Partitioning of CH₄ and CO₂ Production Originating from Rice Straw, Soil and Root Organic Carbon in Rice Microcosms

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Abstract

Flooded rice fields are an important source of the greenhouse gas CH₄. Possible carbon sources for CH₄ and CO₂ production in rice fields are soil organic matter (SOM), root organic carbon (ROC) and rice straw (RS), but partitioning of the flux between the different carbon sources is difficult. We conducted greenhouse experiments using soil microcosms planted with rice. The soil was amended with and without ¹³C-labeled RS, using two ¹³C-labeled RS treatments with equal RS (5 g kg⁻¹ soil) but different δ¹³C of RS. This procedure allowed to determine the carbon flux from each of the three sources (SOM, ROC, RS) by determining the δ¹³C of CH₄ and CO₂ in the different incubations and from the δ¹³C of RS. Partitioning of carbon flux indicated that the contribution of ROC to CH₄ production was 41% at tillering stage, increased with rice growth and was about 60% from the booting stage onwards. The contribution of ROC to CO₂ was 43% at tillering stage, increased to around 70% at booting stage and stayed relatively constant afterwards. The contribution of RS was determined to be in a range of 12–24% for CH₄ production and 11–31% for CO₂ production; while the contribution of SOM was calculated to be 23–35% for CH₄ production and 13–26% for CO₂ production. The results indicate that ROC was the major source of CH₄ though RS application greatly enhanced production and emission of CH₄ in rice field soil. Our results also suggest that data of CH₄ dissolved in rice field could be used as a proxy for the produced CH₄ after tillering stage.

Introduction

Flooded rice fields are an important source of the greenhouse gas CH₄ [1,2]. Methane and CO₂ are end products of anoxic degradation of organic matter in rice field soil [3]. The organic matter is mainly derived from three sources [4]: (1) soil organic matter (SOM), (2) root organic carbon (ROC) including root exudates and sloughed-off dead root, and (3) dead plant organic matter, such as rice straw (RS), which is often applied in large amounts (up to 12 t ha⁻¹ annually) to maintain soil fertility [5–7]. Methane production is partitioned mainly between these three types of organic matter. Knowledge of partitioning is important for improving process-based modeling of CH₄ emission from rice fields [8,9], which is the basis for predicting methane flux and assessing the impact of agricultural management and global change.

Quantification of carbon partitioning can in principle be achieved by pulse-labeling of rice plant with ¹³C or ¹⁴C. Recently, free-air CO₂ enrichment (FACE) using ¹³C-depleted CO₂ was used for determining the contribution of ROC to production of CO₂ and CH₄ in rice field soil [13]. However, pulse-labeling only assesses the immediate contribution of root exudates, while the contribution of sloughed-off dead root cells cannot be fully accounted for [13–16]. Since FACE experiments apply elevated CO₂ concentrations, photoassimilation of CO₂ may be enhanced and thus increase the contribution of plants and soil organic matter to carbon flux [17–19]. Furthermore, most studies of carbon flux partitioning in rice fields have been done without application of straw, so that full partitioning of the origin of carbon flux into SOM, ROC and RS was not possible [4]. However, application of RS should be taken into account, since RS may not only be used as substrate for CH₄ production, but might also enhance CH₄ production from other carbon sources [20,21].

The partitioning of the CH₄ production from different sources of organic carbon (SOM, ROC, RS) can be achieved, if these have different isotopic signatures. However, a major difficulty during partitioning the sources of CH₄ is caused by the carbon isotopic fractionation during the conversion of organic matter to CH₄, which is typically 10–70% [22]. Nevertheless, the relative contribution of acetoclastic versus hydrogenotrophic methanogenesis to CH₄ production has been determined successfully in environments such as rice field soil [23] and lake sediments [24], after the isotopic fractionation factors in both methanogenic pathways were determined. The δ¹³C values of CH₄ from the two pathways are substantially different, since the isotopic fractionation factors of the two pathways are largely different [22,24,25]. Analogously, it is possible to partition the sources of CH₄ if the δ¹³C of CH₄ derived from each carbon source in the rice field soil is known. Normally, the CH₄ derived from SOM, ROC and RS has similar δ¹³C values, since all the organic matter has eventually been derived from rice plant material [23,26]. However, this

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problem may be solved by cultivation of rice in soil amended with 13C-labeled RS.

