Comparison of isotope methods for partitioning methane production and soil C priming effects
during anaerobic decomposition of rice residue in soil

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Abstract: Management of rice residue in paddies often results in higher methane (CH₄) emissions, but the carbon (C) sources contributing to higher emissions are not well characterized. We examined the relative contribution of soil and residue C sources for CH₄ production in three different soils using both ¹³C-labeled or unlabeled (natural abundance) rice and maize residues. The results were compared to a recently proposed natural ¹³C abundance soil C partitioning method (CNAM) (Conrad et al. 2012b). As anticipated, residue addition increased CH₄ production, with the timing and magnitude of emission varying across soils, likely due to differences in soil C and amount of electron acceptors. The CH₄ derived from residue-C, when estimated using the typical ¹³C natural abundance method yielded either negative values or those exceeding 100%. Similarly, the CNAM frequently overestimated the contribution of residue C compared to the ¹³C enriched residue method (ERM). The over estimation is likely due to C isotopic fractionation during the utilization of rice and maize residues in the different soils. Substantial C isotope fractionation would bias the fundamental assumption of the CNAM. The ERM produced a consistent partitioning result of 50 to 70% of CH₄ derived from residue additions. When typical minimum, average, and maximum values of natural ¹³C fractionation factors were applied to the ERM the partitioning outcome was unaffected. The priming effect of residue addition on CH₄ production was regulated by electron acceptor concentrations in various soils and was positively correlated to the degree of residue decomposition. The priming effect of residue, therefore, depends both on soil and residue properties, with the ERM showing to be more accurate to account for the partitioning of CH₄ from various C pools compared to CNAM.

Keywords: methane production partitioning; C isotopic fractionation; priming effect; anaerobic decomposition;

1. Introduction

The demonstration of soil C priming from crop residues under aerobic decomposition has been studied extensively (Chen et al., 2014; Keiluweit et al., 2015; Kuzyakov, 2010; Qiao et al., 2014; Zhu and Cheng, 2011). However, the priming effect in anaerobic systems, such as rice fields, has received much less attention. Paddy soils are important sources of atmospheric methane (CH_4), a potent greenhouse gas with a global warming potential 28 to 34 times greater than CO_2 over 100 years (Myhre et al., 2013), contributing up to 19% of global total emissions (Forster et al., 2007). Crop residue management (retention) often leads to increased CH_4 emissions (Bossio et al., 1999; Ye et al., 2015), but the role it plays in the priming of soil organic carbon (SOC) as a source for CH_4 production is not well established, due not only to the lack of studies but the lack of a reliable method to partition sources of CH_4 (Conrad et al., 2012b; Yuan et al., 2012).

Quantifying the sources of CH_4 production is essential to determine the timing, size, and duration of the priming effect contributing to increased CH_4 production. Unlike aerobic SOC mineralization, the reactions leading to CH_4 production include a series of related microbial processes (Conrad, 1999; Reddy and DeLaune, 2008), resulting in relatively larger C isotopic fractionation factors, which typically range from 1.01 to 1.07 (Conrad, 2005). Therefore, accurate partitioning of CH_4 production into its sources requires consideration of the associated fractionation factors (Conrad et al., 2012b), which, however, is largely neglected by the most widely used approaches to partition C fluxes, such as ^{13}C natural abundance and labelling experiments with ^{14}C or ^{13}C (Kuzyakov, 2006; Rochette et al., 1999).

Recently, Conrad et al. (2012b) proposed a ^{13}C natural abundance method (CNAM) for differentiating among soil C substrates contributing to CH_4 production, which does not consider

C isotope fractionation. This method assumes that overall C isotope fractionation associated with CH₄ production is reflected proportionately in CH₄ generated from natural abundance ¹³C values in rice (~δ¹³C -28 ‰) and maize (~δ¹³C -12.5 ‰) residues that are sufficiently different to SOC (~δ¹³C -27 ‰) found in wetland environments. The equation used to calculate CH₄ production from rice residue is subtracted from an analogous equation for maize residue (Equation 2). As such, the resulting equation (Equation 3) consists only of variables that are easily measured (¹³C content of residue source and the emitted CH₄), and therefore, the amount of CH₄ derived from residue can be calculated regardless of the fractionation occurring in this process.

