

# Partitioning of CH<sub>4</sub> and CO<sub>2</sub> 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 CH<sub>4</sub>. Possible carbon sources for CH<sub>4</sub> and CO<sub>2</sub> 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 <sup>13</sup>C-labeled RS, using two <sup>13</sup>C-labeled RS treatments with equal RS (5 g kg<sup>-1</sup> soil) but different δ<sup>13</sup>C of RS. This procedure allowed to determine the carbon flux from each of the three sources (SOM, ROC, RS) by determining the δ<sup>13</sup>C of CH<sub>4</sub> and CO<sub>2</sub> in the different incubations and from the δ<sup>13</sup>C of RS. Partitioning of carbon flux indicated that the contribution of ROC to CH<sub>4</sub> production was 41% at tillering stage, increased with rice growth and was about 60% from the booting stage onwards. The contribution of ROC to CO<sub>2</sub> 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 CH<sub>4</sub> production and 11–31% for CO<sub>2</sub> production; while the contribution of SOM was calculated to be 23–35% for CH<sub>4</sub> production and 13–26% for CO<sub>2</sub> production. The results indicate that ROC was the major source of CH<sub>4</sub> though RS application greatly enhanced production and emission of CH<sub>4</sub> in rice field soil. Our results also suggest that data of CH<sub>4</sub> dissolved in rice field could be used as a proxy for the produced CH<sub>4</sub> after tillering stage.

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## Introduction

Flooded rice fields are an important source of the greenhouse gas CH<sub>4</sub> [1,2]. Methane and CO<sub>2</sub> 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<sup>-1</sup> 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 CH<sub>4</sub> 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 <sup>13</sup>CO<sub>2</sub> or <sup>14</sup>CO<sub>4</sub> [10–12]. Recently, free-air CO<sub>2</sub> enrichment (FACE) using <sup>13</sup>C-depleted CO<sub>2</sub> was used for determining the contribution of ROC to production of CO<sub>2</sub> and CH<sub>4</sub> 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 CO<sub>2</sub> concentrations, photoassimilation of CO<sub>2</sub> 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 CH<sub>4</sub> production, but might also enhance CH<sub>4</sub> production from other carbon sources [20,21].

The partitioning of the CH<sub>4</sub> 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 CH<sub>4</sub> is caused by the carbon isotopic fractionation during the conversion of organic matter to CH<sub>4</sub>, which is typically 10–70% [22]. Nevertheless, the relative contribution of acetoclastic versus hydrogenotrophic methanogenesis to CH<sub>4</sub> 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 δ<sup>13</sup>C values of CH<sub>4</sub> 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 CH<sub>4</sub> if the δ<sup>13</sup>C of CH<sub>4</sub> derived from each carbon source in the rice field soil is known. Normally, the CH<sub>4</sub> derived from SOM, ROC and RS has similar δ<sup>13</sup>C values, since all the organic matter has eventually been derived from rice plant material [23,26]. However, this

problem may be solved by cultivation of rice in soil amended with  $^{13}\text{C}$ -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  $\delta^{13}\text{C}$  of  $\text{CH}_4$  derived from ROC. We therefore prepared rice microcosms with two treatments of  $^{13}\text{C}$ -labeled RS, both having the same amount of RS ( $5 \text{ g kg}^{-1}$  soil, equals about  $5 \text{ t ha}^{-1}$ ) but different content of  $^{13}\text{C}$ . We determined the produced  $\text{CH}_4$  and  $\text{CO}_2$  by collecting soil cores and incubating samples anoxically [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 ( $<2 \text{ mm}$ ) prior to use. The characteristics of the soil have been described previously [28]. Planting pots (upper diameter =  $19 \text{ cm}$ ; lower diameter =  $14 \text{ cm}$ ; height =  $16 \text{ cm}$ ) were filled with  $2 \text{ kg}$  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 ( $50 \text{ ml}$  of a solution containing per liter:  $10 \text{ g}$  urea,  $7.6 \text{ g}$   $\text{KH}_2\text{PO}_4$ ) was added to each pot as basal fertilizer. For both RS treatments,  $10 \text{ g}$  powder of RS was added to each pot and mixed thoroughly into the soil slurry. The  $\delta^{13}\text{C}$  values of RS added in treatment I and II were  $213.0\%$  and  $474.7\%$ , respectively. These  $\delta^{13}\text{C}$  values were obtained by adding desired amount of  $^{13}\text{C}$ -labeled ( $\delta^{13}\text{C} = 1859.9\%$ ) and unlabeled ( $\delta^{13}\text{C} = -27.6\%$ ) RS separately into each pot. The  $^{13}\text{C}$ -labeled RS was prepared by growing rice plants in the greenhouse until the late vegetative stage. The plants were covered with a  $18\text{-L}$  acrylic chamber,  $1\%$   $^{13}\text{CO}_2$  (final concentration;  $99 \text{ atom}\%$ , Sigma, Germany) was added to the headspace, incubated for 5 days ( $12 \text{ h}$  light,  $25^\circ\text{C}$ ), and then harvested. The unlabeled RS was from rice plant grown in the same manner without feeding on  $^{13}\text{CO}_2$ . 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  $5 \text{ cm}$  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  $12\text{-h}$  photoperiod and a  $28/22^\circ\text{C}$  day/night temperature cycle. The day of transplantation was taken as day zero. On day 21, a second dose of  $30 \text{ ml}$  fertilizer solution was added to each microcosm. At each sampling time (day 41, 55, 70 and 90), 12 rice microcosms were sacrificed (4 replicates for control and for each treatment). For unplanted microcosms, the preparation was the same as for planted ones, but without rice plant in the pots. In total, 12 pots were prepared with 4 pots each for the unamended control, RS treatment I and RS treatment II.