The aim of this study was to determine the partitioning of the carbon flux involved in methanogenic degradation of carbon sources by determining the δ13C of CH4 derived from ROC. We therefore prepared rice microcosms with two treatments of 13C-labeled RS, both having the same amount of RS (5 g kg⁻¹ soil, equals about 5 t ha⁻¹) but different content of 13C. We determined the produced CH4 and CO2 by collecting soil cores and incubating samples anaerobically [27].

Materials and Methods

Planted and unplanted rice microcosms

Soil samples were provided by the Italian Rice Research Institute in Vercelli. Soil was taken from a drained paddy field in spring 2009 and was air dried and stored at room temperature. The soil was sieved (<2 mm) prior to use. The characteristics of the soil have been described previously [28]. Planting pots (upper diameter = 19 cm; lower diameter = 14 cm; height = 16 cm) were filled with 2 kg dry soil and turned into a slurry with demineralized water.

For planted rice microcosms, in total 48 pots were prepared, 16 pots for the unamended control, and 16 pots each for RS treatment I and RS treatment II. Fertilizer solution (50 ml of a solution containing per liter: 10 g urea, 7.6 g KH2PO4) was added to each pot as basal fertilizer. For both RS treatments, 10 g powder of RS was added to each pot and mixed thoroughly into the soil slurry. The δ13C values of RS added in treatment I and II were 213.0 and 474.7, respectively. These δ13C values were determined by adding desired amount of 13C-labeled (δ13C = 1859.9‰) and unlabeled (δ13C = −27.6‰) RS separately into each pot. The 13C-labeled RS was prepared by growing rice plants in the greenhouse until the late vegetative stage. The plants were covered with a 18-L acrylic chamber, 1% 13CO2 (final concentration; 99 atom%, Sigma, Germany) was added to the headspace, incubated for 5 days (12 h light, 25°C), and then harvested. The unlabeled RS was from rice plant grown in the same manner without feeding on 13CO2. These rice plants were dried and ground to powder. After 3 days of incubation in the greenhouse, all the pots were planted with one 12-day old rice seedling (Oryza sativa var. KORAL type japonica), and were flooded with demineralized water to give a water depth of 5 cm above the soil surface. The water depth was maintained throughout the experimental period. The rice microcosms were incubated in the greenhouse with a relative humidity of 70%, a 12-hour day/night temperature cycle. The pot comprising of 48 pots was kept in an incubator at a temperature of 27.6°C.

Pore water samples were collected into Venioject blood-collecting tubes (Terumo Europe N.V., Belgium) from the rhizosphere (3 cm depth) and bulk (9 cm depth) soil of rice microcosms using Rhizon pore water samplers (Rhizosphere Research Products, the Netherlands). After heavy shaking by hand, the headspace of the tubes was sampled using a pressure lock syringe and directly analyzed for CH4 and CO2 and δ13C. The CH4 and CO2 concentration in the soil pore water was calculated as described previously [27].

Plant height, tiller number and aboveground biomass were determined. For dry weight determination, samples were dried for 48 h at 60°C.

CH4 and CO2 production

Production rates of CH4 and CO2 and respective δ13C values were determined by collecting soil core samples in rice microcosms on day 41, 55, 70 and 90 of incubation in the greenhouse [27]. After cutting off the rice plant, the surface water layer was removed. Soil cores were taken in each pot with stainless steel corer (Ø 22 mm, 210 mm in length). Two to three soil cores (about 100 g in total) were collected from each pot and transferred into a 250-ml bottle. The soil samples were turned into slurry using N2-gassed deionized sterile water so that the ratio of dry weight of soil to water was 1:1. After flushing the samples with N2, the bottles were sealed with butyl rubber stoppers and, after shaking, flushed again with N2 to remove residual O2 and CH4. Incubation was performed statically at 25°C in the dark for 24 h. Headspace samples were taken every 12 h after shaking the bottles, and analyzed for concentration of CH4 and CO2 and their δ13C. The CH4 and CO2 production from planted soil microcosms was due to decomposition of SOM plus ROC (unamended control) or of SOM, ROC plus RS (RS treatments). CH4 production rates were calculated by linear regression of the CH4 increase with incubation time, and expressed in mmol CH4 m⁻² h⁻¹ of soil. The CO2 production rates were determined analogously.