The CNAM provides a convenient approach to partition CH₄ production sources between SOC and plant residues, and can be potentially employed in field investigations of the priming effect induced by residue management under anaerobic processes (Conrad et al., 2012b). This method was developed in mineral soils with electron acceptors intentionally exhausted to allow methanogens to prevail. Under natural conditions the availability of electron acceptors and C substrates varies dramatically in space and time. Furthermore, the presence of electron acceptors generally limits electron and C flows to CH₄ production (Reddy and DeLaune, 2008), and most importantly, their presence affects the decomposition patterns of plant materials and other organic constituents in soils (Chidthaisong and Conrad, 2000a; Chidthaisong and Conrad, 2000b). In addition, the chemical composition of rice (a C₃ plant) and maize (a C₄ plant) residues can be significantly different (Singh et al., 2011). It is therefore hypothesized that in the presence of electron acceptors the decomposition of plant residue source that contributes to CH₄ production is different according to the residue source, and consequently the final C isotopic fractionation is also likely different. The objective of this study was 1) to compare the CNAM

with both a typical ^{13}C -natural abundance method and a ^{13}C -labeling approach to calculate the relative contribution of C sources to CH_4 production during the anaerobic decomposition of plant residues and SOC, and 2) to quantify the timing, magnitude, and duration of the priming effect on CH_4 production in paddy soils.

2. Materials and Methods

2.1. Residue and soil preparation

Two independent experiments were conducted. In Experiment I, a rice residue (Rice 1) and maize residue (Maize 1) were incubated in a peat paddy soil (Soil 1) for 32 days. Experiment II used a combination of two rice residues (Rice 2 and 3), two maize residues (Maize 2 and 3), and two soils (Soil 2 and 3). Rice 1 and Maize 1 were collected from fields at Twitchell Island, CA, USA (38°06' N, 121°39' W) in 2012, while both Rice 2 and 3 were collected from fields at the California Rice Experiment Station (RES) near Biggs, CA, USA (39°27' N, 121°44' W) in 2014. Maize 2 and 3 were collected from fields near Stockton, CA, USA (37°57' N, 121°11' W) in 2014. All residues were harvested at the end of the growing season. A ^{13}C -enriched rice residue (Rice 4) was also included in both experiments. Rice 4 was obtained by growing rice plants to maturity in the greenhouse using a sealed chamber to deliver ^{13}C - CO_2 intermittently as the source of ^{13}C enrichment (Bird et al., 2003). Residues were dried at 60 °C to a constant weight and stored at room temperature (20±1°C) until used. The residues were cut into small pieces (<1cm) and ground coarsely with a household coffee grinder prior to the laboratory incubation.

Soil 1 was sampled from a rice paddy at Twitchell Island during harvesting of Rice 1, and used in Experiment I after the removal of vegetation, visible roots, and dead plant residues. Soil 2 was collected from a rice paddy adjacent to Soil 1 (< 50 m), but contained less SOC and total

iron, while Soil 3 was collected from a rice paddy at the RES. Both Soil 2 and Soil 3 were collected at the end of the 2014 growing season, air-dried, sieved (2 mm), and stored at room temperature ($20\pm 1^{\circ}\text{C}$) until used. The soils at the Twitchell Island site are silt loams classified as euic, thermic Typic Haplosaprists, while Soil 3 is a clay soil classified as fine smectitic, thermic Xeric Epiaquerts and Duraquerts. Selective analyses of residue and soil are given in Tables 1 and 2.

2.2. Laboratory incubation

In Experiment I, 10 g Soil 1 at 45-48% moisture content was mixed with 50 mg residue (Rice 1, Maize 1, or Rice 4) in a 160 mL serum bottle, followed by 10 mL deionized (DI) water to submerge the soil. The bottles were crimped sealed with butyl rubber septa, purged with N_2 gas for 10 minutes to create anaerobic conditions, and incubated at $20\pm 1^{\circ}\text{C}$ in the dark for 32 days. Control samples without residue addition were prepared concurrently. Each treatment had 4 replicates.

In Experiment II, 250 g of Soil 2 and 3 were saturated with DI water (48% water content for Soil 2 and 37% for Soil 3) in a 1 L Mason jar and pre-incubated at $20\pm 1^{\circ}\text{C}$ in the dark for 7 days. The jars contained lids with a foam plug to allow for air exchange. After pre-incubation, 10 g of well-mixed bulk soils were transferred to a 160 mL serum bottle and mixed with 50 mg residue (Rice 2, Rice 3, Rice 4, Maize 2, or Maize 3), followed by 10 mL DI water. The incubation conditions were the same as in Experiment I with 4 replicates of each treatment prepared. Controls were prepared as above.

