

Potential contribution of acetogenesis to anaerobic degradation in methanogenic rice field soils

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ABSTRACT

Acetate is an important intermediate in the anaerobic degradation of organic matter. It is not only produced by fermentation but also by the reduction of CO₂ via the acetyl-CoA pathway (acetogenesis). However, the interplay of this process in methanogenic rice field soils is not fully understood. Chemolithotrophic acetogenesis results in a rather strong depletion of the ¹³C of acetate. Therefore, we measured the δ¹³C of acetate, CO₂ and CH₄ that were produced during methanogenic degradation of organic matter in rice paddy soils from two geographical origins (Philippines and Italy) and used three different strategies to estimate the contribution of acetogenesis to acetate formation: (1) Incubation of soil slurries under elevated concentrations of H₂/CO₂ to specifically activate the H₂-dependent communities; (2) incubation at three different temperatures (15, 30, 50 °C) to shift the conditions for H₂ consumption; (3) incubation in the presence of inhibitors presumed to inhibit acetogenesis (KCN) or methanogenesis (BES). Only incubations under elevated H₂/CO₂ resulted in ¹³C-depleted acetate (δ¹³C of –68 to –65‰) compared to the control (δ¹³C of –25‰). Temperature and presence of inhibitors also affected the δ¹³C of acetate, CO₂ and/or CH₄, but δ¹³C of acetate was never as low as after addition H₂/CO₂. A significant ¹³C enrichment of acetate at 15 °C in presence of BES and KCN indicated that H₂-dependent acetogenesis is a favoured process at low temperature. Copy numbers in the Philippine soil of the *fts* gene coding for the formyl-tetrahydrofolate synthetase of the acetyl-CoA pathway were on a similar order of magnitude (10⁶ per gram dry soil) irrespectively of the different incubation conditions. Our results indicate that chemolithotrophic acetogenesis was operative in methanogenic rice soil at 15 °C but was more important at elevated H₂/CO₂ concentrations.

1. Introduction

The anaerobic degradation of organic matter accounts for a significant part of the global carbon cycle and involves the production of the greenhouse gas CH₄ (Reeburgh, 2003). While the terminal processes liberating CH₄ have been thoroughly investigated using isotopic techniques (Conrad, 2005), the contribution of other pathways during the anaerobic degradation is not well investigated. In this study we aimed to specify the contribution of acetogenesis to the acetate pool in rice paddy soils from two geographical origins (Philippines and Italy).

The unifying trait of acetogenic bacteria is that acetate is their major product, which is mainly formed by the acetyl-CoA pathway reducing CO₂ to acetate. However, the usage of the acetyl-CoA pathway in acetogens is versatile (Drake et al., 2002; Schuchmann and Muller, 2016): acetogens can compete with primary fermenters for monomeric compounds, e.g., glucose, fructose or xylose; they can act as secondary fermenters using lactate, ethanol or even methoxylated aromatics;

many acetogens can grow chemolithotrophically converting H₂ and CO₂ to acetate; and some acetogens can even grow in syntrophic associations at the expense of acetate running the acetyl-CoA pathways in the reverse direction (Hattori et al., 2000; Hattori, 2008).

Even though acetate is the major end product of acetogens, their environmental role is hard to address since many anaerobic bacteria also produce acetate by other biochemical pathways than the acetyl-CoA pathway. To investigate the function of chemolithotrophic acetogens in rice field soils we studied their contribution to the acetate pool using isotope techniques. This is a reasonable approach since studies of microbial pure cultures have shown that the stable carbon isotope fractionation of most fermentative processes is almost negligible (< 5‰) (Blair et al., 1985; Penning and Conrad, 2006; Botsch and Conrad, 2011), while in contrast the fractionation by chemolithotrophic acetogenesis (4 H₂ + 2 CO₂ → CH₃COOH + 2 H₂O) is strong (–38‰ to –68‰) (Gelwicks et al., 1989; Preuss et al., 1989; Blaser et al., 2013). One has to keep in mind that the acetate formed by heterotrophic

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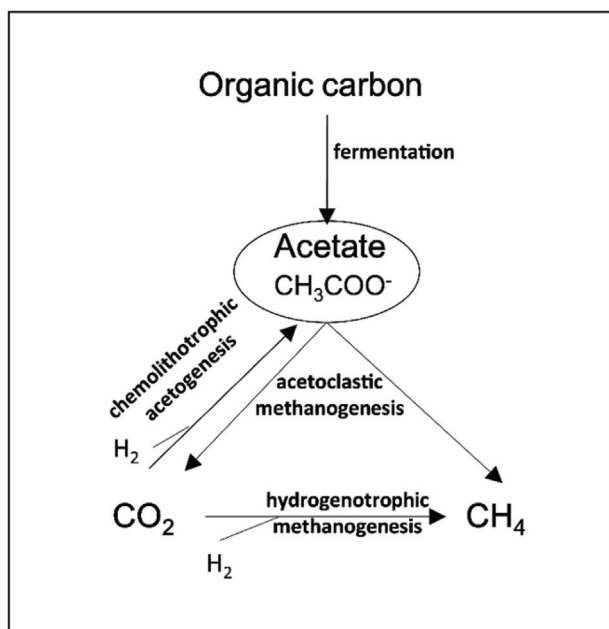


Fig. 1. Scheme illustrating acetate as important intermediate in the anaerobic degradation of organic matter: fermentation or heterotrophic acetogenesis, chemolithotrophic acetogenesis, acetoclastic methanogenesis, hydrogenotrophic methanogenesis.

acetogenesis originates partially from fermentation of organic matter and partially from the reduction of CO_2 (Freude and Blaser, 2016).

However, while the differentiation of aceticlastically and hydrogenotrophically formed CH_4 is rather straight forward (Conrad, 2005; Blaser and Conrad, 2016), the application of such techniques for the investigation of chemolithotrophic acetogenic bacteria is much more complex. In contrast to CH_4 , which is an end product in most anaerobic sediments, acetate is produced and consumed by different metabolic pathways, which all imprint a signature on the stable carbon isotope composition ($^{13}\text{C}/^{12}\text{C}$) of acetate (Fig. 1) (Conrad et al., 2014).