### $\text{CH}_4$ flux, soil pore water and plant parameters

Rates of  $\text{CH}_4$  emission was measured on day 41, 55, 70 and 90 of incubation in the greenhouse as described previously [27]. For flux measurements, planted rice microcosms were covered by flux chambers, and gas samples were taken every  $30 \text{ min}$  for  $2 \text{ h}$ .  $\text{CH}_4$  emission rates were determined from the slope of the linearly increasing  $\text{CH}_4$  mixing ratio and expressed in  $\text{mmol CH}_4 \text{ m}^{-2} \text{ h}^{-1}$ .

Samples for the determination of the isotopic signature ( $\delta^{13}\text{C}\text{CH}_4$ ) of the emitted  $\text{CH}_4$  were taken in glass containers ( $100 \text{ ml}$ ). The first sample was taken directly after closure of the chambers, the second sample was taken at the end of the  $2\text{-h}$  closure period. The isotopic signature of the emitted  $\text{CH}_4$  was calculated according to [27].

Pore water samples were collected into Venoject blood-collecting tubes (Terumo Europe N.V., Belgium) from the rhizosphere ( $3 \text{ cm}$  depth) and bulk ( $9 \text{ cm}$  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  $\text{CH}_4$  and  $\text{CO}_2$  and  $\delta^{13}\text{C}$ . The  $\text{CH}_4$  and  $\text{CO}_2$  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  $48 \text{ h}$  at  $60^\circ\text{C}$ .

### $\text{CH}_4$ and $\text{CO}_2$ production

Production rates of  $\text{CH}_4$  and  $\text{CO}_2$  and respective  $\delta^{13}\text{C}$  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 ( $\text{O}$   $22 \text{ mm}$ ,  $210 \text{ mm}$  in length). Two to three soil cores (about  $100 \text{ g}$  in total) were collected from each pot and transferred into a  $250\text{-ml}$  bottle. The soil samples were turned into slurry using  $\text{N}_2$ -gassed deionized sterile water so that the ratio of dry weight of soil to water was  $1:1$ . After flushing the samples with  $\text{N}_2$ , the bottles were sealed with butyl rubber stoppers and, after shaking, flushed again with  $\text{N}_2$  to remove residual  $\text{O}_2$  and  $\text{CH}_4$ . Incubation was performed statically at  $25^\circ\text{C}$  in the dark for  $24 \text{ h}$ . Headspace samples were taken every  $12 \text{ h}$  after shaking the bottles, and analyzed for concentration of  $\text{CH}_4$  and  $\text{CO}_2$  and their  $\delta^{13}\text{C}$ . The  $\text{CH}_4$  and  $\text{CO}_2$  production from planted soil microcosms was due to decomposition of SOM plus ROC (unamended control) or of SOM, ROC plus RS (RS treatments).  $\text{CH}_4$  production rates were calculated by linear regression of the  $\text{CH}_4$  increase with incubation time, and expressed in  $\text{nmol CH}_4 \text{ g}_{\text{dw}}^{-1} \text{ h}^{-1}$  of soil. The  $\text{CO}_2$  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  $60\text{-g}$  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  $5 \text{ cm}$  depth. Using this procedure about  $2.1\%$  of the total amount of soil in the pot was collected during each sampling. The  $\text{CH}_4$  and  $\text{CO}_2$  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  $\text{CH}_4$  and  $\text{CO}_2$  using a gas chromatograph (GC) equipped with flame ionization detector (FID) [29]. Stable isotopic analysis of gas samples ( $\text{CH}_4$  and  $\text{CO}_2$ ) from pore water and soil core incubation were performed directly using the GCC-IRMS, samples from flux measurements (low in  $\text{CH}_4$ ) 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  $\text{CO}_2$  ( $99.998\%$  purity; Messer-Griessheim, Düsseldorf, Germany) calibrated with the working standard methyl stearate (Merck). The latter was intercalibrated at the Max-Planck-Institute for Biogeo-

