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Fractionation of stable carbon isotopes during acetate consumption by methanogenic and sulfidogenic microbial communities in rice paddy soils and lake sediments

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Running head: Isotope fractionation by anaerobic acetate consumption

Abstract. Acetate is an important intermediate during the degradation of organic matter in anoxic flooded soils and sediments. Acetate is disproportionated to CH_4 and CO_2 by methanogenic or is oxidized to CO_2 by sulfate-reducing microorganisms. These reactions result in carbon isotope fractionation, depending on the microbial species and their particular carbon metabolism. To learn more about the magnitude of the isotopic enrichment factors (ε) involved, acetate conversion to CH_4 and CO_2 was measured in anoxic paddy soils from Vercelli (Italy) and the International Rice Research Institute (IRRI, the Philippines) and in anoxic lake sediments from the north east (NE) and the south west (SW) basins of Fuchskuhle (Germany). Acetate consumption was measured using samples of paddy soil or lake sediment suspended in water or in phosphate buffer (pH 7.0), both in the absence and presence of sulfate (gypsum), and of methyl fluoride (CH_3F), an inhibitor of aceticlastic methanogenesis. Under methanogenic conditions, values of ε_{ac} for acetate consumption were always in a range of -21‰ to -17‰, but higher in the lake sediment from the SW basin (-11‰). Under sulfidogenic conditions ε_{ac} values tended to be slightly lower (-26‰ to -19‰) especially when aceticlastic methanogenesis was inhibited. Again, ε_{ac} in the lake sediment of the SW basin was higher (-18‰ to -14‰). Determination of ε_{CH4} from the accumulation of ^{13}C in CH_4 resulted in much lower values (-37‰ to -27‰) than from the depletion of ^{13}C in acetate (-21‰ to -17‰), especially when acetate degradation was measured in buffer suspensions. The microbial communities were characterized by sequencing the bacterial 16S rRNA genes as well as the methanogenic *mcrA* and sulfidogenic *dsrB* genes. The microbial communities were quite different between lake sediments and paddy soils, but were similar in the sediments of the two lake basins and in the soils from Vercelli and IRR, and were similar after preincubation without and with addition of sulfate (gypsum). The different microbial compositions could hardly serve for the prediction of the magnitude of enrichment factors.

1 Introduction

Organic matter degradation under anaerobic conditions results in the production of CO_2, when electron acceptors such sulfate (sulfidogenic conditions) are available, and in the production of CH_4 and CO_2, when they are absent (methanogenic conditions). Carbon dioxide is not only end product, but can also serve as an intermediate. It can for example be converted by chemolithotrophic microorganisms to CH_4 or to acetate. These conversion reactions of CO_2 have an isotope effect and result in products that are strongly depleted in ^{13}C, expressing isotope enrichment factors (ε) being on the order of -70 to -50‰ (Blaser and Conrad, 2016). However, the conversion of acetate to CO_2 by sulfidogenic reactions or to CH_4 plus CO_2 by methanogenic reactions can also have quite substantial enrichment factors, often being on the order of about -20‰ (Goevert and Conrad, 2009; Goevert and Conrad, 2010).

Knowledge of enrichment factors is important for the quantification of the pathways involved in anaerobic degradation of organic matter. For example, the relative contribution of hydrogenotrophic and aceticlastic methanogenesis to total CH_4 production can be determined in situ from analytical values of ^{13}C in organic matter, CO_2 and CH_4, if the enrichment factors (ε) are known for the reduction of CO_2 to CH_4 (hydrogenotrophic methanogenesis) and the cleavage of acetate to CH_4 and CO_2 (aceticlastic methanogenesis)(Conrad, 2005). While ε of the former reaction can be experimentally determined by incubation in the presence of CH_3F (Chan et al., 2005; Conrad et al., 2007; Holmes et al., 2014; Penning et al., 2006b), that of the latter reaction is not so easy to determine. So far reference values are mainly available from relatively few experiments with pure microbial cultures. This is true for both aceticlastic methanogenic archaea (Gelwicks et al., 1994; Goevert and Conrad, 2009; Krzycki et al., 1987; Penning et al., 2006a; Valentine et al., 2004; Zyakun et al., 1988) and acetate-oxidizing sulfidogenic bacteria (Goevert and Conrad, 2008; Goevert and Conrad, 2010; Londry and DesMarais, 2003).

There are quite some studies of methanogenic and sulfidogenic microbial communities in environmental samples, notably in paddy soils from Italy and the Philippines (Liu et al., 2019; Liu et al., 2018a; Liu et al., 2018b; Wörner et al., 2016) and in sediments from Lake Fuchskuhle (Chan et al., 2002). However, there are hardly studies of environmental samples,

in which ε values of acetate consumption were determined together with the composition of the methanogenic and sulfidogenic microbial communities (Blair and Carter Jr., 1992; Chidthaisong et al., 2002; Conrad et al., 2009; Goevert and Conrad, 2009; Penning et al., 2006a). In order to constrain the magnitude of such ε values, we decided to investigate the stable carbon isotope fractionation during consumption of acetate in methanogenic and sulfidogenic rice paddy soils and anaerobic lake sediments.

2 Materials and Methods

2.1 Environmental samples

The soil samples were from the research stations in Vercelli, Italy and the International Rice research Institute (IRRI) in the Philippines. Sampling and soil characteristics were described before (Liu et al., 2018b). The 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).

2.2 Paddy soils

Two different experimental setups were used using soil suspensions in water (unbuffered suspensions) or in 20 mM potassium phosphate buffer (pH 7.0)(buffered suspensions). For the unbuffered suspensions, paddy soil was mixed with autoclaved anoxic H_2O at a ratio of 1:1 and incubated under N_2 at 25°C (which is close to the in-situ temperature) for 4 weeks. Then, 5 ml preincubated soil slurry was incubated at 25°C with 45 ml 5.6 mM sodium acetate in a 150-ml bottle under an atmosphere of N_2. The bottles were (i) unamended; (ii) amended with 4.5 ml CH_3F; (iii) amended with 200 µl of a gypsum ($CaSO_4.2H_2O$) suspension (corresponding to a concentration of 2.5 M sulfate) giving a final concentration of 10 mM sulfate. The experiment was performed in 4 replicates.

For the buffered suspensions, paddy soil was mixed with autoclaved anoxic H_2O at a ratio of 1:1 and incubated under N_2 at 25°C for 4 weeks. In a second incubation, paddy soil was mixed with autoclaved anoxic H_2O at a ratio of 1:1, was amended with 0.07 g $CaSO_4.2H_2O$, and then incubated under N_2 at 25°C for 4 weeks. These two preincubated soil slurries were

sampled and stored (in one replicate) at -20°C for later molecular analysis. The preincubated soil slurries were also used (in 3 replicates) for the following incubation experiments. Three different sets of incubations were prepared. In the first set (resulting in methanogenic conditions), 5 ml soil slurry preincubated without sulfate was incubated at 25°C with 40 ml 20 mM potassium phosphate buffer (pH 7.0) in a 150-ml bottle under an atmosphere of N_2. The bottles were the amended with (i) 5 ml H_2O; (ii) 5 ml H_2O + 4.5 ml CH_3F; (iii) 5 ml 50 mM sodium acetate; (iv) 5 ml 50 mM sodium acetate + 4.5 ml CH_3F. In the second set (resulting in mainly methanogenic conditions), again 5 ml soil slurry preincubated without sulfate was incubated at 25°C with 40 ml 20 mM potassium phosphate buffer (pH 7.0) in a 150-ml bottle under an atmosphere of N_2. The amendments were the same as above, but with the addition of 200 µl of a $CaSO_4$ suspension giving a final concentration of 10 mM sulfate. In the third set (resulting in sulfidogenic conditions), 5 ml soil slurry preincubated with sulfate was incubated at 25°C with 40 ml 20 mM potassium phosphate buffer (pH 7.0) in a 150-ml bottle under an atmosphere of N_2. The amendments were the same as above, but with the addition of 200 µl of a $CaSO_4$ suspension corresponding to a concentration of 2.5 M (giving a final concentration of 10 mM sulfate).

2.3 Lake sediments

The lake sediments (NE and SW basins) of Lake Fuchskuhle were sampled and stored (in one replicate) at -20°C for later molecular analysis. For methanogenic conditions, 5 ml lake sediment was incubated in 3 replicates at 10°C (which is close to the in-situ temperature) with 45 ml 20 mM potassium phosphate buffer (pH 7.0) in a 150-ml bottle under an atmosphere of N_2. The bottles were the amended with (i) 5 ml H_2O; (ii) 5 ml H_2O + 4.5 ml CH_3F; (iii) 5 ml 50 mM sodium acetate; (iv) 5 ml 50 mM sodium acetate + 4.5 ml CH_3F.

Part of the 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. The preincubated lake sediments were sampled and stored (in one replicate) at -20°C for later molecular analysis. For sulfidogenic conditions, 5 ml of the preincubated sediment was incubated in 3 replicates at 10°C with 40 ml 20 mM potassium phosphate buffer (pH 7.0) in a 150-ml bottle under an

atmosphere of N_2. The bottles were 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.

2.4 Extraction of DNA and amplicon sequencing

The lake sediments or paddy soils in phosphate buffer were collected by centrifugation (11000 × g, 4°C, 5 min). Genomic DNA were extracted with NucleoSpin Soil Kit (Macherey-Nagel, Düren, Germany) by following the user's manufacture. DNA were checked by gel electrophoresis (1% agarose in TEA buffer, stained with GelRed) and quantified by Qubit 2.0. The amplification of 16S rRNA, *mcrA* and *dsrB* gene were done as described previously (Liu and Conrad, 2017). This publication also shows in Table S9 all the primers used. In brief, First step PCR, for 16S rRNA, h515-Y / h926R primers were used with the following PCR protocol: 94°C for 3 min; 15 cycles with 94°C for 30 s, 52°C for 30 s and 68°C for 60 s; 68°C for 10 min and hold at 8°C. For *mcrA*, hmlas-mod-F / hmcra-rev-R primers were used with the following PCR protocol: 94°C for 4 min; 15-18 cycles with 94°C for 30 s , 60 by 1°C to 55°C for 30 s and 68°C for 60 s; 68°C for 10 min and hold at 8°C. For *dsrB* hDSR1762Fmix / hDSR2010Rmix primers were used with the following PCR protocol: 94°C for 3 min; 25 cycles with 94°C for 30 s, 60°C by 1°C to 50 °C for 30 s and 68°C for 60 s; 68°C for 10 min and hold at 8°C.