Hence, the stable carbon isotopic composition of acetate is basically governed by three factors: (1) the isotopic composition of its precursors, (2) the isotopic fractionations (enrichment factors) associated with its formation and consumption, and (3) the relative rates of all acetate producing and consuming processes which influence its pool size. The knowledge of all three factors is of great importance for the evaluation of ^{13}C values of acetate determined in environmental samples. The isotopic composition of the precursors can be measured, since CO_2 is the exclusive C substrate for chemolithotrophic acetate formation, while soil organic carbon is the substrate for fermentatively produced acetate. We used three strategies to estimate the contribution of chemolithotrophic acetogenesis to acetate formation: (1) Incubation of soil slurries under elevated concentrations of H_2/CO_2 to specifically activate the H_2 -dependent communities; (2) incubation at three different temperatures to shift the conditions for H_2 consumption; (3) incubation in the presence of inhibitors to control the competitive role of chemolithotrophic acetogens and methanogens under natural H_2 concentrations.

In many anoxic systems the supply of H_2 is low, and hence microbial populations will consume H_2 to the threshold of bioenergetic limitation (Cordruwisch et al., 1988; Hoehler et al., 2002). In such systems, the competition of acetogens and methanogens for H_2 is of great ecological importance (Kotsyurbenko et al., 2001). Even though methanogens are expected to outcompete acetogens for H_2 because of thermodynamic reasons (Hoehler et al., 2002), there are additional constraints. Thus, at low temperatures (5–15 °C) acetogens have often been found to be better adapted than methanogens and outcompete them for H_2 (Schulz and Conrad, 1996; Schulz et al., 1997; Kotsyurbenko et al., 2001; Glissmann et al., 2004; Nozhevnikova et al., 2007; Liu and Conrad,

2011). Then, CH_4 has been generated by a two-step process: First acetogens reduce CO_2 to acetate which in turn is cleaved by aceticlastic methanogens. In contrast at elevated temperatures (> 55 °C), CH_4 production has frequently been found to be driven by syntrophic acetate oxidation coupled to hydrogenotrophic methanogenesis (Hattori et al., 2000; Hattori, 2008; Conrad and Klose, 2011). Syntrophic acetate oxidation is the reversal of chemolithotrophic acetogenesis and presumably exhibits a low carbon isotope fractionation (Hattori et al., 2000; Hattori, 2008; Conrad and Klose, 2011). Thermophilic syntrophic acetate oxidizers were found to be able also operating in the reverse mode (i.e., chemolithotrophic acetogenesis) if supplied with H_2 plus CO_2 (Liu and Conrad, 2011). To elucidate the environmental relevance of acetogenically formed acetate we therefore used different temperature regimes to shift conditions and estimate the contribution of acetogenic and methanogenic pathways.

Finally, different inhibitors may constrain the influence of methanogens and acetogens. While 2-bromoethanesulfonate (BES) has been shown to be an effective inhibitor for methanogens (Sparling and Daniels, 1987), the inhibition of acetogenic bacteria is much more difficult. We used cyanide (KCN) as possible inhibitor, as it interferes with the electron transport via cytochromes (Cooper and Brown, 2008). Cytochromes are used by several acetogens, which rely on a proton gradient for energy translocation (e.g. *Moorella thermoacetica* (Pierre et al., 2008) or *Clostridium aceticum* (Poehlein et al., 2015)). Other acetogens however rely on ferredoxins instead of cytochromes for electron transportation (e.g. *Acetobacterium woodii* (Poehlein et al., 2012), *Clostridium ljungdahlii* (Kopke et al., 2010), *Thermoacetogenium phaeum* (Oehler et al., 2012) and *Thermoanaerobacter kivui* (Hess et al., 2014)). Likewise KCN directly inhibits carbon monoxide dehydrogenase (CODH), which is one of the key enzymes of the reductive acetyl CoA pathway (Ensign et al., 1989). Therefore, pathways involving CODH should be more susceptible to cyanide inhibition than fermentation processes.

Distinct isotopic fractionations have been observed in various pure microbial cultures of acetate-producing and consuming cultures (Meinschein et al., 1974; Rinaldi et al., 1974; Blair et al., 1985; Krzycki et al., 1987; Gelwicks et al., 1989, 1994; Preuss et al., 1989; Penning et al., 2005; Penning et al., 2006; Penning and Conrad, 2006; Londry et al., 2008; Govert and Conrad, 2009; Blaser et al., 2013; Freude and Blaser, 2016). Most of them focused on carbon isotope fractionation during the consumption of acetate, in particular by aceticlastic methanogenic archaea which show isotopic enrichment factors of –21‰ to –35‰ in *Methanosarcinaceae* (Krzycki et al., 1987; Gelwicks et al., 1994; Londry et al., 2008; Govert and Conrad, 2009) and –7‰ to –10‰ in *Methanosaetaceae*, respectively (Valentine et al., 2004; Penning et al., 2006). So far only few studies have analyzed the stable carbon isotopic composition of acetate in natural sediments under in situ conditions. The isotopic composition of acetate has been shown to be highly variable in natural sediments, ranging from –85‰ up to –3‰ (Blair et al., 1987; Blair and Carter, 1992; Heuer et al., 2006, 2009, 2010; Conrad et al., 2014). Detailed analyses to study the contribution of acetogenesis have been performed using freshwater sediments under elevated H_2 concentrations using BES to inhibit methanogenesis and at low temperature (8 °C) to follow acetate concentrations and their $\delta^{13}\text{C}$ values (Heuer et al., 2010). A similar approach using peat soil under elevated H_2 concentrations was used to monitor the acetogenic community (Haedrich et al., 2012). However, the contribution of acetogenic bacteria in rice paddy soils using inhibitors or natural abundance of stable carbon isotopes has barely been investigated.

Molecular studies have shown that acetogens can be found in almost every anoxic and also some oxic environments (Leaphart and Lovell, 2001; Leaphart et al., 2003; Conrad, 2005; Henderson et al., 2010; Hunger et al., 2011). They reach 10^6 to 10^8 gene copies per gram dry weight (Xu et al., 2009). However, acetogens cannot be targeted using 16 S rRNA-based approaches, because they do not form a

phylogenetically coherent group (Drake et al., 2008). Targeting the *fhs* gene (coding for the 10-formyl tetrahydrofolate synthetase (FTHFS) of the acetyl-CoA pathway) as functional marker for acetogenesis provides only limited insight into the genetic potential for acetogenesis because the primer set also targets sequences of purinolytic clostridia, sulfate reducers, methanogens, and other organisms that possess the FTHFS enzyme (Drake et al., 2006). Nevertheless this functional marker has frequently been used to analyze acetogenic communities in different environmental samples (Leaphart and Lovell, 2001; Lovell and Leaphart, 2005; Henderson et al., 2010; Haedrich et al., 2012).