2.3. Soil and residue chemistry

Total C and N, and $\delta^{13}\text{C}$ and $\delta^{15}\text{N}$ values of the soils and residues were analyzed at the Stable Isotope Facility of the University of California Davis on a PDZ Europa ANCA-GSL

elemental analyzer interfaced to a PDZ Europa 20-20 IRMS (Sercon Ltd., Cheshire, UK). Soil pH was measured in a soil to water ratio of 1:3 after equilibration for 30 min. Dissolved organic C (DOC) was quantified by UV-persulfate oxidation (Teledyne-Tekmar Phoenix 8000) after extracting the soils with 0.5 M K_2SO_4 for 1 h, followed by centrifugation and filtration through Whatman 57 filter paper. Subsamples of the filtrates were also used to measure extractable NO_3^- (Doane and Horwath, 2003) and NH_4^+ (Verdouw et al., 1978). Total extractable Fe was measured by hydrochloric acid extraction and Ferrozine determination as described by Ye et al. (2015). For extractable SO_4^{2-} , soils were extracted with deionized water for 30 min, followed by centrifugation and filtration through Whatman GA55 glass fiber filters. Concentration of soluble sulfur was determined by inductively coupled plasma atomic emission spectrometry (ICP-AES) (iCAP 6500, Thermo Elemental), and reported as SO_4^- -S assuming that all sulfur present in soil extracts is in the sulfate form (EPA, 2001).

2.4. CO_2 and CH_4 measurements

Headspace samples (0.5~1 mL) were collected frequently throughout the incubation. Methane and CO_2 were quantified on a gas chromatograph (GC) (GC-2014, Shimadzu), equipped with a thermal conductivity detector (for CO_2) and a flame ionization detector (for CH_4). Total CO_2 and CH_4 production was calculated from both gas and liquid phases, adjusting for solubility, temperature, and pH (Stumm and Morgan, 1995). On days 6, 10, 15, 22 and 32, headspace ^{13}C - CH_4 was determined at the Stable Isotope Facility of the University of California Davis on a Thermo GC combustion isotope ratio mass spectrometer (GC/C-IRMS) consisting of a Trace GC Ultra gas chromatograph (Thermo Electron Corp., Milan, Italy) coupled to a Delta V Advantage IRAM through a GC/C-III interface (Thermo Electron Corp., Bremen, Germany).

2.5. Calculations

The typical ^{13}C natural abundance method was used to partition CH_4 production in soils (all soils are C_3 -plant derived) amended with maize residue as follows,

$$F_1 = (\delta^{13}\text{C}_{\text{MS, CH}_4} - \delta^{13}\text{C}_{\text{SOC, CH}_4}) / (\delta^{13}\text{C}_{\text{MS}} - \delta^{13}\text{C}_{\text{SOC}}) \quad (1)$$

where F_1 is the fraction of total CH_4 production derived from residue, $\delta^{13}\text{C}_{\text{MS, CH}_4}$ and $\delta^{13}\text{C}_{\text{SOC, CH}_4}$ are $\delta^{13}\text{C}$ - CH_4 values in soils incubated with and without maize residue, respectively; while $\delta^{13}\text{C}_{\text{MS}}$ and $\delta^{13}\text{C}_{\text{SOC}}$ are $\delta^{13}\text{C}$ values for maize residue and soil. This method uses the natural variation in ^{13}C isotopic composition and has been widely used to partition C fluxes from various sources (Kuzyakov, 2006). It was assumed that the C fractionation factor associated with CH_4 production is the same for residue and SOC. The ^{13}C -enrichment method used an analogous equation to quantify the contribution of ^{13}C -enriched rice residue to the total CH_4 production (F_2). However, we further considered potential differences in associated isotopic fractionation between CH_4 production from ^{13}C -enriched rice residue and from SOC by applying typical minimum (-10‰), averaged (-40‰), and maximum (-70‰) enrichment factors (Conrad, 2005) into the following equation (i.e. equation 6 in Conrad et al. (2012b)),

$$F_2 = (\delta^{13}\text{C}_{\text{RS, CH}_4} - \delta^{13}\text{C}_{\text{SOC, CH}_4}) / (\delta^{13}\text{C}_{\text{RS}} + \epsilon_{\text{RS, CH}_4} - \delta^{13}\text{C}_{\text{SOC, CH}_4}) \quad (2)$$

where $\delta^{13}\text{C}_{\text{RS, CH}_4}$ are $\delta^{13}\text{C}$ - CH_4 values in soils incubated with rice residue, $\delta^{13}\text{C}_{\text{RS}}$ are $\delta^{13}\text{C}$ values for rice residue, and $\epsilon_{\text{RS, CH}_4}$ is the isotopic enrichment factor.

The $\Delta^{13}\text{C}$ values, i.e. $\delta^{13}\text{C}$ - CH_4 relative to $\delta^{13}\text{C}$ value of the organic matter from which it was derived ($\Delta^{13}\text{C} = \delta^{13}\text{C}_{\text{CH}_4} - \delta^{13}\text{C}_{\text{Straw or SOC}}$), and the priming effect was calculated as described by Conrad et al. (2012b).

