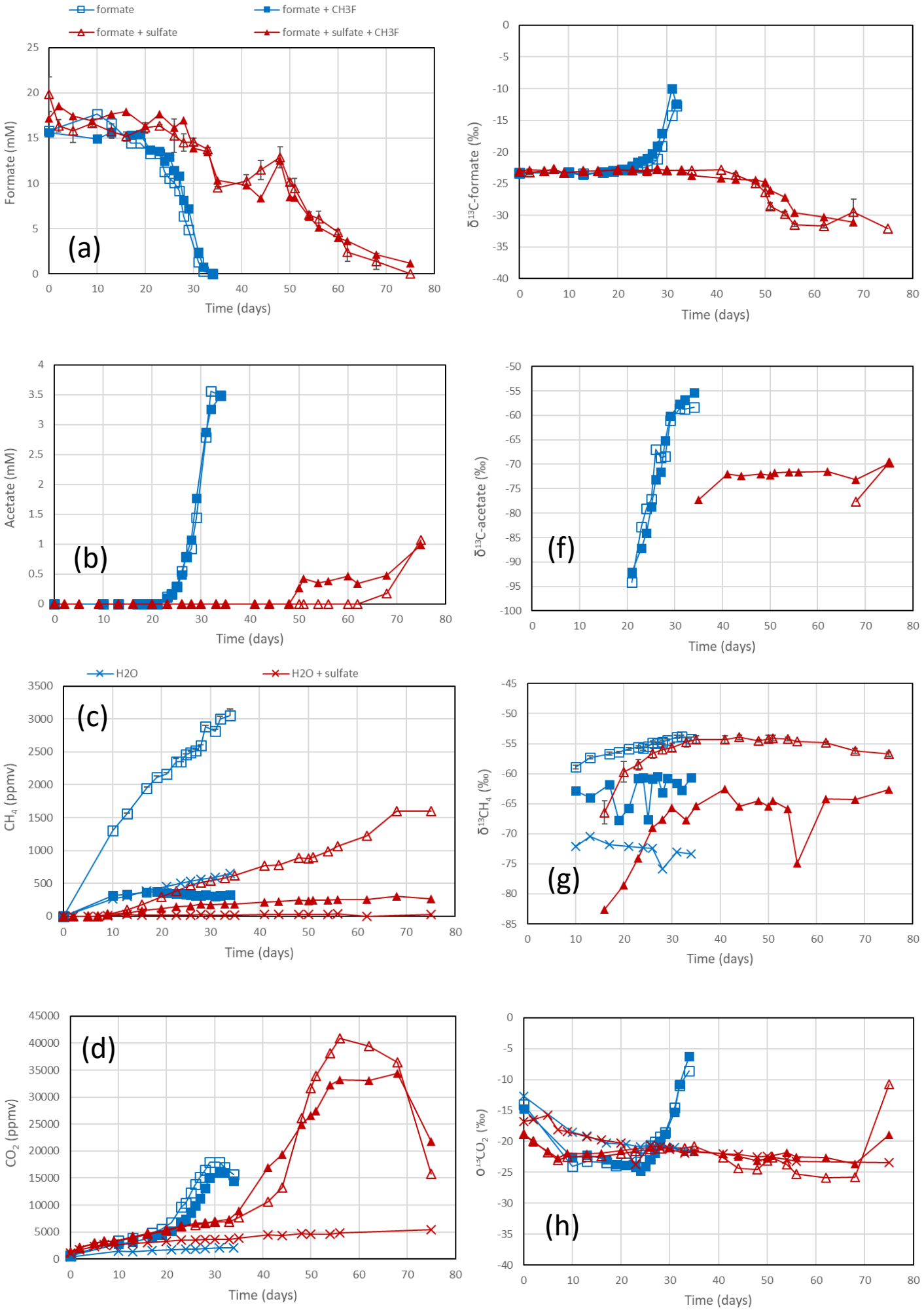

formate
formate + CH3F
formate + sulfate
formate + sulfate + CH3F
Formate (mM)
Time (days)
(a)
δ13C-formate (‰)
Time (days)
Acetate (mM)
Time (days)
(b)
δ13C-acetate (‰)
Time (days)
(f)
H2O
H2O + sulfate
CH4 (ppmv)
Time (days)
(c)
δ13CH4 (‰)
Time (days)
(g)
CO2 (ppmv)
Time (days)
(d)
δ13CO2 (‰)
Time (days)
(h)