In this study we used two rice field soils from Italy and the Philippines to determine the stable carbon isotopic composition of acetate and assess the contribution of chemolithotrophic acetogenesis to methane production. We used different approaches: (1) Incubation under H₂/CO₂ served for determining the potential of the soils for chemolithotrophic acetogenesis. (2) Incubation under 15, 30 and 50 °C, as well as (3) in the presence of inhibitors presumed to inhibit acetogenesis (KCN) or methanogenesis (BES).

2. Material and methods

2.1. Incubation experiments

Soil samples were obtained from rice fields at the Rice Research Institute in Vercelli, Italy (Pump and Conrad, 2014), and at the International Rice Research Institute in Los Banos, the Philippines (Heinz et al., 2013). The main physicochemical characteristics of these soils have recently been summarized (Liu and Conrad, 2017): The Italian soil is a sandy loam with a pH of 5.75, total C of 1.1% and total N of 0.08%; the Philippines rice paddy soil is a silt loam with a pH of 6.3, total C of 1.9% and total N of 0.2%. Soil slurries from Philippines rice fields were prepared in 26-ml pressure tubes using 4 g dry soil and 6 ml anoxic sterile water. The tubes were closed with black rubber stoppers, flushed with N₂, pressurized to 0.5 bar overpressure, and pre-incubated at 25 °C for 2 weeks to deplete alternative electron acceptors and initiate methanogenesis. Then the soil was subsequently incubated at 15, 30 and 50 °C. The experiments consisted of a series of four parallel incubations in which inhibitors and substrates were added to soil slurries in the following way: (1) Control, no substrate or inhibitor was added and the sediment slurry was incubated under N₂ headspace. (2) Methanogenesis was inhibited by 20 mM bromoethanesulfonate (BES) and the sediment slurry was incubated under N₂ headspace. (3) Methanogenesis was inhibited by BES (20 mM) and acetogenesis was inhibited by 40 μM KCN. (4) Use of H₂/CO₂ (80/20, v/v) headspace gas and methanogenesis was inhibited by 20 mM BES. The tubes with soil slurry were prepared in numerous parallels (about 468 tubes), of which triplicates were sacrificed for determination of CH₄ accumulation, concentrations of acetate and of their δ¹³C values after different times of incubation. The δ¹³C of the organic matter in the Philippines soil was determined to be −24.0‰ using an elemental analyzer coupled to an IRMS at the center for stable isotope research and analysis (KOSI) at University of Göttingen.

Soil slurries from Italian rice fields were prepared from sieved (1 mm mesh) dry soil. Since the Italian soil was sandier than the Philippine soil, the water to soil ratio was slightly different. In 60-ml serum bottles 5 g dry soil was mixed with 5 ml water. The serum bottles were closed with black rubber stoppers and pressurized to 0.5 bar overpressure with either N₂/CO₂ (80/20) or H₂/CO₂ (80/20). The slurries were prepared in numerous parallels (about 360 bottles) and incubated at three different temperatures (15 °C, 30 °C, and 50 °C) in the dark without shaking. In half the samples 20 mM BES was added to specifically inhibit methanogenesis. At each time point three individual bottles were sacrificed for the determination of the concentration and carbon isotope signal of CH₄, CO₂, H₂, and acetate. The δ¹³C of the organic matter in the Vercelli soil was determined to be −26.5‰ using an elemental analyzer coupled to an IRMS at the center for stable

isotope research and analysis (KOSI) at University of Göttingen.

2.2. Chemical analysis

Methane and CO₂ were quantified in a gas chromatograph equipped with a methanizer and flame ionization detector (Conrad and Klose, 1999). The partial pressures of CH₄ and CO₂ measured in the headspace of the incubations were converted into molar quantities using the molar volumes of an ideal gas at the different temperatures. The amounts of dissolved CO₂ were calculated using the Henry constants at the different temperatures (Stumm and Morgan, 1981). The small amounts of dissolved CH₄ were neglected. The isotopic composition (δ¹³C) of CH₄ and CO₂ was determined in a Finnigan gas chromatograph combustion isotope ratio mass spectrometer system (Thermoquest, Bremen, Germany) (Fey et al., 2004).

Concentrations of acetate in the liquid phase of the soil incubations were analyzed by HPLC equipped with an ion-exclusion column (Aminex HPX-87-H, BioRad, München, Germany) and coupled to an UV-Vis detector (Sykam, Fuerstenfeldbruck, Germany) (Krumbock and Conrad, 1991). The isotopic measurements and quantification of acetate were performed on a HPLC system (Spectra System P1000, Thermo Fisher Scientific, San Jose, CA, USA; Mistral, Spark, Emmen, The Netherlands) equipped with an ion-exclusion column (Aminex HPX-87-H, BioRad, München, Germany) and coupled to Finnigan LC IsoLink (Thermo Fisher Scientific, Bremen, Germany) (Krummen et al., 2004). Isotope ratios were detected on an IRMS (Finnigan MAT Delta-plus Advantage).

2.3. Quantification of *fhs* gene

DNA was extracted from soil samples using the NucleoSpin kit (Machery-Nagel GmbH & Co. KG, Düren, Germany) according to the manufacturer's instructions. All the oligonucleotide primers were synthesized by Sigma-Aldrich Chemie GmbH (Germany), and all reaction components were from Sigma-Aldrich Chemie GmbH (Germany). The qPCR was conducted in 96-well micro titer plates (BioRad, München, Germany) using a CFX Connect Real-Time System (BioRad). Forward primer *fhs1* (5'-GTWTGG GCWAARGGYGGMGAAGG-3') and reverse primer FTHFS-r (5'-GTATTGDGTYTTRGCCATACA-3') were used to amplify *fhs* gene encoding formyltetrahydrofolate synthetase (FTHFS) to quantify acetogenic bacteria (Leaphart et al., 2003; Xu et al., 2009). qPCR was conducted according to the description by Xu et al. (2009). PCR amplification mixture solutions (25 μl) contained 12.5 μl Sybr-Green Ready Mix, 5 μl MgCl₂ (25.0 mM), 0.25 μl of each primer (50 mM) and 1 μl DNA templates. In brief, the program begins with denaturation at 94 °C for 4 min. This step is followed by a short touchdown program consisting of 9 cycles of 94 °C for 45 s, 63 °C for 45 s (decreasing by 1 °C per cycle), and 72 °C for 1 min. A production program consisting of 30 cycles of 94 °C for 45 s, 55 °C for 45 s, and 72 °C for 1 min is used next, followed by a final extension step of 72 °C for 5 min. Melting curve analysis from 65.0 to 98.0 °C at the heating rate of 0.2 °C/s was performed at the end of 39 cycles.