In the second step PCR, barcode-head primers were used for the PCR products of 16S rRNA, mcrA and *dsrB* obtained from the first step with the following PCR protocol: 94°C for 3 min; 10-20 cycles with 94°C for 30 s, 52°C for 30 s and 68°C for 60 s; 68°C for 10 min and hold at 8°C.

PCR amplicons were purified by AMP xx for both the first and second step PCR. After quantification, the individual barcoded amplicons of 16S rRNA gene and *dsrB* were mixed in equimolar concentrations, with 16S rRNA gene amplicons added in double amounts. Library was sequenced on an ILLUMINA HISEQ 2000 system using 2×250 cycle combination mode

by Max Planck-Genome-Centre (Cologne, Germany). For *mcrA*, individual barcoded amplicons were mixed in equimolar concentrations and library was sequenced on an ILLUMINA MISEQ system using 2 × 300 cycle combination mode by Max Planck-Genome-Centre (Cologne, Germany).

2.5 Amplicon sequence data processing

Amplicon Sequence data were analyzed according to pipeline as described previously (Liu and Conrad, 2017). In brief, paired-end reads were first merged by USEARCH and 16S rRNA gene and *dsrB* datasets were separated by primer sequences using CUTADAPT and demultiplexed using QIIME1. Datasets of *mcrA* were demultiplexed using QIIME1directly. All reads were subjected to quality control, *de novo* chimera filtering, singleton filtering and OTU clustering according to the UPARSE pipeline. Species level OTUs for 16S rRNA gene were obtained at 97% sequence identity. Approximate species-level dsrB and *mcrA* OTUs were obtained with the gene-specific OTU threshold 0.90 (Pelikan et al., 2016) and 0.84 (Yang et al., 2014). Taxonomic identities of the OTUs of 16S rRNA gene were assigned with the Ribosomal Database Project Classifier against the SILVA 123 SSU Ref database (Pruesse et al., 2007). Nucleotide sequences of *dsrB* and *mcrA* were initially translated into amino acid sequences using FrameBot (Wang et al., 2013). For *dsrB* gene, amino acid sequences were aligned to the DsrAB reference sequence alignment (Pelikan et al., 2016) using MAFFT (Katoh and Standley, 2013). Subsequently, the taxonomic classification of each *dsrB* OTU was analysed using the Evolutionary Placement Algorithm (EPA) in RAXML (Berger et al., 2011). For *mcrA* gene, amino acid sequences of each OTUs and updated full length *mcrA* amino acid sequences from NCBI were imported into a *mcrA* reference ARB database developed by Angel et al. (2012). The taxonomic classification of each *mcrA* OTU was analysed by phylogenetic tree construction using Maximum parsimony implemented in ARB software (Ludwig et al., 2004).

186 *2.6 Chemical and isotopic analyses*

187 Chemical and isotopic analyses were performed as described in detail previously (Goevert
188 and Conrad, 2009). Methane was analyzed by gas chromatography (GC) with flame
189 ionization detector. Carbon dioxide was analyzed after conversion to CH_4 with a Ni catalyst.
190 Stable isotope analyses of $^{13}C/^{12}C$ in gas samples were performed using GC-combustion
191 isotope ratio mass spectrometry (GC-C-IRMS). Acetate was measured using high-
192 performance liquid chromatography (HPLC) linked via a Finnigan LC IsoLink to an IRMS.
193 The isotopic values are reported in the delta notation ($\delta^{13}C$) relative to the Vienna Peedee
194 Belemnite standard having a $^{13}C/^{12}C$ ratio ($R_{standard}$) of 0.01118: $\delta^{13}C = 10^3$ ($R_{sample}/R_{standard} -$
195 1). The precision of the GC-C-IRMS was ± 0.2‰, that of the HPLC-IRMS was ± 0.3‰. The
196 carbon of the sodium acetate that was used in the incubation experiments had the following
197 $\delta^{13}C$ values: total acetate, -24.4‰; acetate-methyl, -27.9‰; acetate-carboxyl, -20.9‰.
198
199 *2.7 Calculations*

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

201 $$\alpha_{A/B} = (\delta_A + 1000)/ (\delta_B + 1000) \quad (1)$$

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

205 $$\delta_r = \delta_{ri} + \varepsilon [\ln(1-f)] \quad (2)$$

206 and from the product formed

207 $$\delta_p = \delta_{ri} - \varepsilon (1 - f) [\ln(1-f)]/f \quad (3)$$

208 where δ_{ri} is the isotopic composition of the reactant (acetate) at the beginning, δ_r is the
209 isotopic composition of the residual acetate and and δ_p that of the product (CH_4), both at the
210 instant when f is determined. f is the fractional yield of the products based on the
211 consumption of acetate ($0 < f < 1$). Linear regression of $\delta^{13}C$ of acetate against $\ln(1 - f)$ yields
212 ε_{ac} as the slope of best fit lines. Similarly, linear regression of $\delta^{13}C$ of CH_4 against $(1 - f)$
213 $[\ln(1-f)]/f$ yields ε_{CH4} as the slope of best fit lines. The regressions of $\delta^{13}C$ of acetate were
214 done for data in the range of $f < 0.5$. The linear regressions of $\delta^{13}C$ of CH_4 were done either

215 for the entire data range of again only for $f < 0.5$. The linear regressions were done
216 individually for each experimental replicate (n = 3-4) and were only accepted if $r^2 > 0.8$ for
217 paddy soils or $r^2 > 0.7$ for lake sediments. The ε values resulting from the replicate
218 experiments were then averaged (± SE).
219 For mass balance calculations, total inorganic carbon (TIC) was calculated as the sum of
220 gaseous CO_2, dissolved CO_2 and bicarbonate using the measured data of gaseous CO_2, the pH
221 and the relevant solubility and equilibrium constants (Stumm and Morgan, 1996).
222
223 **3 Results**
224 *3.1 Incubation of unbuffered suspensions of rice field soils*
225 Incubation of unbuffered suspensions (soil + H_2O) of rice field soil from the International
226 Rice Research Institute (IRRI) of the Philippines with acetate under anoxic conditions
227 resulted in the depletion of acetate and the release of CH_4 and CO_2 (Fig. 1A, C). In the
228 presence of CH_3F, an inhibitor of aceticlastic methanogenesis (Janssen and Frenzel, 1997),
229 acetate was no longer consumed, and production of CH_4 and CO_2 was inhibited. However,
230 addition of sulfate had only little effect on acetate consumption and the production of CH_4 and
231 CO_2 (Fig. 1A, C). Both in the presence and the absence of sulfate, $\delta^{13}C$ of the residual acetate
232 and the produced CH_4 increased, whereas $\delta^{13}C$ in CO_2 stayed relatively stable (Fig. 1B, D). In
233 the presence of CH_3F, the $\delta^{13}C$ in CH_4 was much more negative than in the absence. The
234 results were similar for soil from the Rice Research Station in Vercelli (Italy) (Fig.S1).
235 Mariotti plots of the ^{13}C of acetate as function of the fractions (*f*) of acetate consumed
236 resulted in similar curves for all four replicates of the incubations of IRRI soil without (Fig.
237 2A) and with (Fig. 2B) sulfate amendment. The lines were straight for $f < 0.5$ (<50% of
238 acetate consumed). The same was the case for Mariotti plots of $^{13}CH_4$, the product of acetate
239 consumption (Fig. 2C, D). The enrichment factors ε, which were calculated from the Mariotti
240 plots were in a range of -22‰ to -19‰, irrespectively whether they were determined in the
241 presence or the absence of sulfate and whether they were determined from acetate depletion
242 or from CH_4 formation (Fig. 3; Table S1). Similar Mariotti plots were obtained for Vercelli
243 soil (Fig. S2), which resulted in ε values ranging between -20‰ to -17‰, except the ε

244 determined for CH_4 production in the presence of sulfate, which was only -14 ± 1.4‰ (Fig.
245 3).
246 Mass balance calculations showed that on a molar basis the accumulated CH_4 amounted to
247 about 90% of the consumed acetate (the methyl group) in the absence and to about 71% in the
248 presence of sulfate in the IRRI soil and to 97% and 76%, respectively, in the Vercelli soil.
249
250 *3.2 Incubation of buffered suspensions of rice field soils*
251 The experiments with rice field soils were repeated using soil slurries suspended in
252 phosphate buffer. This was done to run the experiment at a constant pH 7.0. In Vercelli soil,
253 acetate was consumed and CH_4 and CO_2 were produced (Fig. 4A, C, E). The $\delta^{13}C$ of the
254 residual acetate and the produced CH_4 increased as acetate consumption proceeded (Fig. 4B,
255 D). The $\delta^{13}C$ of the produced CO_2 first decreased and later increased (Fig. 4F). This happened
256 also, when the soil suspensions were incubated in the presence of sulfate after preincubation
257 with sulfate (Fig. 4F), but CH_4 production was lower and CO_2 production was higher in the
258 presence than in the absence of sulfate (Fig. 4C, E). Production of CH_4 ceased in the presence
259 of sulfate after about 10 d (Fig. 4C). Addition of CH_3F completely inhibited CH_4 production
260 both in the presence and absence of sulfate (Fig. 4C). It also inhibited CO_2 production but
261 only in the absence of sulfate (Fig. 4E). In the presence of sulfate, CH_3F only delayed but did
262 not inhibit acetate consumption and CO_2 production (Fig. 4A, E), and also did not prevent the
263 increase of $\delta^{13}C$ in the residual acetate (Fig. 4B).
264 Mass balance calculations showed that while acetate (the methyl group) was almost
265 completely degraded to CH_4 in the absence of sulfate, it accounted, after a delayed inhibition,
266 for only about a third in the presence of sulfate (Fig. 5). When the soil suspensions were
267 incubated in the presence of sulfate but without preincubation, CH_4 production was only
268 slightly less than in the incubations without sulfate (Fig. 5). The complete set of these
269 experiments is shown in Fig. S3. When CH_4 production was inhibited, CO_2 production
270 apparently was a substitute, since the consumed acetate was always rather well balanced by
271 the production of both CH_4 and TIC together (Fig. S4). The same experimental setup was
272 used for IRRI soil. The results were similar and are shown in the supplementary (Fig. S5, S6,

S7). In IRRI soil suspensions, the mass balance between the production of CH_4 + TIC and the acetate consumed was improved when the production was corrected with the background production in a control without addition of acetate (Fig. S8).