chemistry, Jena, Germany (courtesy of Dr. W.A. Brand) against NBS 22 and USGS 24, and reported in the delta notation vs. V-PDB:  $\delta^{13}\text{C} = 10^3 (R_{\text{sa}}/R_{\text{st}} - 1)$ , with  $R = {}^{13}\text{C}/{}^{12}\text{C}$  of sample (sa) and standard (st), respectively. The precision of repeated analysis was  $\pm 0.2\%$ , when 1.3 nmol  $\text{CH}_4$  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  $\text{CH}_4$  production from ROC ( $f_{\text{ROC}}$ ).** The fraction of  $\text{CH}_4$  derived from ROC ( $f_{\text{ROC}}$ ) can be determined from the following mass balance equation:

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

where  $\delta^{13}\text{C}_{\text{CH}_4} = \delta^{13}\text{C}$  of  $\text{CH}_4$  produced (or dissolved) in the planted rice microcosms at each sampling time;  $\delta^{13}\text{C}_{\text{CH}_4-\text{ROC}} = \delta^{13}\text{C}$  of  $\text{CH}_4$  formed from ROC (determination see below);  $\delta^{13}\text{C}_{\text{CH}_4-\text{SOR}} = \delta^{13}\text{C}$  of  $\text{CH}_4$  formed from SOM plus RS, i.e. the  $\text{CH}_4$  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:

$$f_{\text{ROC}} = \frac{\delta^{13}\text{C}_{\text{CH}_4-\text{I}} - \delta^{13}\text{C}_{\text{CH}_4-\text{SOR-I}}}{\delta^{13}\text{C}_{\text{CH}_4-\text{ROC}} - \delta^{13}\text{C}_{\text{CH}_4-\text{SOR-I}}} \quad (2)$$

$$f_{\text{ROC}} = \frac{\delta^{13}\text{C}_{\text{CH}_4-\text{II}} - \delta^{13}\text{C}_{\text{CH}_4-\text{SOR-II}}}{\delta^{13}\text{C}_{\text{CH}_4-\text{ROC}} - \delta^{13}\text{C}_{\text{CH}_4-\text{SOR-II}}} \quad (3)$$

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

$$\delta^{13}\text{C}_{\text{CH}_4-\text{ROC}} = \frac{\delta^{13}\text{C}_{\text{CH}_4-\text{I}}\delta^{13}\text{C}_{\text{CH}_4-\text{SOR-II}} - \delta^{13}\text{C}_{\text{CH}_4-\text{II}}\delta^{13}\text{C}_{\text{CH}_4-\text{SOR-I}}}{\delta^{13}\text{C}_{\text{CH}_4-\text{I}} - \delta^{13}\text{C}_{\text{CH}_4-\text{SOR-I}} - \delta^{13}\text{C}_{\text{CH}_4-\text{II}} + \delta^{13}\text{C}_{\text{CH}_4-\text{SOR-II}}} \quad (4)$$

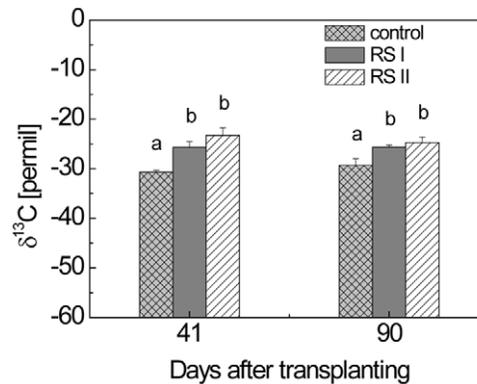
Then,  $f_{\text{ROC}}$  can be calculated from either equation (2) or (3).