Fig. 3

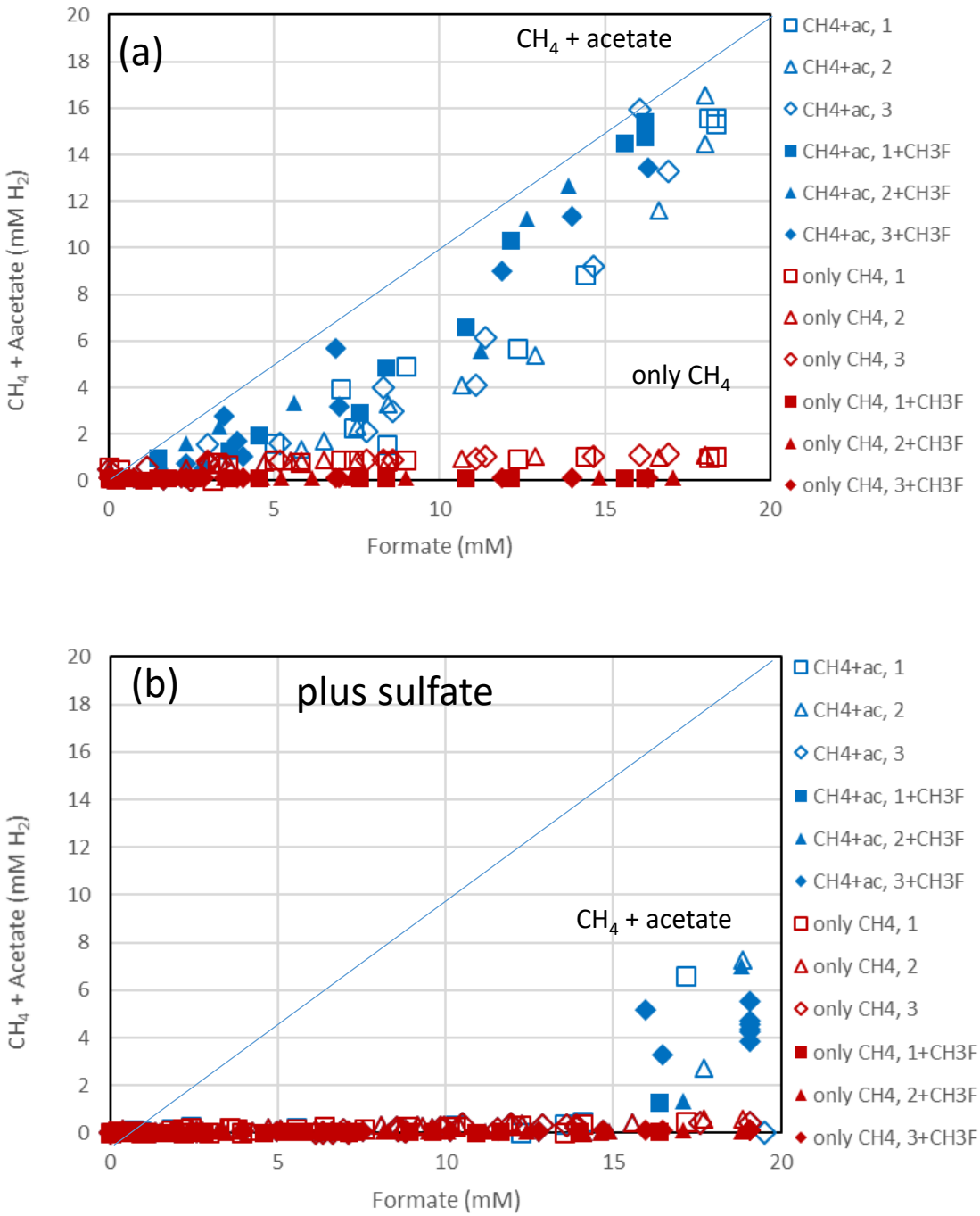
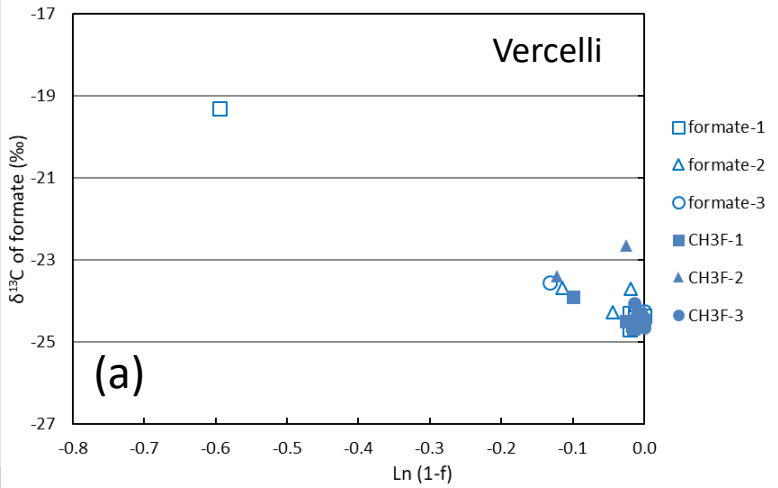