For unplanted soil microcosms, the methods for collection and incubation of soil core samples were similar, but these pots were not sacrificed, but at each sampling day (day 41, 55, 70 and 90), a 60-g soil core was taken from the pot. After removal of the soil core the residual soil in the pot was compacted, and water was added to maintain a water level of 5 cm depth. Using this procedure about 2.1% of the total amount of soil in the pot was collected during each sampling. The CH4 and CO2 production from unplanted soil microcosms was only due to decomposition of SOM (unamended control) or of SOM plus RS (RS treatments).

Analytical techniques

The gas samples were analyzed for CH4 and CO2 using a gas chromatograph (GC) equipped with flame ionization detector (FID) [29]. Stable isotopic analysis of gas samples (CH4 and CO2) from pore water and soil core incubation were performed directly using the GCC-IRMS, samples from flux measurements (low in CH4) were preconcentrated on a Precon (Finnigan, Bremen, Germany). The principal operation of the GCC-IRMS has been previously described [30,31]. The isotope reference gas was CO2 (99.998% purity; Messer-Griesheim, Dusseldorf, Germany) calibrated with the working standard methyl stearate (Merek). The latter was intercalibrated at the Max-Planck-Institute for Bioge-
chemistry, Jena, Germany (courtesy of Dr. W.A. Brand) against
NBS 22 and USGS 24, and reported in the delta notation vs.
V-PDB: $\delta^{13}C = 10^3 \left( R_{\text{sample}}/R_{\text{standard}} - 1 \right)$, with $R = 13C/12C$ of sample (sa) and standard (st), respectively. The precision of repeated analysis
was $\pm 0.2\%$, when 1.3 nmol CH$_4$ were injected [23]. The
determination of the stable isotopic signatures of dried plant and
soil samples was carried out at the Institute for Soil Science and
Forest Nutrition (IBW) at the University of Göttingen, Germany.

Calculations

1. Fraction of CH$_4$ production from ROC ($f_{\text{ROC}}$). The
fraction of CH$_4$ derived from ROC ($f_{\text{ROC}}$) can be determined
from the following mass balance equation:

$$\delta^{13}C_{\text{CH}_4} = f_{\text{ROC}} \delta^{13}C_{\text{CH}_4-\text{ROC}} + (1-f_{\text{ROC}}) \delta^{13}C_{\text{CH}_4-\text{SOR}}$$

(1)

where $\delta^{13}C_{\text{CH}_4} = \delta^{13}C$ of CH$_4$ produced (or dissolved) in the
planted rice microcosms at each sampling time; $\delta^{13}C_{\text{CH}_4-\text{ROC}} = \delta^{13}C$ of CH$_4$
formed from ROC (determination see below); $\delta^{13}C_{\text{CH}_4-\text{SOR}} = \delta^{13}C$ of CH$_4$
formed from SOM plus RS, i.e. the CH$_4$ produced (or dissolved) in the
unplanted soil treated with RS. The equation can be transformed into the following two equations
for RS-treatment I and II, respectively:

$$f_{\text{ROC}} = \frac{\delta^{13}C_{\text{CH}_4-I} - \delta^{13}C_{\text{CH}_4-\text{SOR-I}}}{\delta^{13}C_{\text{CH}_4-I} - \delta^{13}C_{\text{CH}_4-\text{SOR-I}}}$$

(2)

$$f_{\text{ROC}} = \frac{\delta^{13}C_{\text{CH}_4-II} - \delta^{13}C_{\text{CH}_4-\text{SOR-II}}}{\delta^{13}C_{\text{CH}_4-II} - \delta^{13}C_{\text{CH}_4-\text{SOR-II}}}$$

(3)

Since $f_{\text{ROC}}$ and $\delta^{13}C_{\text{CH}_4-\text{ROC}}$ should be the same in treatment I
and II, $\delta^{13}C_{\text{CH}_4-\text{ROC}}$ can be calculated by solving equations (2) and (3):

$$\delta^{13}C_{\text{CH}_4-\text{ROC}} = \frac{\delta^{13}C_{\text{CH}_4-I} - \delta^{13}C_{\text{CH}_4-\text{SOR-I}} - \delta^{13}C_{\text{CH}_4-II} + \delta^{13}C_{\text{CH}_4-\text{SOR-II}}}{\delta^{13}C_{\text{CH}_4-I} - \delta^{13}C_{\text{CH}_4-\text{ Sor-I}} - \delta^{13}C_{\text{CH}_4-II} + \delta^{13}C_{\text{CH}_4-\text{SOR-II}}}

(4)

Then, $f_{\text{ROC}}$ can be calculated from either equation (2) or (3).