2.4. DNA extraction and terminal restriction fragment length polymorphism (T-RFLP) analysis

For genomic DNA extraction, 1 g wet weight of soil sample was processed using the PowerSoil DNA Isolation Kit (MO-BIO, USA), according to the manufacturer's instructions. Extracted DNA was checked for quality and concentration using a Nanodrop spectrophotometer (Nano-Drop Technologies, Wilmington). The extracted DNA was used to characterize the *mcrA* gene by T-RFLP (Terminal-restriction length polymorphism) (Liu et al., 1997; Chin et al., 1999) using the primers *mcrAf* (TAY GAY CAR ATH TGG YT) and *mcrAr* (ACR TTC ATN GCR TAR TT) (Springer et al., 1995) with FAM (6-carboxyfluorescein)-label at the forward primer. The *mcrA* gene amplicons were digested with

Sau96I (Fermentas), and the products were size-separated in an ABI 3130 DNA sequencer (Applied Biosystems, Darmstadt, Germany). For downstream analysis only fragments between 80 and 520 bp were considered to avoid analysis of false signals originated from primer residuals, primer dimers and undigested PCR product. The normalization and standardization of the T-RFLP profiles was done according to the method of Dunbar et al. (2001). The possible phylogenetic affiliations were determined using previously published data (Chin et al., 1998; Lueders et al., 2001; Ramakrishnan et al., 2001; Kemnitz et al., 2004; Conrad et al., 2008).

2.5. Enzyme assay

Exoglucanase activity was determined as a proxy for fermentative degradation. The potential activity of exo- β -1,4-glucanase was analyzed using fluorogenic methylumbelliferone (MUF)-labeled glucoside (MUF_{GLU}) (Glissmann and Conrad, 2002). Aliquots (0.1 ml) of the incubated soil slurry were mixed with 0.167 ml sodium-phosphate-buffer (50 mM, pH 7.0) and 0.333 ml MUF-labeled substrate (2 mM). Solutions of 0.1 ml gluconolactone (GLN, 330 mM) for inhibiting glycosidases or 0.1 ml distilled water were added. The samples were incubated at the same temperature as used for the pre-incubation of soil slurries. The hydrolysis reaction was stopped after 30 min (MUF_{GLU}) by addition of 1.67 ml ethanol (96%). After mixing thoroughly, the samples were centrifuged (20 min, 5000 rpm) and 200 μ l of the supernatant was mixed with 800 μ l distilled H₂O and 250 μ l NaOH-glycine-buffer (1 M, pH 11.0). Fluorescence was then measured against a blank in a fluorescence-spectrometer (excitation: 365 nm, emission: 450 nm; infinite M200 PRO, Tecan Austria GmbH, Grödig, Austria). For blanks, ethanol and NaOH-glycine-buffer were added before addition of the MUF-labeled substrate, to account for the initial fluorescence of non-hydrolyzed MUF-substrate. The experiment was done twice with three replicates each.

3. Results

3.1. Incubation of Vercelli rice field soils

Vercelli rice field soils were incubated in triplicates at 15, 30 and 50 °C (Fig. 2). The four different treatments (H₂/CO₂, H₂/CO₂ + BES, N₂/CO₂, N₂/CO₂ + BES) are depicted in the four columns of Fig. 2. When H₂ was offered as additional substrate a fast turnover of H₂/CO₂ was observed (Fig. 2A, B, H, I); which was accompanied by drastic increase in the $\delta^{13}\text{C}$ of the CO₂ (Fig. 2C, J). In contrast the incubations under N₂/CO₂ and N₂/CO₂ + BES, resulted in a net production of CO₂ at temperatures above 30 °C and were characterized by comparably small changes in the $\delta^{13}\text{C}$ value of CO₂ (Fig. 2N, O, T, U).

The two major products were CH₄ and acetate. Under H₂/CO₂ the highest CH₄ concentrations were observed in the 50 °C incubations reaching almost 100 $\mu\text{mol g-dw}^{-1}$ (Fig. 2D). This was tenfold higher compared to the incubations under H₂/CO₂ at 15 and 30 °C (Fig. 2D) and two orders of magnitude higher compared to the incubations under N₂/CO₂ (Fig. 2K). In the control incubations under N₂/CO₂ the CH₄ production reached 16 $\mu\text{mol g-dw}^{-1}$ in the 30 °C incubations (Fig. 2P) and > 1 $\mu\text{mol g-dw}^{-1}$ in the 50 °C incubations under N₂/CO₂ + BES (Fig. 2V). Concentrations and $\delta^{13}\text{C}$ of CH₄ could only be detected in the non-inhibited incubations (Fig. 2E, Q). A strong change in the $\delta^{13}\text{C}$ values of CH₄ (−68‰ to −38‰) was observed for incubations under H₂/CO₂ at 30 °C. Values $\delta^{13}\text{C}$ of CH₄ of roughly −100‰ in the incubations under N₂/CO₂ at 50 °C indicate a strong contribution of hydrogenotrophic methanogenesis in these samples (Fig. 2Q).

The highest acetate concentrations were reached for incubations under H₂/CO₂ at 30 °C (Fig. 2F) and for incubations under H₂/CO₂ + BES at 50 °C (Fig. 2L). The control incubations under N₂/CO₂ and N₂/CO₂ + BES resulted in almost two orders of magnitude lower acetate concentrations. The $\delta^{13}\text{C}$ of acetate under H₂/CO₂ always

showed transiently very low values of −67.9‰, −60.2‰ and −45.6‰ for 50 °C, 30 °C and 15 °C, respectively (Fig. 2G). In the presence of H₂/CO₂ + BES similar results were obtained (Fig. 2M). Such very low values of $\delta^{13}\text{C}$ of acetate were completely missing in the control incubations under N₂/CO₂ and N₂/CO₂ + BES (Fig. 2S, X).

We evaluated the composition of the methanogenic community using terminal restriction length polymorphism (TRFLP) profiling of the *mcrA* gene (Fig. S1). The TRFLP showed that in the presence of BES the composition stayed constant and was similar to that of the original soil. In the uninhibited samples, however, *Methanocella* (238-bp TRF) strongly increased in the incubations at 50 °C similarly as found before (Conrad et al., 2009). At 50 °C, the methanogenic community did not contain T-RFs characteristic for aceticlastic *Methanosarcina* or *Methanosaeta*. At the other temperatures, *Methanobacteria* (468-bp TRF) increased and *Methanosarcina* (390-bp TRF) decreased when the soil was incubated under H₂/CO₂ compared to N₂/CO₂.