Mariotti plots of acetate consumption and CH_4 production in both Vercelli soil (Fig. S9) and IRRI soil (Fig. S10) could be created for all the different incubation conditions, in which acetate was consumed, i.e. in the absence of sulfate (control), in the presence of sulfate, and in the presence of sulfate after preincubation with sulfate. Enrichment factors (ε) were calculated for fractions of acetate consumption with $f < 0.5$ (Fig. 3; Table S1). The ε values for acetate consumption were similar for the experiments without and with sulfate and ranged between -21‰ and -17‰. However, the ε values for CH_4 production were systematically lower, ranging between -37‰ and -23‰ (Fig. 3; Table S1). Since acetate consumption in the presence of sulfate was also possible when CH_4 production was inhibited by CH_3F, Mariotti plots could also be created for these conditions (Fig. S11). The resulting ε values were similar than those in the absence of sulfate and ranged for Vercelli soil between -24‰ and -22‰ (Fig. 3; Table S1). Only in the IRRI soil ε values were higher (-10‰), but only when the soil had been preincubated with sulfate (Fig. 3; Table S1).

3.3 Incubation of buffered suspensions of lake sediments

Experiments with lake sediments were done analogous to those with rice filed soils. Slurries of sediment from the NE and SW basins of Lake Fuchskuhle were suspended in phosphate buffer pH 7.0 in the absence and the presence of sulfate (after preincubation with sulfate) and without and with addition of CH_3F. In the sediment from the NE basin acetate was consumed after a lag phase, first (after about 40 d) in the incubations with sulfate, then (after about 60 d) also in the incubations without sulfate (Fig. 6A). Addition of CH_3F only partially inhibited the acetate consumption in the absence of sulfate, and did not at all inhibit the acetate consumption in the presence of sulfate (Fig. 6A). However, CH_3F almost completely inhibited the production of CH_4, and also inhibited almost completely the increase of the $\delta^{13}C$ in the residual acetate when sulfate was absent (Fig. 6B). Presence of sulfate also strongly inhibited CH_4 production (Fig. 6C). The small amounts of CH_4 produced showed a

302 rather constant $\delta^{13}C$ of about -40‰ in the absence and of -90 to -80‰ in the presence of
303 CH_3F (Fig. 6D). Without sulfate, by contrast, the $\delta^{13}C$ in CH_4 was first about -70‰ and then
304 with acetate consumption progressively increased to about -40‰ in the absence and decreased
305 to about -90‰ in the presence of CH_3F (Fig. 6D). Mass balance calculations showed that CH_4
306 production in the presence of sulfate accounted on a molar basis only for about 5% of the
307 acetate consumed, while in the absence of sulfate CH_4 production accounted for about 45%
308 (Fig. 5). In the sediment from the SW basin, the contribution of CH_4 production to acetate
309 consumption was even lower (about 30%)(Fig. 5). These low values are noteworthy in
310 comparison to those found in the rice field soils (Fig. 5). In the presence of sulfate, acetate
311 was almost exclusively converted to CO_2, which strongly increased during the time of acetate
312 consumption exhibiting a relatively good mass balance (Fig. S4, S8). This was also the case in
313 the incubations without sulfate, indicating that a rather large fraction of the acetate was
314 converted to CO_2 rather than CH_4 (Fig. S4, S8). The $\delta^{13}C$ of the produced CO_2 strongly
315 decreased from about -30‰ to about -55‰ during the period of acetate consumption (Fig. 6F)
316 and then slowly increased back to -30‰, when about 50% of the acetate had been consumed
317 (Fig. 6A). The experimental results were similar in the incubations with sediment from the
318 SW basin, which are shown in the supplement (Fig. S12, S13).
319 Mariotti plots of acetate consumption could be generated for all incubation conditions both
320 in the NE and in the SW basin of Lake Fuchskuhle (Fig. S14, S15). These plots allowed the
321 calculation of ε values, which were generally higher (-20‰ to -19‰) in the NE (-20‰ to -
322 19‰) than the SW basin (-14‰ to -11‰). For CH_4 production, useful Mariotti plots could
323 only be generated for incubations without sulfate resulting in ε values, which were higher (-
324 28‰ to -27‰) than those calculated from acetate consumption (Fig. 3; Table S1). Mariotti
325 plots of acetate consumption could be generated for incubations with sulfate, in which the
326 very low CH_4 production was further inhibited by CH_3F (Fig. S16). The ε values of these
327 incubations (NE and the SW basin, respectively) were lower (-26‰, and -24‰) than those
328 without CH_3F (-20‰ and -14‰) (Fig. 3; Table S1).

329

330 *3.4 Microbial community composition*

331 The composition of the microbial communities was determined at the beginning of
332 incubation, after preincubation without and with sulfate in the rice field soil and lake sediment
333 suspensions by targeting three different genes, i.e., *mcrA* (methyl CoM reductase), *dsrB*
334 (dissimilatory sulfate reductase), and the bacterial 16S rRNA gene. The compositions of
335 microorganisms represented by all three genes were quite different between the rice field soils
336 and the lake sediments, while the differences within the individual samples of either soils or
337 sediments were smaller (Fig. 7).

338 In the sediments of both basins of Lake Fuchskuhle, the methanogenic archaea
339 (represented by *mcrA*) were dominated by *Methanomicrobiales* and *Methanosaetaceae*, while
340 *Methanomassiliicoccales* and *Methanosarcinaceae* contributed less (Fig. 7A). In the rice field
341 soils, the methanogenic taxa were more diverse comprising 6 different orders or families, with
342 putatively aceticlastic *Methanosarcinaceae* being relatively more abundant than
343 *Methanosaetaceae*. In addition, *Methanomicrobiales* contributed only little compared to
344 *Methanobacteriales* and especially *Methanocellales*. In general, there was only a marginal
345 difference in composition between the incubations in the absence and the presence of sulfate.
346 A statistical analysis of these marginal differences was not warranted and was also not
347 possible due to the fact that only one replicate each was assayed.

348 The composition of putative sulfate reducers (represented by *dsrB*) was also only little
349 different between the incubations with and without sulfate addition (Fig. 7B). However, the
350 composition between rice field soils and lake sediments was completely different. While rice
351 field soils were dominated by members of the uncultured-family-level lineages 9 and 5, the
352 lake sediments were dominated by the *Desulfobacca acetoxidans* lineage. Compared to IRRI
353 soil the relative abundance of *Syntrophobacteraceae* was larger in Vercelli soil, where it
354 increased upon treatment with sulfate. In the lake sediments, the relative abundances of
355 *Syntrophobacteraceae* and members of environmental superclusters were similar. In the lake
356 sediments there was only some minor quantitative difference between the two basins, while
357 the difference between Vercelli and IRRI soil was more pronounced. For example, compared

to IRRI soil the relative abundance of *Syntrophobacteraceae* was larger in Vercelli soil, where it increased upon preincubation with sulfate.

The composition of Bacteria in general (represented by the 16S rRNA gene) was again most different between rice field soils and lake sediments, while differences between Vercelli and IRRI soils and also between sediments from the NE and SW lake basins were much less, and differences between preincubations without and with sulfate were marginal (Fig. 7C). While in rice field soils Clostridia were the most abundant group followed by Deltaproteobacteria, it was the other way round in the lake sediments. Rice field soils contained *Bacilli*, while Lake sediments contained *Spirochaetes*, which were respectively negligible.

4 Discussion

4.1 Methanogenic conditions

We measured ε_{ac} values in anaerobic environmental samples, which consumed acetate almost exclusively by methanogenesis. Predominance of methanogenesis occurred in the absence of sulfate, in some incubations of the rice field soils even in the presence of sulfate provided there was no prior incubation in the presence of sulfate. In the rice field soils, CH_4 carbon accounted for more than 90% of the consumed acetate carbon, and CH_3F completely inhibited acetate consumption, the increase of $\delta^{13}C$ in the residual acetate, and also inhibited most of CH_4 production. In conclusion, acetate was exclusively consumed by aceticlastic methanogenesis and only little CH_4 was produced from other sources than acetate, presumably from background organic carbon via hydrogenotrophic methanogenesis as indicated by the negative $\delta^{13}C$ of the produced CH_4. The increase of $\delta^{13}C$ in the residual acetate was expected due to preferred utilization of isotopically light acetate carbon. Such patterns of CH_4 production and change in ^{13}C isotopic signatures have been observed by us before in rice field soils and lake sediments (Conrad et al., 2010; Conrad et al., 2009; Fu et al., 2018; Ji et al., 2018). They are in agreement with the presence of a diverse methanogenic archaeal community consisting of putatively hydrogenotrophic and aceticlastic methanogenic archaea, which have been found in both Vercelli and IRRI soils (Liu et al., 2019; Liu et al.,