The fraction of total CH₄ production derived from residue (F₃) as described by Conrad et al. (2012b) is,

$$F_3 = (\delta^{13}\text{C}_{\text{MS, CH}_4} - \delta^{13}\text{C}_{\text{RS, CH}_4}) / (\delta^{13}\text{C}_{\text{MS}} - \delta^{13}\text{C}_{\text{RS}}) \quad (3)$$

where $\delta^{13}\text{C}_{\text{MS, CH}_4}$ and $\delta^{13}\text{C}_{\text{RS, CH}_4}$ are $\delta^{13}\text{C}$ -CH₄ values in soils incubated with maize and rice residue, respectively; while $\delta^{13}\text{C}_{\text{MS}}$ and $\delta^{13}\text{C}_{\text{RS}}$ are $\delta^{13}\text{C}$ values for maize and rice residue. In Experiment II, two rice (Rice 2 and 3) and two maize residues (Maize 2 and 3) were used. We therefore used all possible combinations of rice and maize residues (i.e. Rice 1 and Maize 1, Rice 1 and Maize 2, Rice 2 and Maize 1, Rice 2 and Maize 2) to calculate CH₄ partitioning with the CNAM.

2.6. Statistical analysis

One-way repeated measures ANOVA (SAS 9.3, SAS Institute) was used to detect the main effect of residue addition and the sources of residues on measured variable in each soil, respectively. Tukey's test was used to test the significant differences among individual treatments. The data were tested for normality and log-transformed if the transform resulted in significant improvements in the overall distribution, except for the data of $\delta^{13}\text{C}$ -CH₄ and $\Delta^{13}\text{C}_{\text{residue, CH}_4}$, which contain mostly negative values. Correlation analysis was conducted with JMP Pro 10. All significant levels were set at $\alpha = 0.05$.

3. Results

3.1. CH₄ production rates

In the absence of residue, CH₄ production was generally less than 0.004, 0.013, and 0.921 $\mu\text{mol g}^{-1} \text{d}^{-1}$ in Soils 1, 2, and 3, respectively, for both experiments (Fig. 1). As expected,

the rates were significantly enhanced by residue addition (Figure 1, Table 3) and showed a typical pattern with a lag period at the beginning, followed by an accelerated phase and then a plateau response. The duration of the lag period and time to reach maximum rates followed the order of Soil 1 > Soil 2 > Soil 3 for each residue type. The CH₄ production rates were always the highest in Soil 3, followed by Soil 2 and then Soil 1 after day 5.

In Soil 1, CH₄ production rates were generally highest after day 5 when maize was added, though the difference was not always significant (Fig. 1a). Similarly, addition of maize residue always induced higher CH₄ production than rice residue in both Soil 2 and 3 after the lag period (Figs. 1b, c). However, there was no difference between each maize residue in either Soil 2 or Soil 3, although the difference between the rice residues was obvious as Rice 4 generally induced higher CH₄ production than Rice 2 and 3 in both soils (Figs. 1b, c).

3.2. $\delta^{13}\text{C}$ -CH₄ and $\Delta^{13}\text{C}_{\text{residue, CH}_4}$ values

The ^{13}C values of CH₄ ranged from -61 to -28‰, from -74 to -44‰, and from -87 to -45‰ in the maize, rice, and no residue treatments, respectively (Figs. 2a, c, e). In Soil 1, $\delta^{13}\text{C}$ -CH₄ was less negative with maize than with rice residue, but the difference was not observed towards the end of the incubation on days 22 and 32 (Fig. 2a). In Soils 2 and 3, the addition of maize residue always induced ^{13}C -enriched CH₄ production compared to unenriched rice residue (Figs. 2c, e). Moreover, there was consistently no difference in the $\delta^{13}\text{C}$ -CH₄ between soils amended with same residue type in both Soil 2 and 3 (Figs. 2c, e).

In Soil 1, $\Delta^{13}\text{C}_{\text{residue, CH}_4}$ values were always higher in the maize treatment than in rice, increasing from day 13 to 32, after the lag period had passed (Fig. 2b). However, this pattern was not observed in either Soil 2 or 3, where the values fluctuated during the same period of time

(Figs. 2d, f). Despite these variations, $\Delta^{13}\text{C}_{\text{residue, CH}_4}$ values were generally the same when Soil 2 and 3 were amended with same type of residue (Figs. 2d, f), though the difference was marginal in Soil 2 for Rice 2 and 3 on day 5 and for Maize 2 and 3 on day 15.

3.3. Partitioning CH_4 production into its sources

With the typical ^{13}C -natural abundance method (Equation 1), maize residue addition often contributed negatively to total CH_4 production in Soil 1, while in Soils 2 and 3, maize contributed over 100% (Table 4). However, these obvious discrepancies were not observed when ^{13}C -enriched rice residue was used, with the fraction of total CH_4 derived from rice residue ranging from 52 to 63%, 61 to 71%, and 52 to 67% in Soil 1, 2, and 3, respectively (Fig. 3). When C isotopic fractionation was considered by applying typical minimum (-10‰), average (-40‰), and maximum (-70‰) ^{13}C enrichment factors, Equation 2 yielded similar results with the ^{13}C -enrichment approach, with an average of 2%, 1%, and 3% difference, respectively, in the value of the contribution of residue (Fig. 4).