3.2. Incubation of Philippines rice field soils

For Philippines rice field soils the overall experimental setup was slightly modified. In addition to BES, KCN was added as potential inhibitor of acetogenic bacteria (Fig. 3 fourth column: N₂ + BES + KCN). Substrate stimulation by H₂ addition was used only in the presence of BES (Fig. 3 first column: H₂/CO₂ + BES). Since the addition of CO₂ as additional substrate for acetogens had seemingly no effect on the production of acetate, the background controls were performed using N₂ instead of N₂/CO₂ (Fig. 2 column two and three: N₂ and N₂ + BES).

Overall the results of the incubations were comparable to those obtained with Vercelli rice field soil. Again CO₂ served as a substrate if H₂ was added (Fig. 3A) resulting in increase of $\delta^{13}\text{C}$ of CO₂ over time at all temperatures (Fig. 3B). All control incubations under N₂ showed an increase of CO₂ over time independently of the addition of inhibitors (Fig. 3G, M, S). The $\delta^{13}\text{C}$ values of CO₂ were rather stable for all control incubations (Fig. 3H, N, T), with exception of those at 50 °C which all showed relatively depleted $\delta^{13}\text{C}$ values around day 19.

The released CH₄ reached the highest concentrations in the controls under N₂ (Fig. 3I). The amounts of CH₄ produced were similar (around 10 $\mu\text{mol g-dw}^{-1}$) as for the Vercelli soil under N₂/CO₂ (compare Fig. 2P). In all other incubations only traces of CH₄ (< 1 $\mu\text{mol g-dw}^{-1}$) were observed (Fig. 3C, O, U). The $\delta^{13}\text{C}$ of CH₄ again showed a depletion in ¹³C for the samples at 50 °C under N₂ (Fig. 3J) (comparable to the signals obtained for Vercelli under N₂/CO₂ at 50 °C, Fig. 2Q).

Acetate production was strong in the presence of H₂/CO₂ + BES (Fig. 3E). Without BES under N₂, acetate was transiently produced (up to 8 $\mu\text{mol g-dw}^{-1}$) at 50 °C but not at 15 and 30 °C (Fig. 2K). Acetate continuously increased in the presence of BES (Fig. 3Q, W). Again a transiently low $\delta^{13}\text{C}$ of acetate was observed for H₂/CO₂ + BES reaching −48.5‰, −60.2‰ and −68.0‰ for 50 °C, 30 °C and 15 °C, respectively (Fig. 3F). In the control incubations under N₂, N₂ + BES and N₂ + BES + KCN the isotopic values were around or above the ¹³C signal of the total organic carbon of the Philippine soil (−24.0‰) (Fig. 3L, R, X). For 30 °C and 50 °C, there was no significant difference between the treatments N₂ + BES and N₂ + BES + KCN ($P > 0.05$) during the whole incubation. However, $\delta^{13}\text{C}$ of acetate was slightly higher for N₂ + BES + KCN than for N₂ + BES during 34 days of incubation at low temperature ($P < 0.05$).

The observation that $\delta^{13}\text{C}$ of acetate was only transiently low and later increased (Fig. 3F) may be explained by later consumption of acetate, favouring acetate with the light isotope, or by later production of acetate with relatively high $\delta^{13}\text{C}$, e.g. fermentation of organic carbon. Since acetate consumption was not obvious (Fig. 3E), we analyzed exoglucanase activity as a proxy for fermentative degradation. Indeed, exoglucanase activity increased at 30 and 50 °C (Fig. 4) as soon as the substrates for reductive acetogenesis (H₂/CO₂) were depleted (compare Fig. 3C). At 15 °C activity was relatively high during the entire incubation (Fig. 4).