387 2018b). The aceticlastic methanogens consisted of species of the genera *Methanosarcina* and
388 *Methanosaeta* (or *Methanothrix* (Oren, 2014)), which differ in the mechanism of acetate
389 activation and the affinity towards acetate (Jetten et al., 1990).
390 Both genera of methanogens were also present in the sediments of Lake Fuchskuhle, which
391 exhibited a similar pattern of acetate consumption and CH_4 production as the anaerobic rice
392 field soils, thus confirming and extending earlier studies (Chan et al., 2002; Conrad et al.,
393 2010). Notably, CH_3F addition again almost completely inhibited CH_4 production from
394 acetate and was accompanied by highly negative $\delta^{13}C$ in the small amounts of residual CH_4,
395 which was presumably produced by hydrogenotrophic methanogenesis. Addition of CH_3F did
396 not completely inhibit acetate consumption, indicating consumption by oxidation rather than
397 aceticlastic methanogenesis. However, CH_3F almost completely inhibited the increase of $\delta^{13}C$
398 in the residual acetate, indicating only a negligible isotope effect. Also, CH_4 production
399 accounted only for less than 50% of the consumed acetate, as production of CH_4 was replaced
400 by CO_2. Hence, part of the acetate was apparently consumed by oxidative processes, even
401 without addition of sulfate. We assume that the oxidation consumption process was driven by
402 humic acids (Lovley et al., 1996). Notably, imbalance in the stoichiometry between CH_4 and
403 consumed acetate is reflected by the fact that the SW basin has a higher humic acid content
404 than the NE basin (Casper et al., 2003).
405 Values of ε_{ac} measured in cultures of methanogenic archaea differ depending on the genus
406 and the corresponding mechanism of acetate activation. Thus, methanogenic archaea of the
407 genus *Methanosarcina*, which activate acetate with acetate kinase and phosphotransacetylase
408 have a relatively negative ε_{ac} with values ranging between -35‰ and -21‰ (Gelwicks et al.,
409 1994; Goevert and Conrad, 2009; Krzycki et al., 1987; Zyakun et al., 1988). By contrast,
410 those of the genus *Methanosaeta*, which activate acetate with acetyl-CoA synthetase, have
411 less negative ε_{ac} with values ranging between -14‰ and -10‰ (Penning et al., 2006a;
412 Valentine et al., 2004). The ε_{ac} values in methanogenic rice field soils were all in a range of -
413 21‰ to -17‰, which is at the less negative end or even a bit less negative than the values
414 reported for pure cultures of *Methanosarcina* but is more negative than the values reported for
415 *Methanosaeta*. Therefore, it is reasonable to conclude that in the methanogenic rice field soils,

416 acetate was consumed mainly by *Methanosarcina* species and only to a minor extent by
417 *Methanosaeta* species. This conclusion is in agreement with the composition of the soil
418 methanogenic archaeal communities, which consisted of both genera. A similar conclusion
419 has been reached in methanogenic rice field soil (Goevert and Conrad, 2009). A similar
420 enrichment factor for acetate consumption has also been measured in the anoxic sediment of
421 Lake Wintergreen (Gelwicks et al., 1994) and again in the present study of the NE basin of
422 Lake Fuchskuhle. However, the sediment of the SW basin of Lake Fuchskuhle exhibited a
423 less negative ε_{ac} of about -11‰, which would be consistent with the activity of aceticlastic
424 *Methanosaeta* species. Indeed, *mcrA* genes of *Methanoseata* species were much more
425 abundant in the sediments of Lake Fuchskuhle than *mcrA* genes of *Methanosarcina* species.
426 Also in Lake Dagow sediments (located in the same region of Germany), methanogenic
427 archaea were dominated by *Methanosaeta* species and exhibited a relatively high ε_{ac} of about
428 -13‰ (Penning et al., 2006a). However, the sediment of the NE basin of Lake Fuchskuhle,
429 which was also dominated by *Methanosaeta* species exhibited more negative ε_{ac} values of
430 about -19‰.
431 Methanogenic consumption results in disproportionation of the acetate molecule with
432 oxidation of the carboxyl group to CO_2 and reduction of the methyl group to CH_4. In context
433 of the isotope fractionation during the conversion of acetate to CH_4 it is the isotopic
434 enrichment factor of the methyl group, $\varepsilon_{ac\text{-}methyl}$, which matters. Studies of fractionation of the
435 acetate-methyl in pure culture studies of aceticlastic methanogenic archaea have shown that
436 $\varepsilon_{ac\text{-}methyl}$ was always a few permil less negative than ε_{ac}. This difference was due to a larger
437 isotope effect for the conversion of acetate-carboxyl than acetate-methyl (Gelwicks et al.,
438 1994; Goevert and Conrad, 2009; Penning et al., 2006a; Valentine et al., 2004). Alternatively
439 to $\varepsilon_{ac\text{-}methyl}$ the enrichment factor for the conversion of acetate-methyl to CH_4 can also be
440 measured from the isotopic composition in CH_4, i.e., ε_{CH4}. Most of the studies of pure
441 methanogenic cultures resulted in ε_{CH4} being similar to $\varepsilon_{ac\text{-}methyl}$ (Gelwicks et al., 1994;
442 Goevert and Conrad, 2009; Penning et al., 2006a), but occasionally ε_{CH4} was a few permil
443 more negative than $\varepsilon_{ac\text{-}methyl}$, both in pure culture (Valentine et al., 2004) and in environmental
444 samples (Goevert and Conrad, 2009). Similarly, values of ε_{CH4} in the unbuffered suspensions

445 of rice field soils were only slightly more negative than values of ε_{ac}. However, in the
446 buffered suspensions of both rice field soils and lake sediments, values of ε_{CH4} were much
447 more negative than those of ε_{ac}, the difference amounting to 9-17‰. These results indicate
448 that the isotope effect for the conversion of the acetate-methyl to CH_4 was much stronger than
449 that for the conversion of acetate-carboxyl to CO_2, which is completely opposite to the results
450 obtained in cultures of methanogenic archaea. This discrepancy in the results is presently
451 without conclusive explanation. The possibility of effects by bicarbonate or CO_2
452 concentrations, being different in the pure microbial cultures, the unbuffered and buffered soil
453 suspensions, or of phosphate effects should be considered. Effects of CO_2 concentrations and
454 buffer systems on fractionation factors have for example been observed in cultures of
455 chemolithoautotrophic *Thermoanaerobacter kivui* (Blaser et al., 2015).
456
457 *4.2 Sulfidogenic conditions*
458 We also measured ε_{ac} values in anaerobic environmental samples, which consumed acetate
459 by sulfate reduction. These conditions were achieved (only in the buffered suspensions) by
460 preincubation with gypsum and measurement of acetate consumption in the presence of
461 sulfate (gypsum). Preincubation was required because of delayed sulfate reduction (Liu et al.,
462 2018a). The relative abundance of both *dsrB* genes and genes of bacterial 16S rRNA were
463 only marginally different between samples preincubated under methanogenic and sulfidogenic
464 conditions, similarly as observed before (Wörner et al., 2016). It is probably the induction of
465 the sulfate reduction activity, which delayed sulphidogenic conditions (Liu et al., 2018a).
466 Sulfidogenic conditions were verified by showing that methanogenesis was almost completely
467 inhibited while acetate consumption operated, also with respect to increase of $\delta^{13}C$ in the
468 residual acetate, and that CH_3F, which is rather specific for acetoclastic methanogenesis, had
469 only a marginal effect on these sulfidogenic activities. Finally, sulfidogenic conditions were
470 verified by the stoichiometry of acetate conversion, which showed only very little CH_4
471 production.
472 Values of ε_{ac} measured in cultures of sulfate-reducing bacteria differ depending on the
473 genus and the corresponding mechanism of acetate dissimilation. Experiments with cultures

of sulfate reducers showed that *Desulfobacca acetoxidans*, which dissimilates acetate via the acetyl-CoA pathway, exhibits of about -19‰ being similar to the ε_{ac} values of aceticlastic *Methanosarcina* species. By contrast, cultures of *Desulfobacter* species, which dissimilate acetate vie the tricarbonic acid cycle, exhibited ε_{ac} values of about +2‰ (Goevert and Conrad, 2008). The ε_{ac} values measured in sulfidogenic anoxic paddy soils were in a range of -24‰ to -22‰, but were only -24‰ to -10‰, when measured in the presence of CH_3F, which guarantees that all aceticlastic methanogenic activities were inhibited. This range of ε_{ac} values compares rather well with the value of -19‰ measured in *Desulfobacca acetoxidans,* which however, was of only low relative abundance in the rice field soil incubations. However, it is well possible that uncultured-family-level lineages, which were the major sulfate reducers, dissimilated by similar pathway than *Desulfobacca acetoxidans* and thus, exhibited similar ε_{ac} values. Also *Syntrophobacteraceae*, which have been found to act as major acetate-utilizing sulfate reducers in Vercelli soil (Liu et al., 2018a) increased in relative abundance after preincubation with sulfate. The lake sediments, by contrast, exhibited a high relative abundance of *Desulfobacca acetoxidans*, and ε_{ac} values (including those with CH_3F) were in a range of -26‰ to -14‰. Unfortunately, there is, to our knowledge, only a paucity of ε_{ac} values measured in cultures of sulfate reducers (Goevert and Conrad, 2008). Therefore, it is not possible to have a better resolution of the role of different taxa and metabolic types of sulfate-reducing bacteria on the fractionation of acetate carbon.

5 Conclusions

Despite a relatively wide variety of environmental conditions and microbial community compositions, the range of fractionation factors (isotopic enrichment factors ε_{ac} for the fractionation of total acetate) was quite moderate. The observed ε_{ac} values were basically within the range that is known from studies of pure cultures of sulfate-reducing bacteria and methanogenic archaea, with a predominance of ε_{ac} values around -20‰, which is consistent with acetate fractionation in both aceticlastic *Methanosarcina* species and acetate-dissimilating sulfate reducers using the acetyl-CoA pathway. In few cases ε_{ac} values were close to -10‰, being consistent with a predominance of aceticlastic *Methanosaeta* species.

However, there is a paucity of data from cultures of acetate-dissimilating sulfate reducing bacteria, for example from *Syntrophobacteraceae*, which presently limits the potential for predicting fractionation of acetate carbon by knowing the microbial community composition. Another point of concern is the use of buffered growth media, which may affect isotope fractionation, such as indicated by the observation that ε_{CH4} values were much more negative than ε_{ac} values when using suspensions in phosphate buffer rather than in water.

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660

661

Figure legends

Fig. 1: Acetate conversion to CH_4 and CO_2 in unbuffered suspensions of paddy soil from the IRRI (the Philippines) without additions (water control), with gypsum, and with CH_3F. The panels show the temporal change of (A) concentrations of acetate; (B) $\delta^{13}C$ of acetate; (C) partial pressures of CH_4 and CO_2 (1 ppmv = 10^{-6} bar); (D) $\delta^{13}C$ of CH_4 and CO_2. Means ± SE, n = 2.