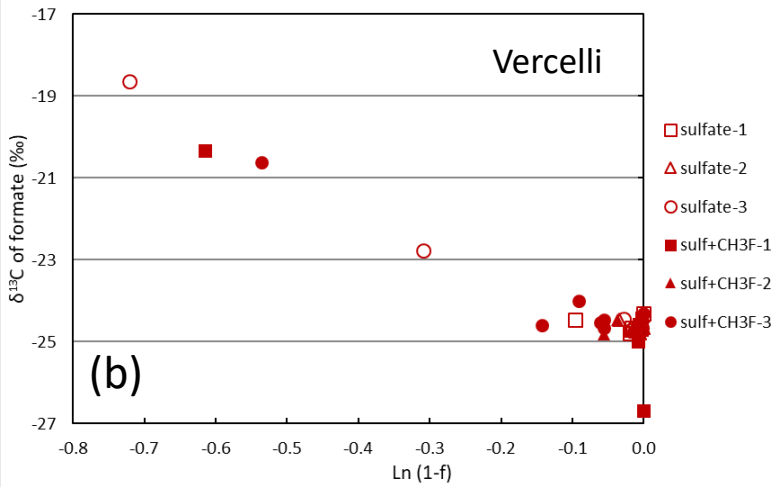
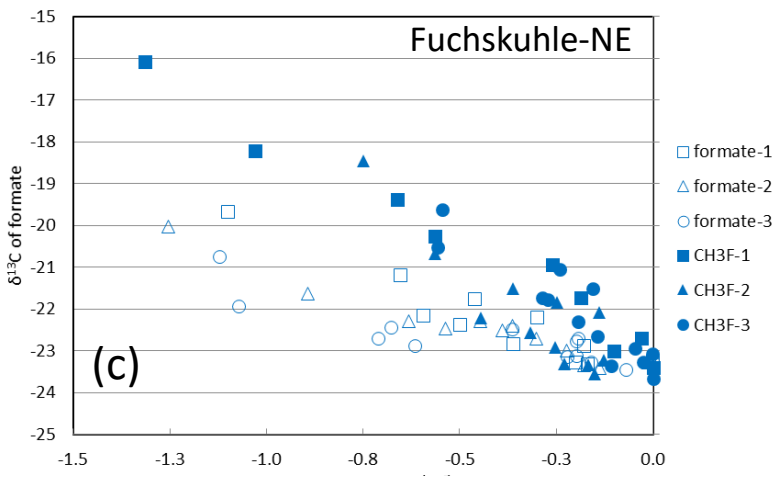