The CNAM frequently yielded values over 100% for CH_4 production derived from different residues, and these values varied significantly across soil types and time (Fig. 3, Table 3). In both Soil 2 and 3 the CH_4 yields were consistently the same when the same rice residue was used with either maize residue in Equation 3 (Figs. 3b, c). However, when the same maize residue was used with either rice residue the yields were not always the same.

3.4. Priming effect

The residue-induced increase in CH_4 production from SOC increased almost linearly from day 6 to 32 in Soil 3, while there was a lag period in both Soil 2 and 1 (Fig. 5a). Consequently, cumulative production was highest in Soil 3, followed by Soil 2 and Soil 1. The

priming effect (i.e. residue-induced increase in CH₄ production from SOC relative to the control production) varied significantly across soils during the entire experiment (Fig. 5b). In Soil 1, the priming effect was minimal from day 6 to 10, but increased rapidly after day 10. During the same period, the priming effect ranged from 33 to 37 in Soil 3, but decreased gradually from day 10 to the end of the experiment. The priming effect in Soil 2 did not change from day 6 to 15, ranging from 10 to 14, but increased sharply from day 15 to 22 and then leveled off. Despite all the differences across soil type and time, cumulative primed CH₄ production was highly correlated to CH₄ derived from residue ($R^2 = 0.97$, $p < 0.01$, Fig. 6).

4. Discussion

4.1. CH₄ production and C isotopic fractionation

Methanogenesis is the terminal step of anaerobic decomposition and is generally inhibited by the presence of various electron acceptors (Reddy and DeLaune, 2008), which largely explains the minimal production of CH₄ observed in all soils during the early stage of the experiment (Fig. 1). As expected, CH₄ production followed a typical pattern with a lag period, followed by an exponential increase and then a plateau response (Segers and Kengen, 1998). The lag period has been widely believed to be dominated by microbial respiration and reduction of electron acceptors and its duration largely depends on overall concentrations of electron acceptors (Reddy and DeLaune, 2008). Addition of rice and maize residue accelerated the consumption of various electron acceptors and consequently increased CH₄ production (Watanabe et al., 1998; Ye et al., 2015). In the present study, the difference in duration of the lag period among the three soils (Fig. 1) essentially implied that Soil 1 contained the highest overall concentrations of electron acceptors, followed by Soil 2 and Soil 3, which was further supported by the measured concentrations of NO₃⁻, extractable Fe and SO₄-S in these soils (Table 2). It is

therefore not surprising that Soil 3 had the highest CH₄ production potential, while Soil 1 the lowest (Fig. 1). Moreover, the methanogenic activity appeared to be limited by substrate supply in the later stage of the experiment, especially in Soil 3, as demonstrated by the plateau phase of CH₄ production following the period of exponential increase (Reddy and DeLaune, 2008; Segers and Kengen, 1998).

Addition of maize residue caused higher CH₄ production than rice residue in all soils, especially in Soils 2 and 3 (Fig. 1), suggesting higher available C in maize, likely due to its lower C to N ratio compared to rice (Table 1) (Singh et al., 2011). This phenomenon was also reported by Conrad et al. (2012a, b). However, unlike the results of Conrad et al. (2012b), the present study shows that the $\delta^{13}\text{C}$ values of CH₄ relative to the $\delta^{13}\text{C}$ values of the different residue from which it was derived (i.e. $\Delta^{13}\text{C}_{\text{residue, CH}_4}$) were generally different between the maize and rice residue treatments in each soil (Figs. 2b, d, f), suggesting that C isotopic fractionation was different when CH₄ was produced from rice than from maize residue. The changes in $\Delta^{13}\text{C}_{\text{residue, CH}_4}$ over time were likely attributable to changes in residue substrate quality (i.e. changes in the $\delta^{13}\text{C}$ values of substrates) and methanogenic pathways (i.e. changes in isotopic fractionation factors) during decomposition (Conrad, 2005; Conrad et al., 2012a; Fernandez et al., 2003). It has been demonstrated previously that various organic constituents of plant materials have different ¹³C composition and different utilization patterns during decomposition, which directly affects C isotopic fractionation (Benner et al., 1987; Fernandez et al., 2003; Werth and Kuzyakov, 2010). Moreover, the type of microbial metabolism (e.g. fermentation vs. acetogenesis, hydrogenotrophic vs. acetoclastic methanogenesis) also affects the magnitude of fractionation (Cifuentes and Salata, 2001; Conrad, 2005; Heuer et al., 2009; Ijiri et al., 2012). In the present study, maize residue was different than rice residue in its total C content and C to N

ratio (Table 1) and extent which they induced methanogenic activity (Fig. 1). It is thus not surprising that $\Delta^{13}\text{C}_{\text{residue, CH}_4}$ values were not the same for CH_4 produced from rice and maize residue (Figs. 2b, d, f). Moreover, we observed no difference in $\Delta^{13}\text{C}_{\text{residue, CH}_4}$ values for CH_4 production in the same soil treated with same residue type (i.e. Rice 2 vs. 3 and Maize 2 vs. 3) (Figs. 2d, f), providing further evidence that the C fractionation factor associated with CH_4 production from maize and rice residues can be substantially different.