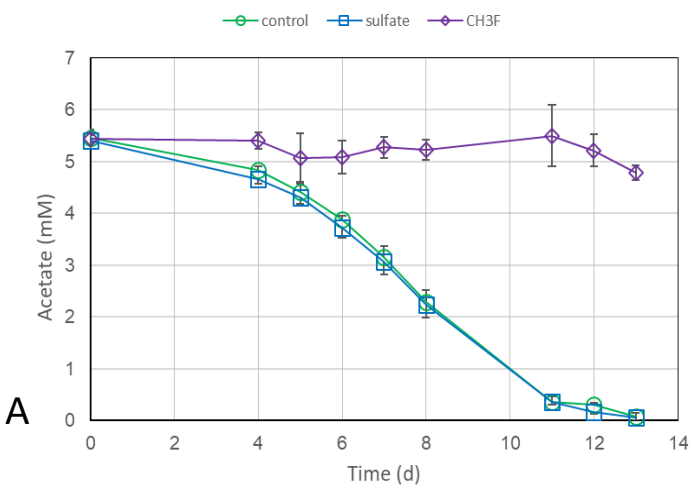
Fig. 2: Mariotti plots of (A, B) acetate consumption and (C, D) CH_4 production in (A, C) the absence (control) and (B, D) the presence of gypsum (+ sulfate) in 4 replicates of unbuffered suspensions of paddy soil from the IRRI.

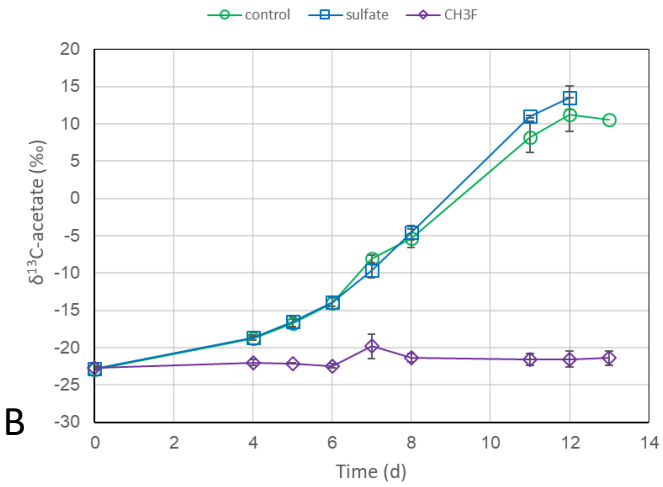
Fig. 3: Isotopic enrichment factors (ε_{ac} or ε_{CH4}, given as negative values) of unbuffered soil suspensions (Vercelli-soil, IRRI-soil) and buffered suspensions of paddy soil (Vercelli, IRRI) or sediments of Lake Fuchskuhle (NE and SW basin). The values of ε_{ac} (acetate) and ε_{CH4} (CH_4) were measured without addition of sulfate (methanogenic conditions), with addition of sulfate during preincubation and the experiment (sulfidogenic conditions) and with sulfate but the preincubation without sulfate (mostly methanogenic conditions). Mean ± SE, n = 3-4.

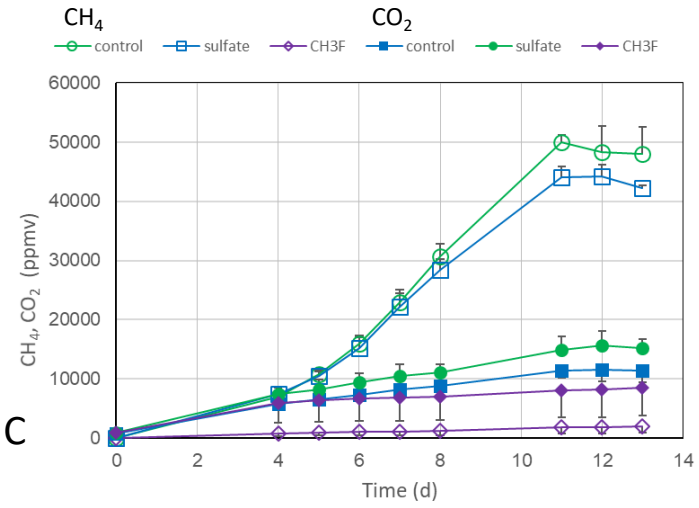
Fig. 4: Acetate conversion to CH_4 and CO_2 in phosphate-buffered (pH 7.0) suspensions of paddy soil from Vercelli (Italy) without additions (control); with CH_3F; with gypsum (preincubation and experiment); with gypsum (preincubation and experiment) + CH_3F. The panels show the temporal change of (A) concentrations of acetate; (B) $\delta^{13}C$ of acetate; (C) partial pressures of CH_4 (1 ppmv = 10^{-6} bar); (D) $\delta^{13}C$ of CH_4; (E) partial pressures of CO_2 (1 ppmv = 10^{-6} bar); (D) $\delta^{13}C$ of CO_2. Means ± SE, n = 3.

Fig. 5: Balance of CH_4 produced against acetate consumed in phosphate-buffered suspensions of paddy soil from Vercelli and IRRI, and of sediments from the NE and SW basin of Lake Fuchskuhle. The figures show individual replicates (n = 3) of the unamended control (methanogenic conditions); of the experiment plus gypsum ($CaSO_4$-1); of preincubation and experiment plus gypsum ($CaSO_4$-2). The diagonal line indicates stoichiometric conversion (disproportionation) of acetate to CH_4 + CO_2.

690 Fig. 6: Acetate conversion to CH_4 and CO_2 in phosphate-buffered (pH 7.0) suspensions of
691 sediment from the NE basin of Lake Fuchskuhle without additions (control); with
692 CH_3F; with gypsum (preincubation and experiment); with gypsum (preincubation and
693 experiment) + CH_3F. The panels show the temporal change of (A) concentrations of
694 acetate; (B) $\delta^{13}C$ of acetate; (C) partial pressures of CH_4 (1 ppmv = 10^{-6} bar); (D) $\delta^{13}C$
695 of CH_4; (E) partial pressures of CO_2 (1 ppmv = 10^{-6} bar); (D) $\delta^{13}C$ of CO_2. Means ±
696 SE, n = 3.
697 Fig. 7: Relative abundance of (A) *mcrA* (methanogens), (B) *dsrB* (sulfate reducers), (C)
698 bacterial 16S rRNA genes; The DNA was extracted after preincubation of phosphate-
699 buffered suspensions of paddy soils (Vercelli, IRRI) and sediments of Lake
700 Fuchskuhle (NE, SW basin) without additions (methanogenic conditions) or
701 preincubated and incubated in the presence of sulfate (sulfidogenic conditions).
702