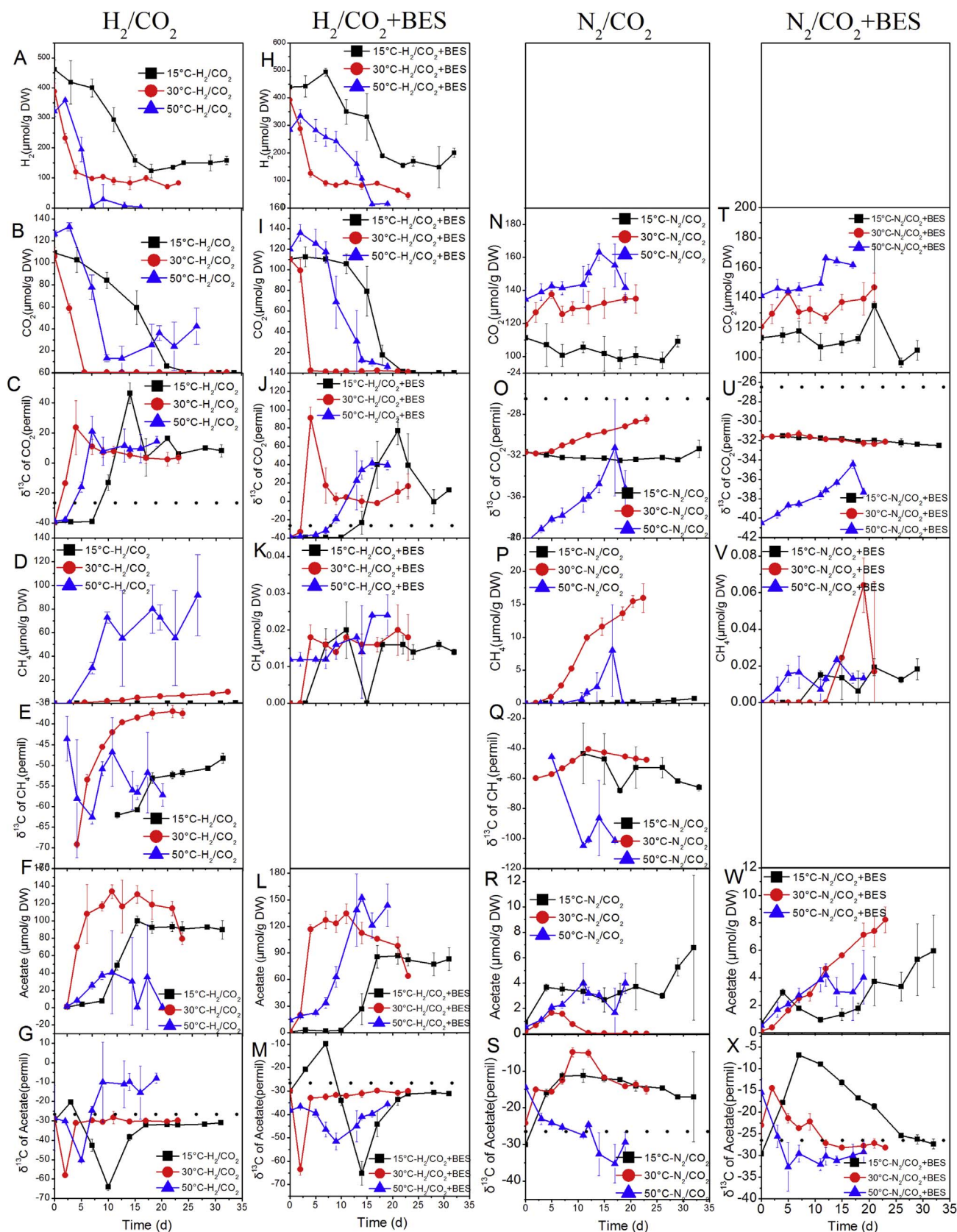


Fig. 2. Turnover of the substrates H₂(A, H) and CO₂ (B, I, N, T), into CH₄ (D, K, P, V) and acetate (F, L, R, W) and δ¹³C values of CO₂ (C, J, O, U), CH₄ (E, Q) and total acetate (G, M, S, X) in Vercelli rice field soil incubated at 15, 30 and 50 °C with BES as an inhibitor of methanogenesis. The δ¹³C of the organic matter in the Vercelli soil (-26.51‰) was represented by dotted line. Mean ± SD, n = 3.

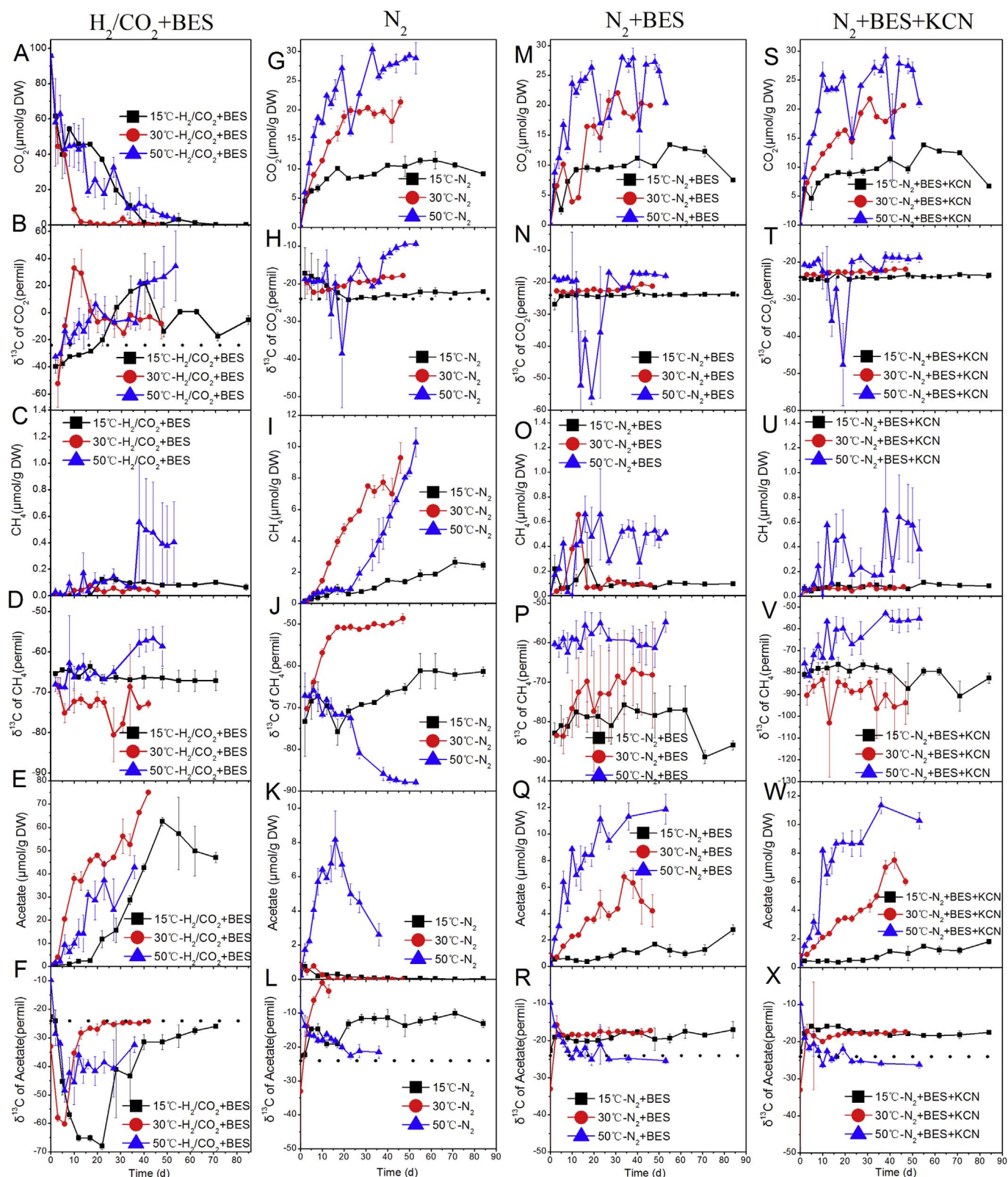


Fig. 3. Turnover of CO₂ (A, G, M, S) into CH₄ (C, I, O, U) and acetate (E, K, Q, W) and δ¹³C values of CO₂ (B, H, N, T), CH₄ (D, J, P, V) and total acetate (F, L, R, X) in Philippines rice field soil incubated at 15, 30 and 50 °C with BES as an inhibitor of methanogenesis or KCN as an inhibitor of acetogenesis. The δ¹³C of the organic matter in the Philippines soil (−24.01‰) was represented by dotted line. Mean ± SD, n = 3.

The quantity of the *fhs* gene was used as a proxy for the number of acetogenic bacteria in Philippines rice field soil (Fig. 5). Irrespectively of temperature or presence of inhibitors the abundance of the *fhs* gene was on the order of 10⁵ and 10⁶ gene copies per gram dry weight soil.

Addition of H₂/CO₂ which resulted in increased acetate production (compare Fig. 3E) did not affect the abundance of *fhs*.

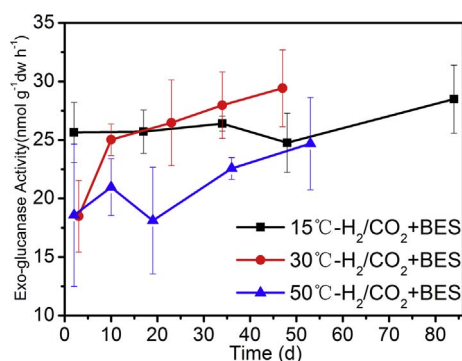


Fig. 4. Exoglucanase activities in Philippines rice field soil under H_2/CO_2 headspace incubated at 15, 30 and 50 °C in the presence of BES. Mean \pm SD, $n = 3$.