2. Fraction of CH$_4$ production from RS carbon ($f_{\text{RS}}$). The
$\delta^{13}C$ values of the CH$_4$ produced (or dissolved) in the two RS
treatments are given by the following two mass balance equations:

$$\delta^{13}C_{\text{CH}_4-I} = f_{\text{RS}} \delta^{13}C_{\text{RS-I}} + f_{\text{SOM}} \delta^{13}C_{\text{SOM}} + f_{\text{ROC}} \delta^{13}C_{\text{ROC}} + \Delta C_{\text{CH}_4}$$

(5)

$$\delta^{13}C_{\text{CH}_4-II} = f_{\text{RS}} \delta^{13}C_{\text{RS-II}} + f_{\text{SOM}} \delta^{13}C_{\text{SOM}} + f_{\text{ROC}} \delta^{13}C_{\text{ROC}} + \Delta C_{\text{CH}_4}$$

(6)

with $f_{\text{RS}}$, $f_{\text{SOM}}$ and $f_{\text{ROC}}$ denote fractions of CH$_4$ produced from
RS, SOM and ROC, respectively; $\delta^{13}C_{\text{RS-I}}$ and $\delta^{13}C_{\text{RS-II}}$ are $\delta^{13}C$ of the rice straw carbon in
treatment I (213.0%) and II (474.7%), respectively; $\delta^{13}C_{\text{SOM}}$ and $\delta^{13}C_{\text{ROC}}$ are $\delta^{13}C$ of SOM
($-25.8\%$) and of the plant biomass (Fig. 1), respectively; $\Delta C_{\text{CH}_4}$
designates the overall isotopic fractionation factors involved in
CH$_4$ production from these organic matters, in case of dissolved
CH$_4$ also those involved in oxidation and transfer of CH$_4$ from soil
to the atmosphere.

$$f_{\text{RS}} + f_{\text{ROC}} + f_{\text{SOM}} = 1$$

(8)

Analogous equations are valid for the fractions of CO$_2$ produced from
ROC, SOM and RS in rice field soil.

Statistical analysis

The significance of differences between treatments over time for
various variables were determined by one-way analysis of variance
(ANOVA) followed by multiple comparisons (Duncan post hoc test)
using SPSS 13.0. To test the significance of the differences
between contributions to produced and dissolved CH$_4$ or CO$_2$,
two-tailed independent t-tests were applied using Microsoft Excel
2007.

Results

1. Stable carbon signature of rice plants

The $\delta^{13}C$ of rice plants in the control and RS treatments were
almost constant with time (Fig. 1). Rice plants in the RS treatments

Figure 1. Values of $\delta^{13}C$ of dried rice plants obtained from
planted microcosms without (control) and with addition of $^{13}C$-
labeled RS. RS I and RS II denote the two treatments, the $\delta^{13}C$ of rice
straw applied was 213.0% and 474.7% for RS I and RS II, respectively;
means ± standard deviation (SD) (n=3). The differences between the
the treatments over time were examined using Duncan post hoc test of a
one-way ANOVA. Different letters on the top of bars indicate significant
difference ($P<0.05$) between the data.
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were enriched in δ13C by about 5‰ compared with the control. The δ13C of rice plants was consistently higher in treatment II than in treatment I, but the difference was not significant.

2. Rates and δ13C of CH4 emitted from planted microcosms

In the rice microcosms without addition of RS, CH4 emission rates increased from the tillering stage (day 41) to the booting stage (day 70) and peaked at the flowering stage (day 90). Application of rice straw increased CH4 emission rates throughout the growth period, but particularly during tillering and booting stage (Fig. 2A). The δ13C of the emitted CH4 became gradually more negative during the cultivation period in all the treatments (Fig. 2B). The δ13C of CH4 was substantially higher in RS treatment II > RS treatment I > control, especially during the tillering stage (Fig. 2B).