control
sulfate
CH3F
Acetate (mM)
Time (d)
A


control
sulfate
CH3F
δ13C-acetate (‰)
Time (d)
B


CH4
CO2
control
sulfate
CH3F
CH4, CO2 (ppmv)
Time (d)
C

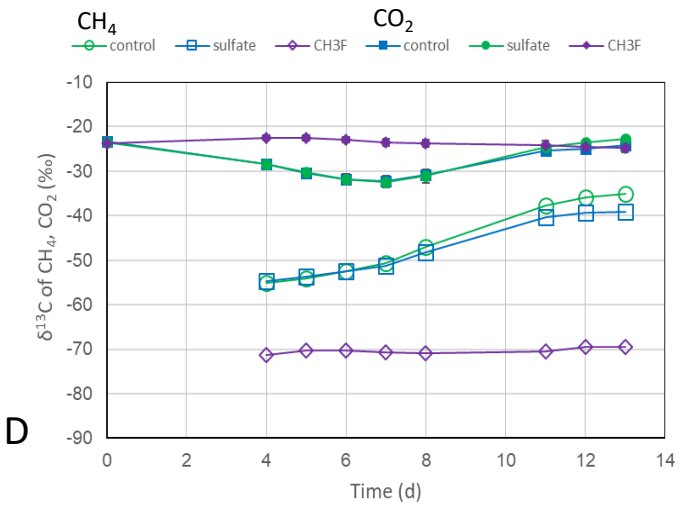

CH4
CO2
control
sulfate
CH3F
δ13C of CH4, CO2 (‰)
Time (d)
D

Fig. 1

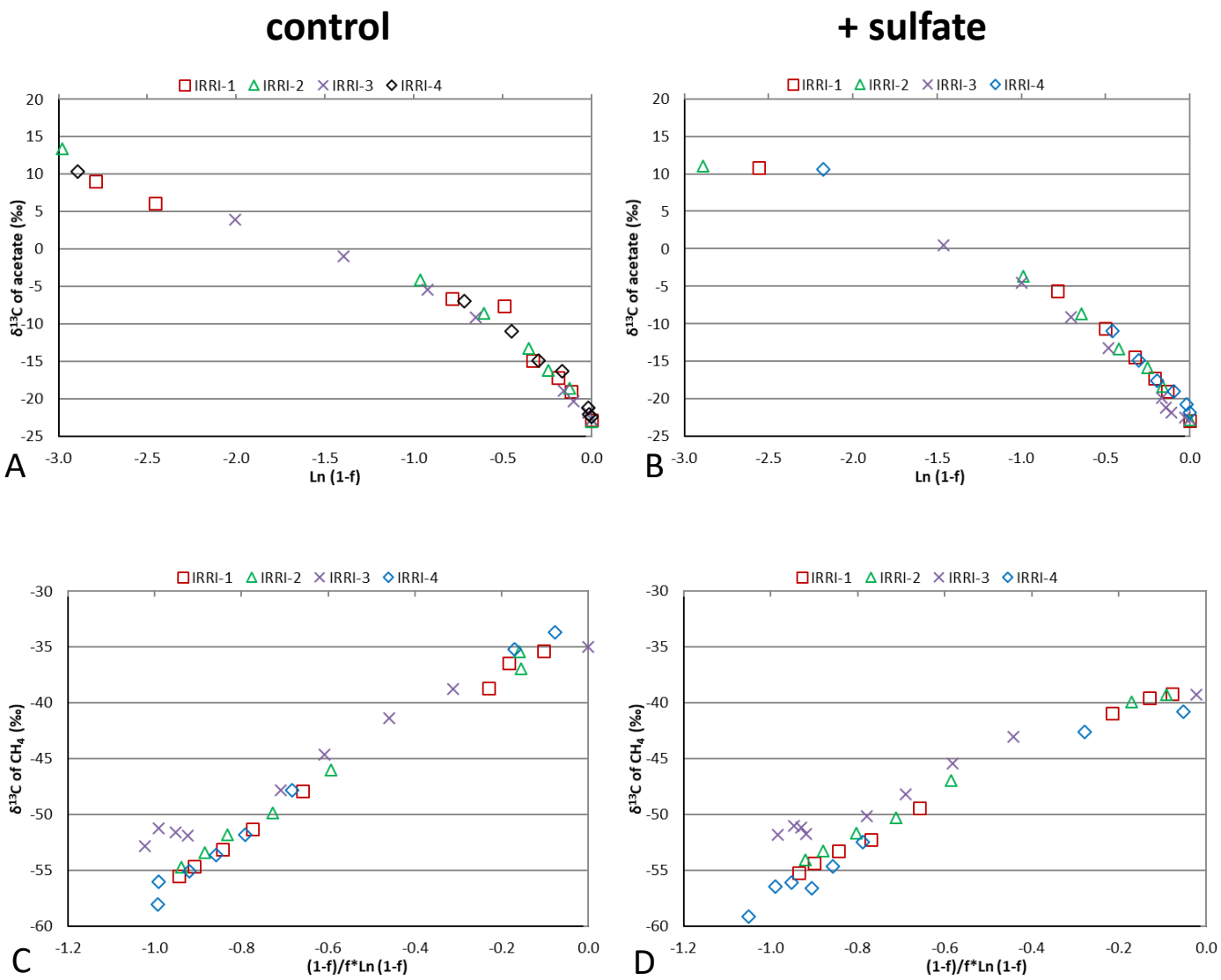

control
+ sulfate
IRRI-1
IRRI-2
IRRI-3
IRRI-4
δ13C of acetate (‰)
Ln (1-f)
A
B
δ13C of CH4 (‰)
(1-f)/f*Ln (1-f)
C
D

Fig. 2

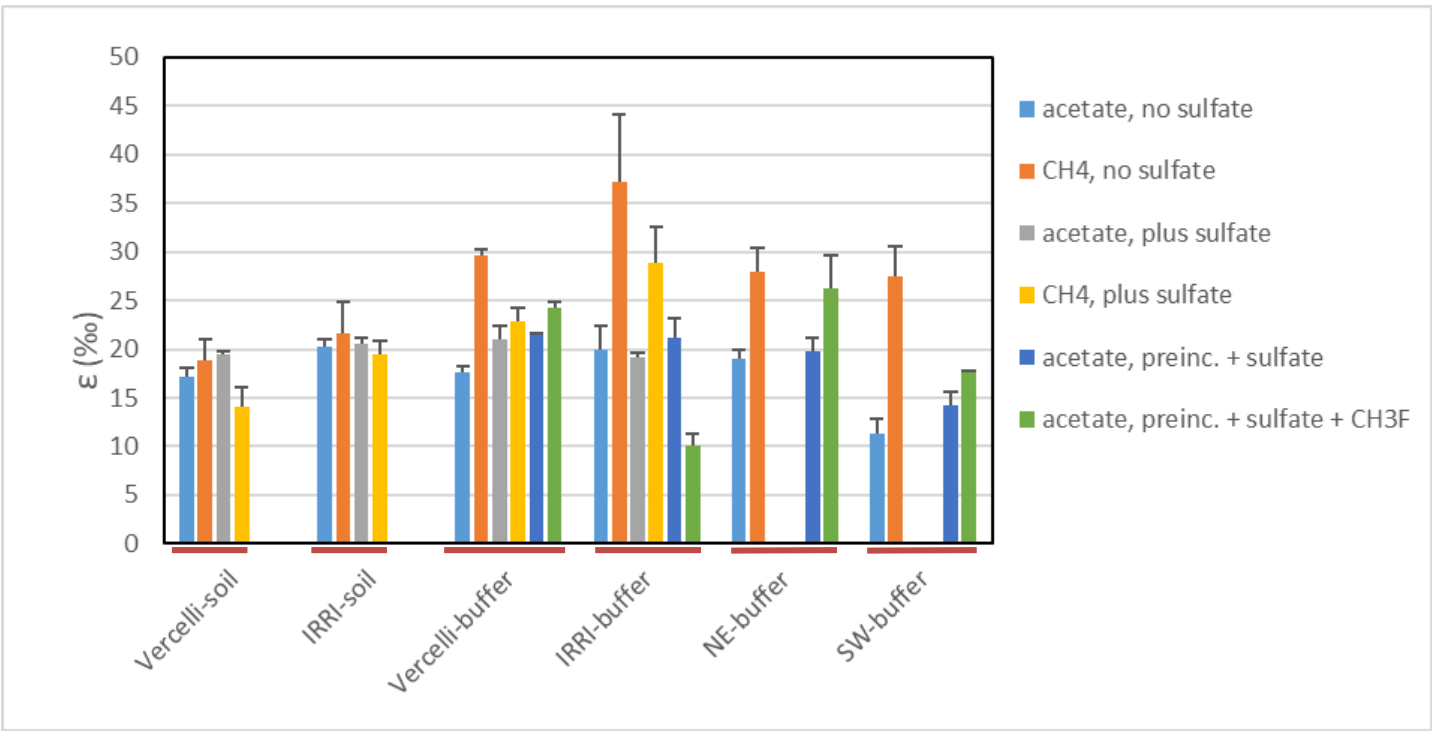
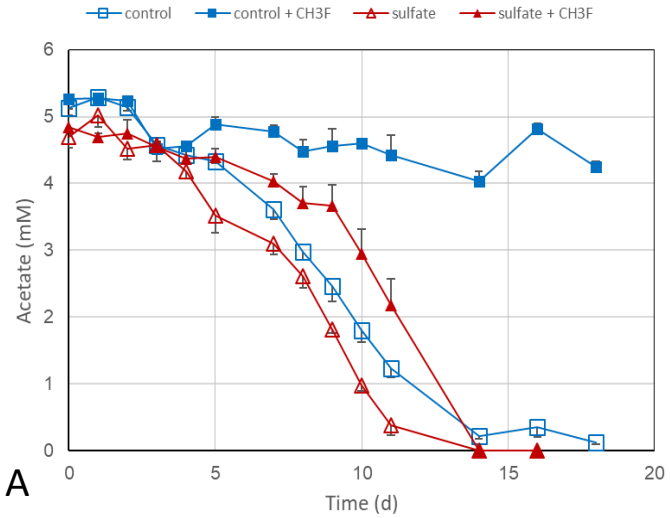
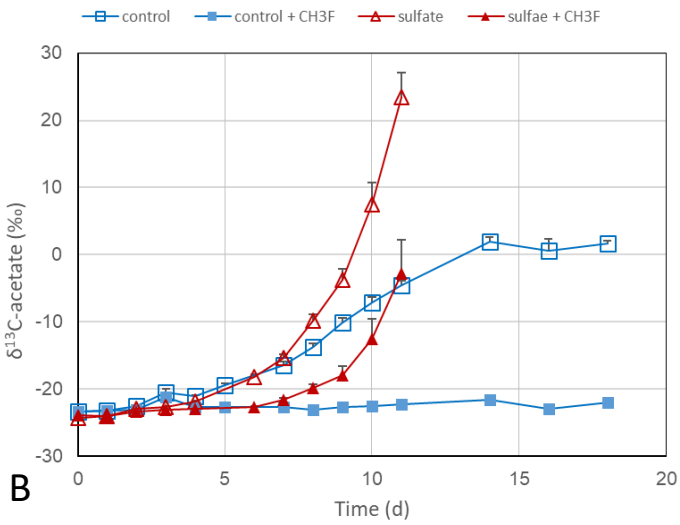

50
45
40
35
30
25
20
15
10
5
0
ε (‰)
Vercelli-soil
IRRI-soil
Vercelli-buffer
IRRI-buffer
NE-buffer
SW-buffer
acetate, no sulfate
CH4, no sulfate
acetate, plus sulfate
CH4, plus sulfate
acetate, preinc. + sulfate
acetate, preinc. + sulfate + CH3F