3.3. Relative contribution of acetogenesis

In order to explore the contribution of acetogenesis at different temperatures, we determined the ratios of the produced acetate to the overall product pool (acetate + CH_4) (Table 1). This may serve as a proxy to understand if the different temperature incubations shifted the competition for hydrogen between methanogens and acetogens. The data indicate a tendency of high contribution of acetogenesis at low temperatures (Vercelli soil) and under conditions where H_2 supply was high, i.e., treatments with H_2/CO_2 and/or with BES (inhibition of H_2 -consuming methanogens).

4. Discussion

The incubations under elevated H_2/CO_2 clearly demonstrated that both soils had the potential for chemolithotrophic acetate production when compared to controls under either N_2 (Philippines) or N_2/CO_2 (Vercelli). Acetate production under H_2/CO_2 was up to two orders of magnitude larger than in the controls. Only under these conditions was isotopically very light acetate (-45.6% to -68%) observed. These values were well within the range of previously reported ones for acetogenic pure cultures, and thus were apparently due to acetate production by chemolithotrophic acetogens. Incubations under H_2/CO_2 resulted in acetate production irrespectively of whether methanogenesis was inhibited (with BES) or not. Inhibition of methanogenesis actually increased acetate production probably since acetate consumption by aceticlastic methanogens was inhibited.

Hence, our observations show that chemolithotrophic acetogenesis is potentially expressed in environmental samples if H_2 and CO_2 are supplied. This is in line with a previous study using Italian rice field soil incubations at 15 °C and 50 °C and monitoring the formation of

Table 1
Percentage of acetate relative to total products (acetate + methane) formed.

Treatment		15 °C	30 °C	50 °C
Vercelli soil	H_2/CO_2	100	93	31
	H_2/CO_2 + BES	100	100	100
	N_2/CO_2	89	9	33
	N_2/CO_2 + BES	92	100	100
Philippine soil	N_2	21	23	15
	N_2 + BES	97	99	96
	N_2 + BES + KCN	95	99	97
	H_2/CO_2 + BES	100	100	99

isotopically labeled acetate in the presence of $^{13}CO_2$ enriched atmosphere (Liu and Conrad, 2011). Under these conditions it was also possible to characterize the acetogenic community to a certain extent using stable isotope probing of ribosomal RNA (Liu and Conrad, 2011). However, the expression was not necessarily paralleled by an increase of copy numbers of the *fhs* gene, suggesting that the populations of chemolithotrophic acetogens did not proliferate significantly. This implies that the consumption of the excess H_2 and CO_2 was most probably regulated by the already expressed *fhs* genes by modulation of the enzyme activity rather than by de-novo synthesis of *fhs* copies. Alternatively the quantified *fhs* genes may not all be affiliated with known acetogens. This has been shown in the previously cited investigation of peat soils (Haedrich et al., 2012), where in general only a small number of known acetogens could be linked to the *fhs* genes found in environmental samples and the vast majority was associated with novel phylogenetic clusters. A broad investigation on the presence of *fhs* genes concluded that 10^5 to 10^9 gene copies per gram could be obtained for different environmental samples (Xu et al., 2009); rice field soil was reported to contain 10^7 *fhs* genes which is slightly higher than our results.

The isotopically depleted acetate was only transiently produced, and production stopped when the added H_2/CO_2 was consumed. Then $\delta^{13}C$ of acetate subsequently increased again and eventually attained a value close to that of soil organic matter. Since acetate was not consumed (at least in the presence of BES), the increase of $\delta^{13}C$ of acetate was probably caused by fermentation of organic substrates. Indeed the fast change in the $\delta^{13}C$ of acetate was accompanied by only a small increase in acetate concentration suggesting that either the freshly released acetate has a very high $\delta^{13}C$ value or that the overall isotopic value of the acetate is influenced by other processes, e.g., exchange reactions of the carboxyl group with the CO_2 pool (Wood, 1952; Schulman et al., 1973; Diekert and Ritter, 1983; Raybuck et al., 1991). In the uninhibited control samples (Vercelli, H_2/CO_2) the addition of substrate also resulted in increased CH_4 production.

Inhibition of methanogenesis effectively increased the production of

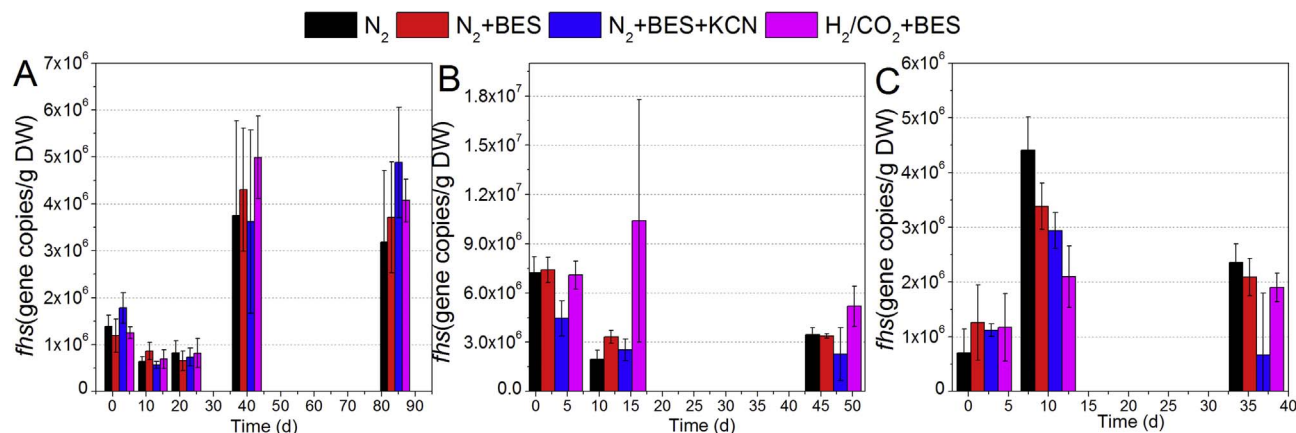


Fig. 5. Copynumbers of *fhs* gene during incubation of Philippines rice field soil at 15 °C (A), 30 °C (B) and 50 °C (C) with BES as an inhibitor of methanogens or KCN as an inhibitor of acetogenesis. Mean \pm SD, $n = 3$.