4.2. Partitioning the C sources in CH_4 production

Partitioning the C flux is required for the quantification of priming effect. In the present study, the well-established ^{13}C -natural abundance method (Equation 1) was used to quantify the sources of CH_4 production during anaerobic decomposition of residue and SOC with the assumption that C fractionation factors associated with CH_4 production from residue and from SOC are the same (Kuzyakov, 2006). Nonetheless, the results yielded from this method were mostly either negative or over 100% in all soils (Table 4). It is plausible that C isotopic fractionation factors during methanogenesis are larger than the difference in natural abundance $\delta^{13}\text{C}$ values (14‰) between maize (a C_4 plant) and paddy soils (C_3 plant-derived) (Figs. 2b, d, f), resulting in these unrealistic results (Conrad et al., 2012b). However, this was not observed when ^{13}C -enriched rice residue (Rice 4) was used (Fig. 3). Further application of typical minimum (-10‰), averaged (-40‰), and maximum (-70‰) enrichment factors with Equation 2 yielded an average of < 3% difference in the fractions of total CH_4 derived from residue, regardless of the values of enrichment factor (Fig. 4). It is thus apparent that the high ^{13}C content of Rice 4 surpassed the relatively small effects of different fractionation factors, ensuring accurate estimation of the contribution of residue to total CH_4 production.

The CNAM frequently yielded over 100% contribution from residue in all soils examined (Fig. 3), a result also observed by Conrad et al. (2012b). In the present study, partitioning was also quantified with random combinations of rice (i.e. Rice 2 and 3) and maize (i.e. Maize 2 and 3) residue in both Soil 2 and 3. However, the results were not always the same in each soil during the entire experiment, especially when different rice residues were used (Figs. 3b, c), further indicating that the CNAM did not provide reliable partitioning outcomes and that the type of residue is in fact important. As discussed above, C isotopic fractionation associated with CH₄ production from rice and maize residue appeared to be substantially different in all soils (Figs. 2b, d, f), which is in contrast to the fundamental assumption underlying the CNAM (Conrad et al., 2012b).

In spite of these potential shortcomings, our results reinforce the notion of Conrad et al. (2012b) that the general approach to quantify the C sources responsible for CH₄ production requires careful consideration of associated enrichment factors as exemplified in Equation 2. The great utility of the CNAM is that it provides a new approach to avoid measurement of ¹³C enrichment factors for CH₄ production from plant residues (Conrad et al., 2012b). However, this method may not provide reliable partitioning outcomes when certain residues have distinct chemical compositions and potentials to simulate methanogenic activities, as was the case in the present study. Application of labeled residues is seemingly a more robust technique (see above) and does not require application of different sources of plant residues in parallel experiments. However, this method requires additional resources of isotope analysis facilities and isotopically labeled residues.

4.3. Priming effects of residue on CH₄ production

Residue incorporation has been widely reported to increase CH₄ emissions in rice fields (Bossio et al., 1999; Chidthaisong and Watanabe, 1997; Sander et al., 2014; Zhang et al., 2013). Nonetheless, less has been done to quantify the priming effect of crop residues during anaerobic decomposition processes and CH₄ production (Conrad et al., 2012b; Ye et al., 2015; Yuan et al., 2014). It has been reported that rice residue addition causes a non-linear response in CH₄ production and timing and direction of priming effect under changing concentrations of electron acceptors and methanogenic pathways (Ye et al., 2015; Yuan et al., 2014). In the present study, the amount of electron acceptors followed the order Soil 1 > Soil 2 > Soil 3 (Table 2), which manifested itself in CH₄ production patterns throughout the experiment (Fig. 1). To understand how this electron acceptor gradient affects the priming effect, we used the partitioning outcomes from the ¹³C-enrichment method to calculate it. The priming effect of residue on CH₄ production from SOC was always positive and decreased when moving up the electron acceptor concentration gradient from Soil 3 to 1 (Fig. 5a). However, when expressed relative to CH₄ production in the controls, the magnitude of priming effect followed the order Soil 3 > Soil 2 > Soil 1 from day 6 to 10, but reversed after day 15 (Fig. 5b), largely mirroring the dynamics of CH₄ production during the same period (Fig. 1). It is therefore apparent that the changes in priming effect essentially resulted from changes in methanogenic activity, which was suppressed by the presence of various electron acceptors at the beginning of the experiment, increased after the complete exhaustion of electron acceptors, but again became limited by substrate supply as the mineralization of SOC declined later in the experiment, a phenomenon described in previous studies (Segers and Kengen, 1998; Ye et al., 2015). Our results suggest that the priming effect of residue on CH₄ production from SOC is essentially substrate-dependent and can be influenced by many microbial processes that underlie the generation and consumption of methanogenic