Fig. 3

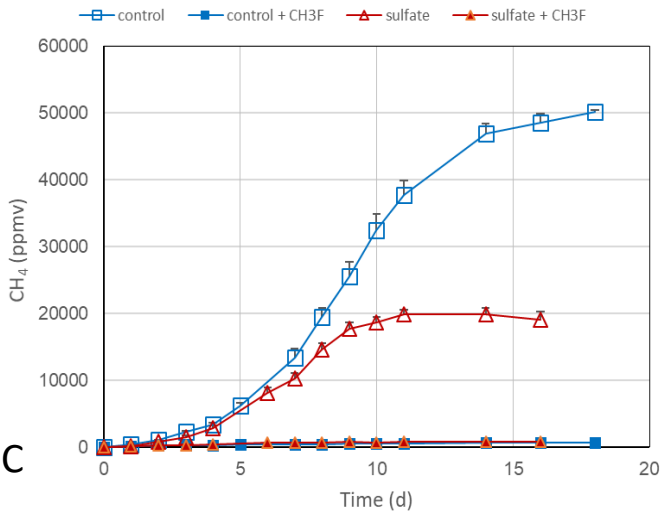
Vercelli, 2nd experiment



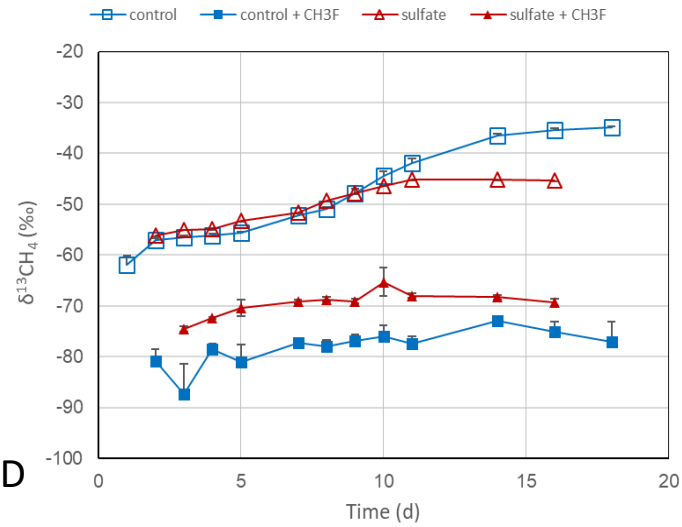
A



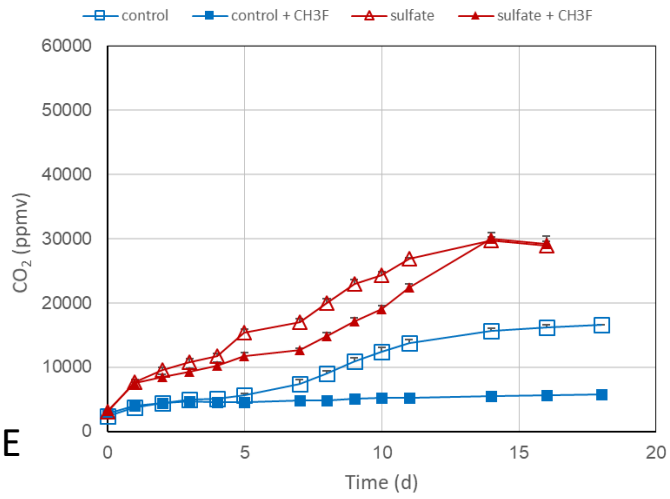
B



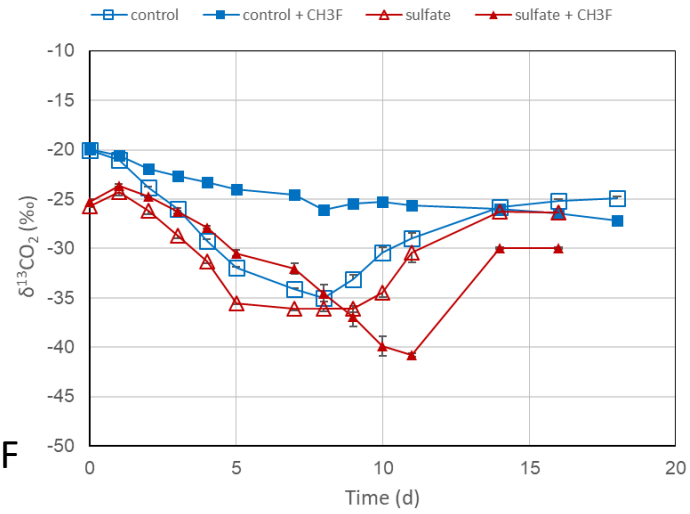
C



D

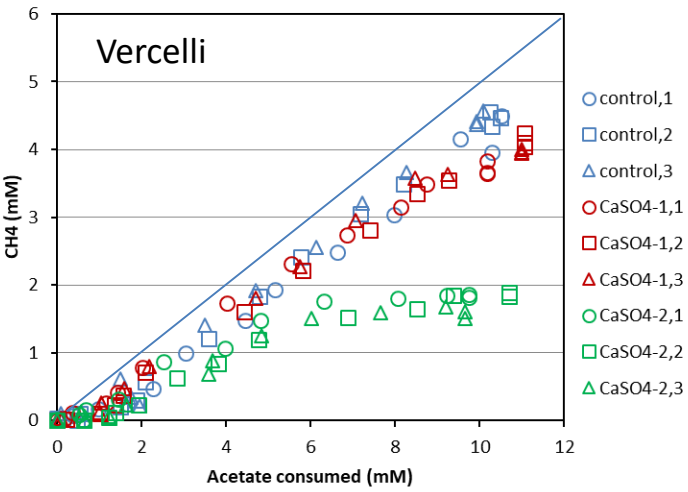


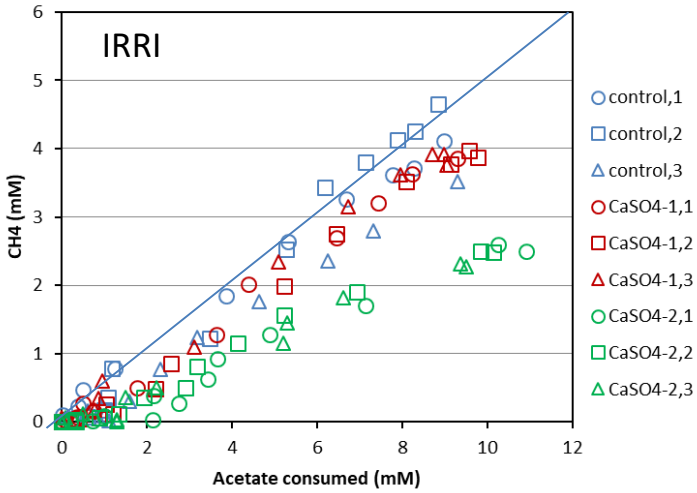
E

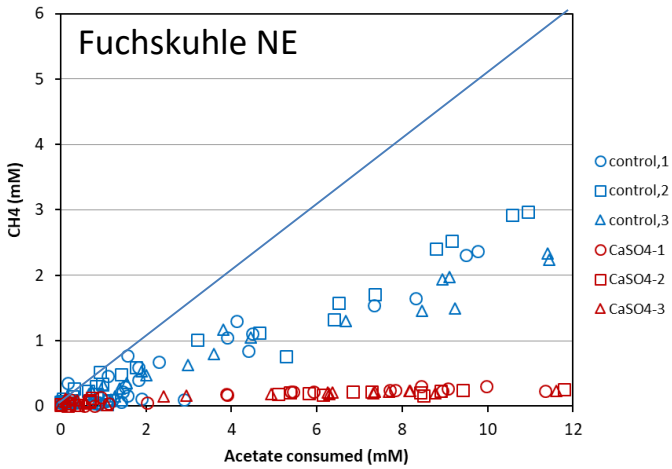


F

Fig. 4


Vercelli
CH4 (mM)
Acetate consumed (mM)
control,1
control,2
control,3
CaSO4-1,1
CaSO4-1,2
CaSO4-1,3
CaSO4-2,1
CaSO4-2,2
CaSO4-2,3


IRRI
CH4 (mM)
Acetate consumed (mM)
control,1
control,2
control,3
CaSO4-1,1
CaSO4-1,2
CaSO4-1,3
CaSO4-2,1
CaSO4-2,2
CaSO4-2,3


Fuchskuhle NE
CH4 (mM)
Acetate consumed (mM)
control,1
control,2
control,3
CaSO4-1
CaSO4-2
CaSO4-3

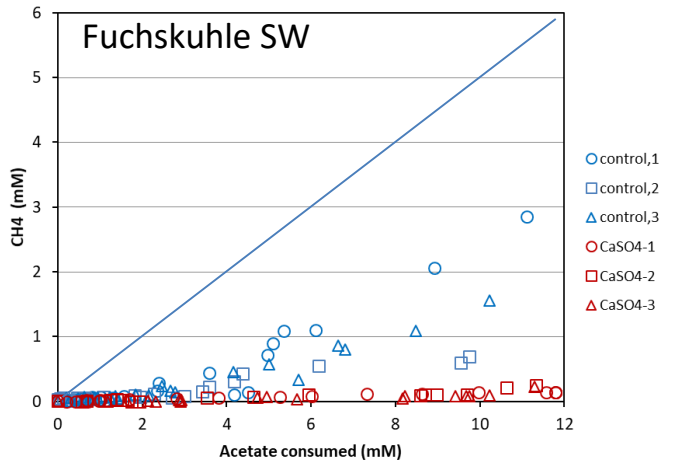

Fuchskuhle SW
CH4 (mM)
Acetate consumed (mM)
control,1
control,2
control,3
CaSO4-1
CaSO4-2
CaSO4-3