3. Concentrations and δ13C values of CH4 and CO2 dissolved in pore water

Concentrations and δ13C values of dissolved CH4 and CO2 were similar in the pore water sampled from 3 cm and 9 cm soil depth. Therefore, only the data from the 9-cm soil layer are shown (Fig. 3A and B). In the planted microcosms, CH4 concentrations increased steadily from the beginning until the ripening stage. Application of rice straw resulted in elevated CH4 concentrations in the beginning but subsequently became similar to the control (Fig. 3A). The δ13C values of the CH4 dissolved in planted and unplanted microcosms were similar with each other in both RS treatments at tillering stage (Fig. 4A). However, while δ13C values decreased with time in the planted microcosms, they did not decrease much in the unplanted microcosms. The δ13C of the dissolved CH4 was consistently higher (less negative) in RS treatment II > RS treatment I > control for both planted and unplanted microcosms (Fig. 4A). The δ13C values of the dissolved CH4 in planted microcosms (Fig. 4A) were similar to those of the emitted CH4 (Fig. 2B).

In the planted microcosms, dissolved CO2 concentrations were between 4.0 and 5.5 mM independently of the treatment and the vegetation period (Fig. 3B). The δ13C of CH4 dissolved in planted microcosms with and without treatment RS was in general higher (less negative) than that of CH4.

4. Rates and δ13C of CH4 and CO2 produced in planted and unplanted microcosms

At each time of sampling, soil cores were collected from microcosms with and without rice plants, in order to determine the rates and the δ13C of the CH4 and CO2 produced. Depending on the microcosm tested, CH4 and CO2 were produced from ROC (planted microcosms), SOM (all microcosms) and RS (RS-treated microcosms). In the planted control without RS treatment, CH4 production rates increased steadily during the vegetation period (Fig. 5A). However, treatment with RS resulted in further increase of CH4 production rates. In the unplanted microcosms, CH4 production rates were also enhanced by RS treatments but were lower than in the planted microcosms with RS treatment. The δ13C of produced CH4 was similar in the planted and unplanted control microcosms without RS (Fig. 4C). Treatment with RS resulted in increase of δ13C values of produced CH4, which was higher in treatment II than treatment I. However, the increase was less in the planted than in the unplanted microcosms (Fig. 4C).

The rates of CO2 production were constant over the vegetation period in the planted microcosms and were similar for the treatments with and without RS, but were at least twice as high in planted as in unplanted microcosms (Fig. 5B). The δ13C values of CO2 exhibited a similar pattern with respect to vegetation period and treatment as that of CH4, but the values were generally higher (Fig. 4D).

5. Partitioning CH4 and CO2 produced in rice microcosms

For calculation of fROC, first of all the δ13C of the CH4 and CO2 produced from ROC had to be determined. The data, which were calculated using eq. (4), are shown in Table 1. The δ13C of CH4 produced from ROC was about −60‰ on average (range of −67 to −49‰) during the whole vegetation period, though fluctuations on individual sampling dates, at tillering stage in particular, were rather high (Table 1). The δ13C values of CO2 produced from ROC were about −31% at tillering stage and increased to around −11% to −4% subsequently (Table 1). Values of fROC were then calculated using eq. (2) and (3). Both equations gave similar values, but those obtained with eq. (2) showed higher standard deviations than those obtained with eq. (3). Only the latter values are shown in Fig. 6 and 7. ROC was found to make a major contribution (41–63%) to CH4 production over the entire vegetation period (Fig. 6A). For CO2 production, ROC had even a higher importance (43–76%) (Fig. 7A).

Figure 2. Seasonal change of (A) CH4 emission rates and (B) δ13C of CH4 emitted in planted microcosms with and without treatment with 13C-labeled RS; means ± SD (n = 4). The differences between the treatments over time were examined using Duncan post hoc test of a one-way ANOVA. Different letters on the top of bars indicate significant difference (P<0.05) between the data.
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The fractions of CH$_4$ and CO$_2$ produced from RS (f$_{RS}$) were calculated using eq. (7). Values of $\delta^{13}$C were obtained from the CH$_4$ (Fig. 4C) and CO$_2$ (Fig. 4D) produced in soil samples from planted microcosms. Values of f$_{RS}$ were determined to be in a range of 12–24% for CH$_4$ production (Fig. 6B) and 11–31% for CO$_2$ production (Fig. 7B).