substrates (Ye et al., 2015; Yuan et al., 2014), which may greatly complicate the evaluation of management outcomes and prediction of CH₄ production and emission. However, despite these uncertainties, SOC-derived CH₄ and the portion primed by residue were well correlated to the cumulative production of CH₄ from residue (Fig. 6). With the exhaustive consumption of various electron acceptors, fermentation is expected to dominate anaerobic decomposition, ultimately generating methanogenic substrates (i.e. acetate and CO₂/H₂) that can be readily utilized (Meronigal et al., 2004). Methanogens appear to have no preference between substrates derived from SOC and residue (Chidthaisong and Watanabe, 1997; Ye et al., 2015). Therefore, the ratio between CH₄ production from SOC and residue was likely affected by the amount of fermentative production between the two C sources. Our results suggest that, despite the large difference in the priming effect and CH₄ production across soil types and time, the priming effect of CH₄ production may be easily estimated from the decomposition of residue (Fig. 6).

5. Conclusion

The ability to quantify the priming effect on CH₄ production depends on reliable methods to partition C sources. It is evident from our results that both the traditional ¹³C natural abundance method and the CNAM approach are not free from bias when calculating substrate partitioning. Since fractionation factors associated with CH₄ production are not constant during decomposition of substrates, use of methods such as these should include temporal awareness of the potential effect on ensuing estimations. The estimations are influenced greatly by the time needed to consume electron acceptors. Application of ¹³C-enriched residue is a more robust technique, especially when isotopically enriched residue is applied, as it largely avoids the issues associated with isotopic fractionation, and, when possible, it can be used as a reference method alongside other approaches (e.g. the CNAM) in obtaining related data. The priming effect of

residue on CH₄ production is a combination of many factors that control microbial processes leading to the production and consumption of methanogenic substrates. By considering the framework presented in our study, future research on the dynamics of fermentative product production, CH₄ production, and associated microbial activity will be more effective in describing how plant residue management affects CH₄ emissions, and more importantly, relevant models and predictions will become more reliable.

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Figure Legends

Figure 1. CH₄ production rates in soils amended with rice (Rice 1, 2, 3), maize (Maize 1, 2, 3), and ¹³C-enriched rice (Rice 4) residues and without residues (Control). a, Soil 1; b, Soil 2; c, Soil 3; Error bars indicate standard errors (n = 4).

Figure 2. The δ¹³C of CH₄ (a, c, e) produced in soils incubated with rice (Rice 1, 2, 3) and maize (Maize 1, 2, 3) residues and without residues (Control), and their values relative to residues from which they derived (Δ¹³C) (b, d, f). a, b: Soil 1; c, d: Soil 2; e, f: Soil 3; Error bars indicate standard errors (n = 4).

Figure 3. Fractions of total CH₄ production derived from residues calculated by the CNAM with different combinations of maize (M1-3) and rice (R1-3) residues and by ¹³C-enrichment method (Enrich). a: Soil 1; b: Soil 2; c: Soil 3; Error bars indicate standard errors (n = 4).

Figure 4. Relationship between estimated fractions of CH₄ production derived from ¹³C-enriched rice residues with (Y axis) and without (X axis) the application of enrichment factors of 10, 40, and 70‰. The solid line describes the 1 to 1 relationship.

Figure 5. Cumulative CH₄ production from SOC as induced by residue (a) and priming effect as relative to the controls (b). Error bars indicate standard errors (n = 4).

Figure 6. Correlation between cumulative production of CH₄ from residue and from soil organic matter (SOC-derived) and from the priming effect (Primed).

Table 1. Total C and N, and the $\delta^{13}\text{C}$ and $\delta^{15}\text{N}$ values of residues and soils. Numbers are means of analytical replicates (n=3) with standard errors in parentheses.