Fig. 5

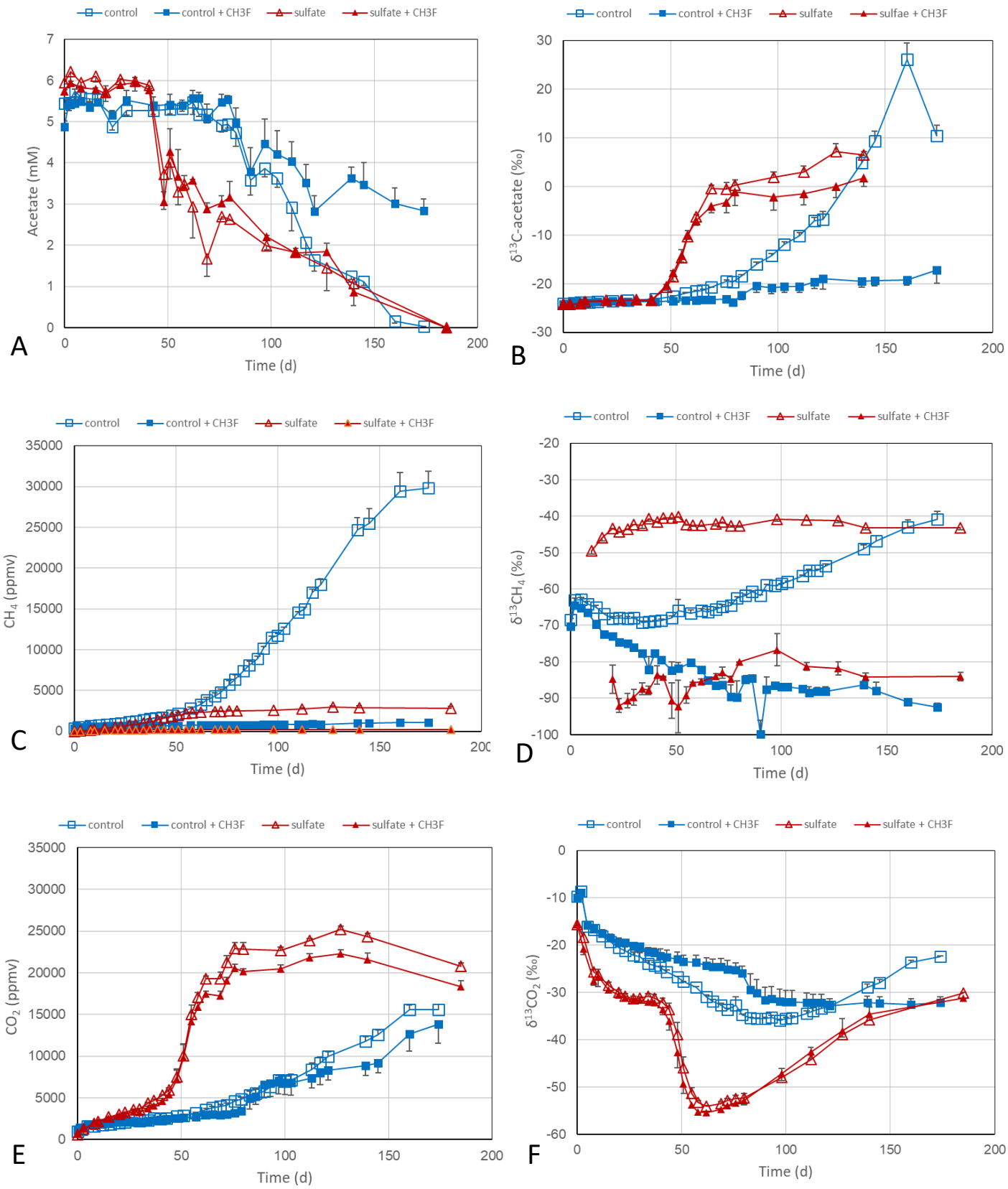
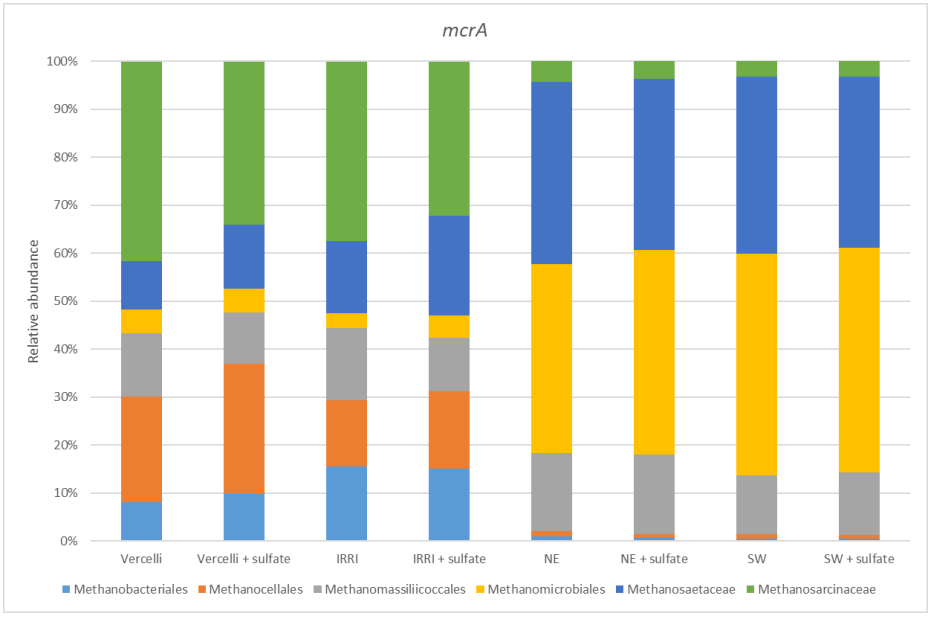
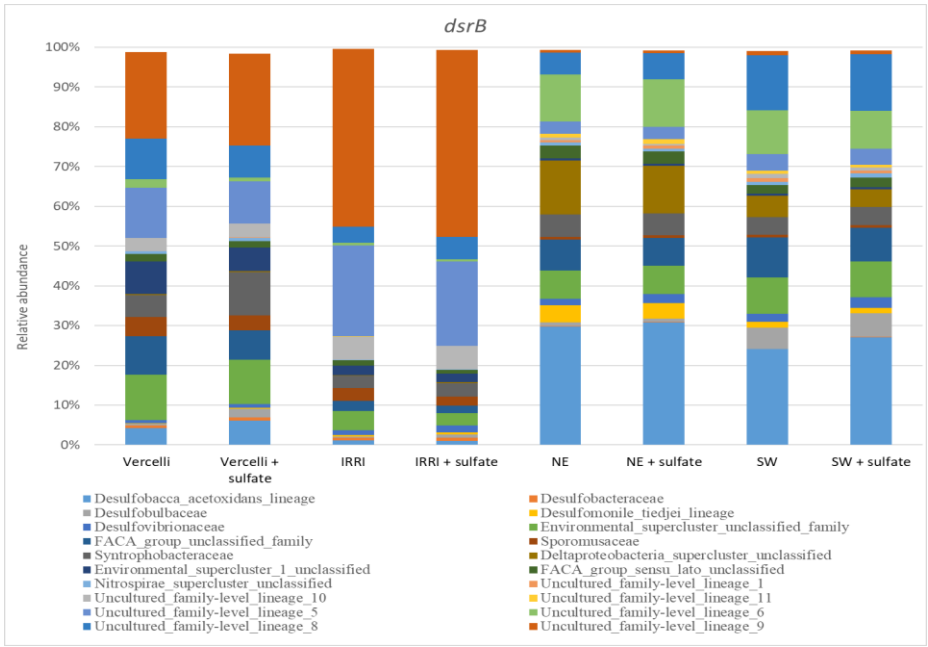

control
control + CH3F
sulfate
sulfate + CH3F
Acetate (mM)
Time (d)
A
sulfae + CH3F
δ13C-acetate (‰)
B
CH4 (ppmv)
C
δ13CH4 (‰)
D
CO2 (ppmv)
E
δ13CO2 (‰)
F

Fig. 6


mcrA
Relative abundance
100%
90%
80%
70%
60%
50%
40%
30%
20%
10%
0%
Vercelli
Vercelli + sulfate
IRRI
IRRI + sulfate
NE
NE + sulfate
SW
SW + sulfate
Methanobacteriales
Methanocellales
Methanomassiliicoccales
Methanomicrobiales
Methanosaetaceae
Methanosarcinaceae


dsrB
Relative abundance
100%
90%
80%
70%
60%
50%
40%
30%
20%
10%
0%
Vercelli
Vercelli + sulfate
IRRI
IRRI + sulfate
NE
NE + sulfate
SW
SW + sulfate
Desulfobacca_acetoxidans_lineage
Desulfobacteraceae
Desulfobulbaceae
Desulfomonile_tiedjei_lineage
Desulfovibrionaceae
Environmental_supercluster_unclassified_family
FACA_group_unclassified_family
Sporomusaceae
Syntrophobacteraceae
Deltaproteobacteria_supercluster_unclassified
Environmental_supercluster_1_unclassified
FACA_group_sensu_lato_unclassified
Nitrospirae_supercluster_unclassified
Uncultured_family-level_lineage_1
Uncultured_family-level_lineage_10
Uncultured_family-level_lineage_11
Uncultured_family-level_lineage_5
Uncultured_family-level_lineage_6
Uncultured_family-level_lineage_8
Uncultured_family-level_lineage_9

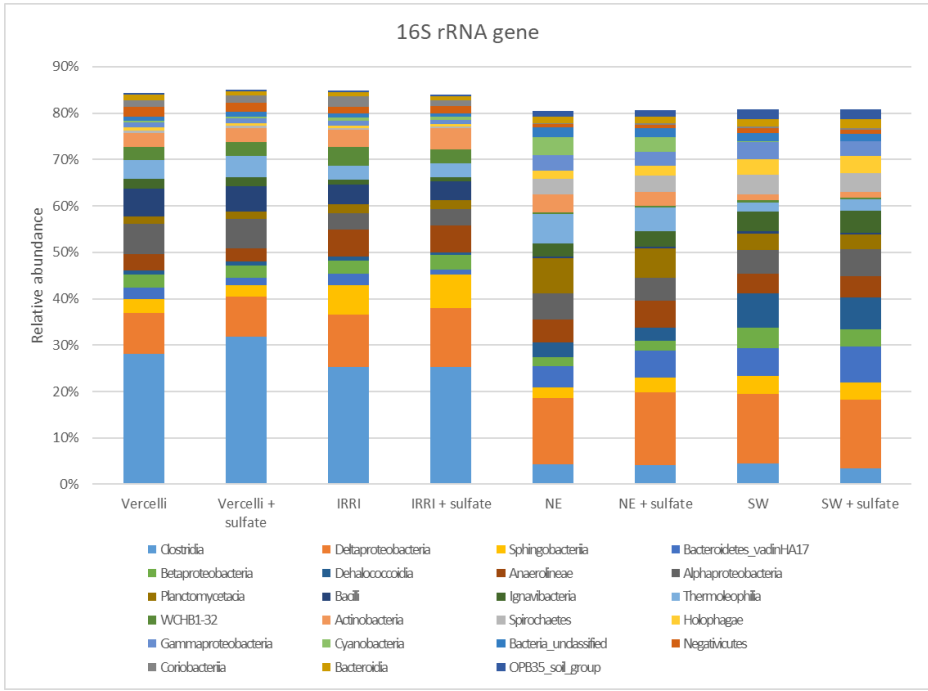

16S rRNA gene
Relative abundance
90%
80%
70%
60%
50%
40%
30%
20%
10%
0%
Vercelli
Vercelli + sulfate
IRRI
IRRI + sulfate
NE
NE + sulfate
SW
SW + sulfate
Clostridia
Deltaproteobacteria
Sphingobacteria
Bacteroidetes_vadinHA17
Betaproteobacteria
Dehalococcoidia
Anaerolineae
Alphaproteobacteria
Planctomycetacia
Bacilli
Ignavibacteria
Thermoleophilia
WCHB1-32
Actinobacteria
Spirochaetes
Holophagae
Gammaproteobacteria
Cyanobacteria
Bacteria_unclassified
Negativicutes
Coriobacteriia
Bacteroidia
OPB35_soil_group

Fig. 7