Finally, f$_{SOM}$ was calculated by difference to f$_{ROC}$ and f$_{RS}$, being in a range of 23–35% of CH$_4$ (Fig. 6C) and 13–26% of CO$_2$ production in soil from planted and straw-treated microcosms (Fig. 7C).

6. Partitioning CH$_4$ and CO$_2$ dissolved in rice microcosms

Similarly as for the production of CH$_4$ and CO$_2$ (see above), the gases dissolved in the rice microcosms were also used for determination of the partitioning of their origin from ROC, RS, and SOM using the equations described above. In this case, values of $\delta^{13}$C were from the CH$_4$ and CO$_2$ dissolved in pore water of planted and unplanted microcosms (Fig. 4A and B). The $\delta^{13}$C of CH$_4$ derived from ROC was $230\%$ at tillering stage when calculated with $\delta^{13}$C of produced CH$_4$ (Table 2), substantially more positive than that calculated with $\delta^{13}$C of produced CH$_4$ (Table 1). The resulting f$_{ROC}$ for CH$_4$ was only 13% (Fig. 6A). In
contrast, the relative contribution of RS $f_{\text{RS}}$ to CH$_4$ dissolved was significantly higher than that for CH$_4$ produced at the tillering stage (Fig. 6B). However, the relative contributions of each carbon source to dissolved and produced CH$_4$ were nearly the same at later season (Fig. 6). For CO$_2$, the $\delta^{13}$C of CO$_2$ derived from ROC was $-49\%$ at tillering stage, more negative than that calculated with $\delta^{13}$C of produced CO$_2$ ($-31\%$), but there was no significant difference between the relative contributions of each carbon source to dissolved and produced CO$_2$ (Fig. 7).

### Discussion

1. **Partitioning of methane production**

Our study comprehensively determined the partitioning of CH$_4$ and CO$_2$ production in a rice ecosystem considering all three major carbon sources (i.e., ROC, RS, SOM). In planted and straw-treated rice microcosms, more than 60% of the CH$_4$ was produced from root organic carbon, except on the first sampling date (tillering stage) when it was 41%. Thus, plant photosynthesis was the most important driver of CH$_4$ production. The same was the case for CO$_2$ production. The results are consistent with the observation that CH$_4$ and CO$_2$ production rates were at least twice as high in microcosms with than without rice plants (Fig. 5A and 5B). At the same time, the substantial lower $\delta^{13}$C of CH$_4$ and CO$_2$ produced in planted versus unplanted microcosms also indicated that ROC-derived CH$_4$ and CO$_2$ production diluted the CH$_4$ and CO$_2$ produced from labeled rice straw (Fig. 4C and 4D). Our results are consistent with two earlier experiments reporting 40–60% of the CH$_4$ production being due to plant derived carbon. These experiments were based on pulse-labeling and FACE techniques [11,13], which potentially influence carbon flux partitioning in a different way than our approach. For instance, pulse-labeling may only account for part of the plant-derived carbon flux and FACE treatment may stimulate carbon flux [13,14]. Nevertheless, the determined relative contribution of plant derived carbon to production of CH$_4$ and CO$_2$ was rather similar despite the different approaches. Hence, the results that plant-derived carbon is the most important carbon source for CH$_4$ production in flooded rice fields is a rather robust finding.

In contrast to ROC, straw contributed only about 20% to CH$_4$ production. A similar low percentage has previously been found in Japanese rice soil microcosms after 50 days of incubation [4]. Immediately after application of the straw, however, its contribution to CH$_4$ production and emission reached almost 100% [4]. This was likely also the case in our experiments. This conclusion is supported by the following observations: (1) On day 41, $\delta^{13}$C of the produced CH$_4$ was $<150\%$; albeit the applied rice straw carbon had a $\delta^{13}$C of 474.7% (Fig. 4C). The difference is much more than theoretically possible from isotope discrimination during methanogenesis. Therefore, we have to assume that the CH$_4$ produced immediately after straw application had a much higher $\delta^{13}$C as it was derived from straw to a large extent. (2) The analogous observation was made with the produced CO$_2$ (Fig. 4D), although isotope discrimination is much smaller for production of CO$_2$ than of CH$_4$. (3) Still after day 40, $\delta^{13}$C of the produced CH$_4$ and CO$_2$ tended to decrease with vegetation time. Hence, we conclude that contribution of decomposition of straw to CH$_4$ production was very high after straw application and then progressively decreased as the carbon compounds of the straw became increasingly less decomposable. Future studies should further refine the seasonal change in flux partitioning. This will help improving the predictions of CH$_4$ emission rates from rice fields by process-based modeling.