(a)
CH4 + acetate
only CH4
CH4 + Aacetate (mM H2)
Formate (mM)
0
2
4
6
8
10
12
14
16
18
20
0
5
10
15
20
CH4+ac, 1
CH4+ac, 2
CH4+ac, 3
CH4+ac, 1+CH3F
CH4+ac, 2+CH3F
CH4+ac, 3+CH3F
only CH4, 1
only CH4, 2
only CH4, 3
only CH4, 1+CH3F
only CH4, 2+CH3F
only CH4, 3+CH3F
(b)
plus sulfate
CH4 + acetate
CH4 + Acetate (mM H2)
Formate (mM)

Fig. 4


Vercelli
(a)
δ13C of formate (‰)
Ln (1-f)
formate-1
formate-2
formate-3
CH3F-1
CH3F-2
CH3F-3


Vercelli
(b)
δ13C of formate (‰)
Ln (1-f)
sulfate-1
sulfate-2
sulfate-3
sulf+CH3F-1
sulf+CH3F-2
sulf+CH3F-3


Fuchskuhle-NE
(c)
δ13C of formate
formate-1
formate-2
formate-3
CH3F-1
CH3F-2
CH3F-3

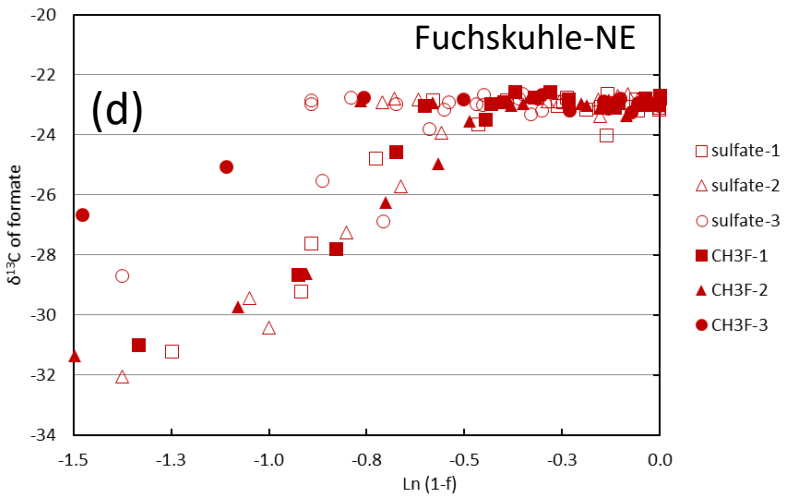

Fuchskuhle-NE
(d)
δ13C of formate
Ln (1-f)
sulfate-1
sulfate-2
sulfate-3
CH3F-1
CH3F-2
CH3F-3

Fig. 5

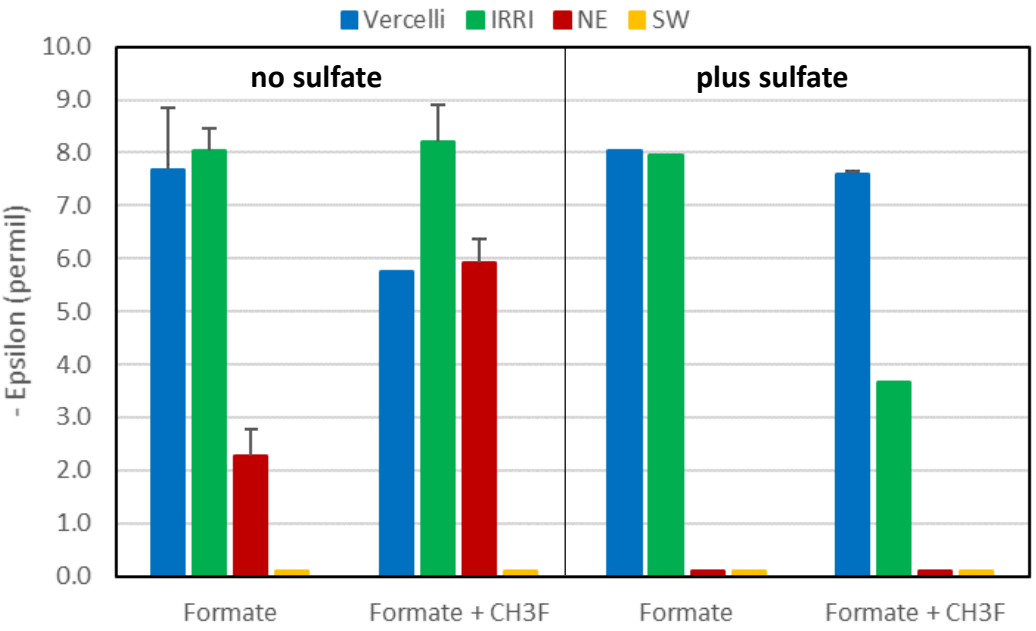

Vercelli
IRRI
NE
SW
no sulfate
plus sulfate
- Epsilon (permil)
10.0
9.0
8.0
7.0
6.0
5.0
4.0
3.0
2.0
1.0
0.0
Formate
Formate + CH3F
Formate
Formate + CH3F