acetate compared to the release of CH₄. Indeed the contribution of CH₄ to the product pool (CH₄ and acetate) was always below 10% when BES was applied. We would expect that treatments with BES result in elevated acetate concentrations, since one of the major acetate sinks (aceticlastic methanogenesis) is inhibited. Likewise the inhibition of the hydrogenotrophic methanogenesis may result in excess H₂/CO₂ as possible substrate for chemolithotrophic acetogenesis. Indeed acetate concentrations under N₂ + BES or N₂/CO₂ + BES were usually higher compared to the control incubations without BES. However, the δ¹³C of acetate was not different between the inhibited vs. control incubations in both soils suggesting that H₂/CO₂ was not the dominant substrate for acetate production. The strongest difference was observed for the 30 °C incubations of Vercelli soil under N₂/CO₂ vs. N₂/CO₂ + BES (Fig. 2S, X). However, the δ¹³C of acetate were either higher or only slightly lower than the δ¹³C of soil organic matter so that acetate production by fermentation or heterotrophic acetogenesis was more likely than by chemolithotrophic acetogenesis. The application of KCN in addition to BES (Fig. 3S, T, U, V, W, X) did not show a significant difference to the incubations without KCN (Fig. 3M, N, O, P, Q, R) at 30 °C and 50 °C, indicating that the inhibition did not work and that the acetogens were not active at medium and high temperatures. However, the addition of KCN resulted in a distinct ¹³C enrichment of acetate at 15 °C in presence of BES, showing that KCN inhibited acetate production by chemolithotrophic acetogenesis at low temperature (Fig. 3R, X). The reason for inhibition by KCN only at low temperature may be that H₂-dependent acetogenesis is a more favoured process at low temperature than at medium and high temperatures.

Incubation temperature has a strong effect on metabolic activity and pathway usage in methanogenic environments. Indeed a previous study had shown that chemolithotrophic acetogenesis operated in Vercelli soil at both 50 °C and 15 °C especially when exogenous H₂ was added, and in addition had shown that different bacterial taxa were involved in this process at the two temperatures (Liu and Conrad, 2011). Previous studies using freshwater sediments under elevated H₂ concentrations using BES to inhibit methanogenesis at low temperature (8 °C) likewise concluded that only under elevated H₂ concentrations and in the presence of BES low δ¹³C values of acetate could be reported. (Heuer et al., 2010). A similar conclusion was reached using peat soil under elevated H₂ concentrations at 15 °C (Haedrich et al., 2012). However, in pure culture studies the isotope fractionation itself is usually not influenced by the incubation temperature (Penger et al., 2014). Hence, the differences in δ¹³C of acetate, which were observed after incubation at different temperatures, must be due to different processes involved in production and consumption of acetate; e.g. by activating different specifically adapted populations. However, the δ¹³C of the acetate was generally much higher than in the presence of H₂/CO₂, usually even higher than the δ¹³C of soil organic carbon, so that a major contribution of chemolithotrophic acetogenesis is unlikely. Therefore, the isotopic signals of the acetate in the incubations without substrate (H₂/CO₂) addition did not allow to differentiate the pathway usage affecting the acetate pool.

Contribution of acetogenesis to the overall products (Table 1) showed that with BES, the ratios were as expected around 100%, since CH₄ was not produced. In the Vercelli incubations under N₂/CO₂ (without BES) the contribution of acetate production at 15 °C (89%) was higher than at 30 °C (9%), where acetate was only transiently formed and then converted to CH₄ (Fig. 2P, S). In the Philippine soils, the ratios of acetate at 15 °C (95%) was lower than at 30 °C (99%) or 50 °C (97%) in the presence of BES and KCN, albeit the difference was only small. Accordingly, δ¹³C of acetate at 15 °C was generally higher than that at 30 °C and 50 °C before Day 28 (Fig. 3X), indicating that the isotopic signature of acetate was dominated by fermentation and heterotrophic acetogenesis rather than by fermentation and chemolithotrophic acetogenesis. This was an evidence for significant chemolithotrophic acetogenesis at low temperature, which resulted in relatively strong δ¹³C-enrichment in the acetate pool under the inhibition of both

methanogenesis and acetogenesis. These results indicate that chemolithotrophic acetogenesis was indeed more important at low than at medium and high temperature.

At high temperature (50 °C), the ratios of acetate to acetate + CH₄ were relatively small (Table 1). Even under H₂/CO₂ the ratios were lower at 50 °C (31%) than at 30 °C (93%) or 15 °C (100%). At this temperature acetogenesis was probably minor compared to acetate consumption by syntrophic acetate oxidizers. Acetate consumption by syntrophic acetate oxidizers at high temperature is consistent with the lower values of δ¹³C of acetate at 50 °C compared to those at 15 or 30 °C (Fig. 2S, 3 L), since literature data indicate that aceticlastic methanogens have a higher fractionation factor for the consumption of acetate than syntrophic acetate oxidizers (Conrad and Klose, 2011). Syntrophic acetate oxidation coupled to hydrogenotrophic methanogenesis was also consistent with the composition of the methanogenic community, which was dominated by hydrogenotrophic *Methanocella* while aceticlastic *Methanosarcina* and *Methanosaeta* were absent. This pattern is consistent with a previous study (Conrad et al., 2009).

5. Conclusion

Our study showed that rice field soils have the potential for chemolithotrophic acetogenesis, but only when H₂/CO₂ are added as external substrates. Only then very low δ¹³C of acetate was observed for all incubations irrespectively of the incubation temperature. However, the very low δ¹³C of acetate was transient and reverted to values closer to the δ¹³C of organic matter as soon as the added H₂/CO₂ was consumed. Without addition of H₂/CO₂, chemolithotrophic acetogenesis was insignificant and could not be detected using isotopic values of acetate. Importance of acetate production may be higher at low versus medium temperatures, but the relatively high δ¹³C of acetate indicated production by fermentation (or maybe by heterotrophic acetogenesis) rather than by chemolithotrophic acetogenesis. It was possible to determine production of acetate together with the isotopic signatures without simultaneous consumption when aceticlastic methanogenesis was inhibited with BES. However, this approach was not possible at high temperatures when acetate was presumably consumed by syntrophic acetate oxidizers which were not inhibited by BES. Addition of KCN as putative inhibitor of acetogens was not helpful. The quantification of *fts*, as characteristic gene for acetogens, also turned out to be inconclusive. Hence, discrimination of acetogenically formed acetate using the described techniques is still a problem for future research. Currently, it is only safe to determine the potential for chemolithotrophic acetogenesis through stimulation by H₂/CO₂ and evaluating the δ¹³C of acetate.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at <http://dx.doi.org/10.1016/j.soilbio.2017.10.034>.

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