| Days after transplanting | 41      | 55      | 70      | 90      |
|-------------------------|---------|---------|---------|---------|
| $\delta^{13}$C$_{\text{CH}_4\text{-ROC}}$ | $-67.4 \pm 66.7$ | $-49.4 \pm 14.2$ | $-61.3 \pm 10.2$ | $-57.2 \pm 17.4$ |
| $\delta^{13}$C$_{\text{CO}_2\text{-ROC}}$ | $-31.3 \pm 65.1$ | $-3.6 \pm 14.6$ | $-10.7 \pm 8.8$ | $-9.7 \pm 10.6$ |

The values were calculated using $\delta^{13}$C of CH$_4$ and CO$_2$ produced in rice field soil; means $\pm$ SD (n = 4).

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Sources of Methane Production in Rice Fields

Figure 5. Production rates of (A) CH$_4$ and (B) CO$_2$ in planted and unplanted microcosms with and without RS application; means $\pm$ SD (n = 4). The differences between the treatments over time were examined using Duncan post hoc test of a one-way ANOVA. Different letters on the top of bars indicate significant difference ($P<0.05$) between the data.

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dissolved in soil pore water were derived from root organic carbon after tillering stage, nearly the same as for produced CH₄ and CO₂ (Fig. 6 and 7).

At tillering stage, however, the relative contribution of ROC to the dissolved CH₄ was significantly lower and that of RS significantly higher when compared to the contribution to the produced CH₄. The difference was probably due to the gas transport limitation of rice plants at the early vegetative stage [32, 40]. The residence time of CH₄ in pore water at tillering stage can amount to several days. Therefore, at day 41 the pore water was probably still highly enriched in ¹³CH₄ which had been produced from RS at earlier time. This conclusion is consistent with the substantially higher δ¹³C values of the dissolved CH₄ than those of the produced CH₄ at day 41 (Fig. 4A and 4C). As a result, the relative contribution of RS to dissolved CH₄ was higher than to produced CH₄ at day 41 and that of ROC was lower (Fig. 6B).

In contrast, at later growth season, the residence time of CH₄ in pore water of planted soil was much shorter (several hours) [32], this was consistent with the rapid decrease of δ¹³C values of dissolved CH₄ and CO₂ after tillering stage. Furthermore, the δ¹³C values of dissolved and produced CH₄ were similar with each other after the tillering stage (Fig. 4A and 4C). Therefore, the relative contributions of each carbon source to dissolved and produced CH₄ were similar to each other (Fig. 6). This suggested that pore water CH₄ could be used as a proxy for produced CH₄ and could be suitable for partitioning the CH₄ production after tillering stage.

3. Stable carbon isotope fractionation during CH₄ production from ROC

The δ¹³C of the CH₄ produced from ROC (δ¹³CCH₄-ROC) were in a range of −67% to −49%. These values are similar to δ¹³CCH₄ values observed in rice field soil or in incubations of soil slurries [23, 33]. Theoretically the value of δ¹³CCH₄-ROC should be equal to the δ¹³C of ROC plus the overall isotopic enrichment factor (εROC,CH₄) for the conversion of ROC to CH₄. The δ¹³CROC should be similar to the δ¹³C of the rice plant biomass (Fig. 1). Using these values and the δ¹³CCH₄-ROC, the overall enrichment factor εROC,CH₄ was in a range of about −24% to −42%. This is a rather large range, but has been observed before (about −20% to −75%) during anaerobic decomposition of straw in paddy soil [41] or anoxic incubations of rice roots [42]. The overall enrichment factor εROC,CH₄ is composed of (1) the enrichment factors involved in the conversion of ROC to the methanogenic substrates (i.e., acetate and H₂/CO₂) and (2) in the enrichment factors involved in the conversion of the methanogenic substrates to CH₄. The latter enrichment factors are the larger ones, in particular those involved in the production of CH₄ from H₂/CO₂ [23, 43]. Whereas acetoclastic methanogenesis has relatively moderate enrichment factors (−10% to −25%), those of hydrogenotrophic methanogenesis are often very large (−25% to −90%) [22]. Our data suggest that CH₄ production from ROC is dominated by hydrogenotrophic methanogenesis, which is consistent with earlier observations studying CH₄ production on rice roots [42, 44, 45].