Fig. 6

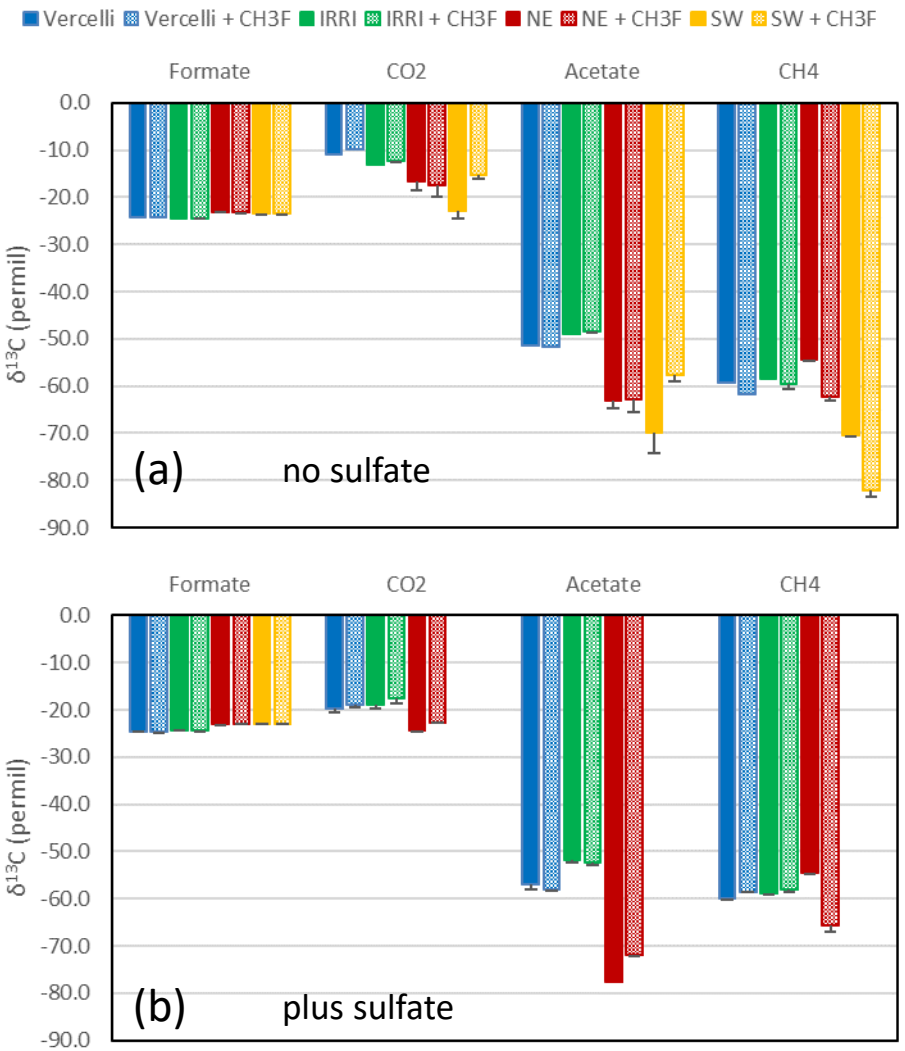

Vercelli
Vercelli + CH3F
IRRI
IRRI + CH3F
NE
NE + CH3F
SW
SW + CH3F
Formate
CO2
Acetate
CH4
δ13C (permil)
0.0
-10.0
-20.0
-30.0
-40.0
-50.0
-60.0
-70.0
-80.0
-90.0
(a)
no sulfate
(b)
plus sulfate

Fig. 7