	$\delta^{13}\text{C}$ (‰)	$\delta^{15}\text{N}$ (‰)	Total C (%)	Total N (%)	C/N ratio
Rice 1	-28.7 (0.02)	5.5 (0.28)	38 (0.1)	0.9 (0.0)	42 (0.0)
Rice 2	-27.5 (0.03)	2.6 (0.56)	36 (0.1)	0.5 (0.0)	76 (2.6)
Rice 3	-27.9 (0.04)	1.7 (0.04)	37 (0.1)	0.5 (0.0)	71 (0.5)
Rice 4	636 (1)	9.8 (0.00)	34 (0.1)	1.5 (0.0)	23 (0.1)
Maize 1	-13.4 (0.02)	5.5 (0.96)	41 (0.2)	0.78 (0.00)	52 (0.0)
Maize 2	-12.6 (0.02)	6.2 (0.01)	41 (0.2)	2.1 (0.0)	20 (0.0)
Maize 3	-12.7 (0.00)	2.2 (0.37)	41 (0.0)	2.6 (0.0)	16 (0.0)
Soil 1	-26.8 (0.02)	1.6 (0.05)	15 (0.2)	1.1 (0.0)	14 (0.1)
Soil 2	-27.2 (0.02)	1.9 (0.02)	6 (0.0)	0.4 (0.0)	15 (0.1)
Soil 3	-27.6 (0.02)	1.9 (0.01)	2 (0.0)	0.1 (0.0)	13 (0.1)

Table 2. Select physicochemical properties of soils. Numbers are means of analytical replicates (n=3) with standard errors in parentheses.

	pH	DOC	NO ₃ ⁻ -N	Extractable Fe [*]	SO ₄ -S	NH ₄ ⁺ -N
	(H ₂ O)	----- (mg kg ⁻¹ dry soil) -----				
Soil 1	5.35	956 (78)	54 (0)	3212 (0)	628 (11)	10 (0.4)
Soil 2	5.48	843 (15)	33 (1)	2726 (14)	434 (5)	8 (0.3)
Soil 3	5.59	103 (2)	0.7 (0.1)	531 (11)	43 (7)	7 (0.1)

^{*}, Fe extracted by 0.5 M hydrochloric acid

Table 3. Results of ANOVA analysis of residue addition effects on CH₄ production rates and $\delta^{13}\text{C}$ values of the produced CH₄ ($\delta^{13}\text{C}$ -CH₄), and of residue source effects on $\delta^{13}\text{C}$ -CH₄ relative to $\delta^{13}\text{C}$ value of residues from which it was derived ($\Delta^{13}\text{C}_{\text{residue, CH}_4}$) and the contributions (Fractions) of residue to the total CH₄ production in Soil 1, 2, and 3 during the 32-day incubation.

Variables	Source	<u>Soil 1</u>			<u>Soil 2</u>			<u>Soil 3</u>		
		df	F	<i>p</i>	df	F	<i>p</i>	df	F	<i>p</i>
CH ₄ production	treatment	3	338	< 0.0001	5	91	< 0.0001	5	89	< 0.0001
	time	18	11	< 0.0001	11	142	< 0.0001	11	883	< 0.0001
	treatment * time	52	11	< 0.0001	55	9	< 0.0001	55	47	< 0.0001
$\delta^{13}\text{C}$ -CH ₄	treatment	3	816	< 0.0001	4	44	< 0.0001	4	118	< 0.0001
	time	5	14	< 0.0001	4	42	< 0.0001	4	93	< 0.0001
	treatment * time	11	12	< 0.0001	16	19	< 0.0001	16	7	< 0.0001
$\Delta^{13}\text{C}_{\text{residue, CH}_4}$	treatment	1	24	0.0023	4	9	0.0016	4	10	< 0.0001
	time	4	8	0.0004	4	42	< 0.0001	4	93	< 0.0001
	treatment * time	4	1	0.4346	16	19	< 0.0001	16	7	< 0.0001
Fraction	treatment	1	13	0.0141	4	34	< 0.0001	4	23	< 0.0001
	time	5	3.4	0.0283	4	154	< 0.0001	4	87	< 0.0001
	treatment * time	3	1	0.4235	16	9	< 0.0001	16	6	< 0.0001

Table 4. Calculated fractions (%) of total CH₄ production derived from residues using the typical ¹³C natural abundance method. Values are mean (n=3) with standard error in parenthesis.

	Day 6	Day 10	Day 15	Day 22	Day 32
<u>Soil 1</u>					
Maize 1	-113 (5)	-91 (13)*	N/A	27 (140)	10 (102)
<u>Soil 2</u>					
Maize 2	57 (10)	52 (21)	272 (8)	319 (59)	304 (42)
Maize 3	61 (12)	54 (25)	256 (0)	310 (59)	304 (40)
<u>Soil 3</u>					
Maize 2	75 (19)	123 (1)	243 (37)	137 (19)	64 (4)
Maize 3	75 (18)	124 (1)	280 (1)	132 (31)	95 (27)

*, samples on Day 11 not 10.

Figure

Fig. 1

Fig. 2

Fig. 3

Fig. 4

Fig. 5

Fig. 6