The δ¹³C of the CO₂ produced from root organic carbon was in a range of −31% to −4% (Table 1). The overall isotopic

Figure 6. Percentage contribution of (A) ROC, (B) SOM and (C) RS to produced and dissolved CH₄ in planted microcosms with RS treatment; means ± SD (n = 4). The differences between contributions to produced and dissolved CH₄ were tested by two-tailed independent t-tests, indicated by * when P<0.05. doi:10.1371/journal.pone.0049073.g006

Figure 7. Percentage contribution of (A) ROC, (B) SOM and (C) RS to produced and dissolved CO₂ in planted microcosms with RS treatment; means ± SD (n = 4). The differences between contributions to produced and dissolved CH₄ were tested by two-tailed independent t-tests, indicated by * when P<0.05. doi:10.1371/journal.pone.0049073.g007
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Table 2. δ13C values of CH4 and CO2 derived from ROC in planted rice microcosms with RS application.

| Days after transplanting | 41 | 55 | 70 | 90 |
|--------------------------|----|----|----|----|
| δ13C(CH4:ROC)            | -29.1±9.5 | -38.7±25.4 | -72.2±28 | -51.0±7.6 |
| δ13C(CO2:ROC)            | -49.2±8.1 | -3.8±22 | -14.2±14.2 | -8.5±6.1 |

The values were calculated using δ13C of CH4 and CO2 dissolved in pore water, mean ± SD (n = 4). doi:10.1371/journal.pone.0049073.t002

enrichment factors involved in CH4 production from organic matter were thus about –6% to +21%. These enrichment factors are much smaller than those involved in CH4 production. Nevertheless, the range is similarly large, which may be due to carbon isotopic fractionation during CO2 consumption by hydrogenotrophic methanogenesis [23] and also during reactions between gaseous CO2 and bicarbonate/carbonate [46].

4. Practical considerations

Our study demonstrated the possibility to determine the partitioning of CH4 and CO2 flux from degradation of straw, soil organic matter, and plant root-derived carbon, by treating soil with 13C-labeled rice straw. The procedure is more practical than labeling of the rice plants with 13CO2 that requires cumbersome incubation techniques or expensive FACE treatment. For calculation of fROC, it was important that the δ13C of the two RS applications were sufficiently different from each other, and in addition were sufficiently different from the δ13C of both ROC and SOM. This was achieved by two RS treatments using the same amount of RS but 13C-labeled to different extent. As a result, the δ13C of emitted CH4 (Fig. 2B), δ13C of dissolved and produced CH4 and CO2 (Fig. 4) were substantially higher than the control without RS, and of course they were always higher in treatment II than treatment I.

Calculation of fRS was simply achieved by using the δ13C values of the applied RS and the CH4 derived from the two RS treatments (Eq. 7) assuming that ROC was not differently affected by the two RS treatments. This assumption was in agreement with the observation that the δ13C values of the rice plants in the two RS treatments were not significantly different (Fig 1). Notably, these values were significantly higher than those in the control microcosms without RS, probably because some of the RS carbon was assimilated (probably via CO2) by the plants [20,21]. However, the difference was only a few permil and did not prevent computation of flux partitioning, since the difference to the δ13C of the labeled RS was quite large.

In summary, application of labeled RS may be a convenient technique to determine flux partitioning in rice fields on a routine basis. The determination requires in total three planted field plots and three unplanted ones, i.e., two RS treatments and one untreated control, everything with appropriate replication. Technical installation is not required. Hence, it should be feasible to increase the data basis on the partitioning of CH4 production from ROC, RS and SOM on a regional and seasonal scale. This will help improving process-based modeling of CH4 emission from rice fields.

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Author Contributions

Conceived and designed the experiments: QY RC. Performed the experiments: QY. Analyzed the data: QY RC. Contributed reagents/materials/analysis tools: JP. Wrote the paper: QY RC.

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