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

$$\delta^{13}\text{C}_{\text{CH}_4-\text{I}} = f_{\text{RS}}\delta^{13}\text{C}_{\text{RS-I}} + f_{\text{SOM}}\delta^{13}\text{C}_{\text{SOM}} + f_{\text{ROC}}\delta^{13}\text{C}_{\text{ROC}} + \Delta\text{CH}_4 \quad (5)$$

$$\delta^{13}\text{C}_{\text{CH}_4-\text{II}} = f_{\text{RS}}\delta^{13}\text{C}_{\text{RS-II}} + f_{\text{SOM}}\delta^{13}\text{C}_{\text{SOM}} + f_{\text{ROC}}\delta^{13}\text{C}_{\text{ROC}} + \Delta\text{CH}_4 \quad (6)$$

with  $f_{\text{RS}}$ ,  $f_{\text{SOM}}$  and  $f_{\text{ROC}}$  denote fractions of  $\text{CH}_4$  produced from RS, SOM and ROC, respectively;  $\delta^{13}\text{C}_{\text{RS-I}}$  and  $\delta^{13}\text{C}_{\text{RS-II}}$  are  $\delta^{13}\text{C}$  of the rice straw carbon in treatment I (213.0%) and II (474.7%), respectively;  $\delta^{13}\text{C}_{\text{SOM}}$  and  $\delta^{13}\text{C}_{\text{ROC}}$  are  $\delta^{13}\text{C}$  of SOM



**Figure 1. Values of  $\delta^{13}\text{C}$  of dried rice plants obtained from planted microcosms without (control) and with addition of  $^{13}\text{C}$ -labeled RS.** RS I and RS II denote the two treatments, the  $\delta^{13}\text{C}$  of rice straw applied was 213.0% and 474.7% for RS I and RS II, respectively; means  $\pm$  standard deviation (SD) ( $n=3$ ). 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. doi:10.1371/journal.pone.0049073.g001

( $-25.8\%$ ) and of the plant biomass (Fig. 1), respectively;  $\Delta\text{CH}_4$  designates the overall isotopic fractionation factors involved in  $\text{CH}_4$  production from these organic matters, in case of dissolved  $\text{CH}_4$  also those involved in oxidation and transfer of  $\text{CH}_4$  from soil to the atmosphere.

Since the terms  $f_{\text{SOM}}$ ,  $\delta^{13}\text{C}_{\text{SOM}}$ ,  $f_{\text{ROC}}$ ,  $\delta^{13}\text{C}_{\text{ROC}}$  and  $\Delta\text{CH}_4$  should be the same in treatment I and II, combination of equations (5) and (6) and solving for  $f_{\text{RS}}$  results in:

$$f_{\text{RS}} = \frac{\delta^{13}\text{C}_{\text{CH}_4-\text{I}} - \delta^{13}\text{C}_{\text{CH}_4-\text{II}}}{\delta^{13}\text{C}_{\text{RS-I}} - \delta^{13}\text{C}_{\text{RS-II}}} \quad (7)$$

of which the  $\delta^{13}\text{C}$  can be determined experimentally. Here,  $\delta^{13}\text{C}_{\text{CH}_4-\text{I}}$  and  $\delta^{13}\text{C}_{\text{CH}_4-\text{II}}$  were determined experimentally, and  $\delta^{13}\text{C}_{\text{RS-I}}$  and  $\delta^{13}\text{C}_{\text{RS-II}}$  were mixtures of labeled and unlabelled RS, of which the  $\delta^{13}\text{C}$  were determined experimentally (see above). Finally, the fraction of  $\text{CH}_4$  production from SOM ( $f_{\text{SOM}}$ ) can be calculated, since

$$f_{\text{RS}} + f_{\text{ROC}} + f_{\text{SOM}} = 1 \quad (8)$$

Analogous equations are valid for the fractions of  $\text{CO}_2$  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  $\text{CH}_4$  or  $\text{CO}_2$ , two-tailed independent t-tests were applied using Microsoft Excel 2007.

## Results

### 1. Stable carbon signature of rice plants

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

were enriched in  $\delta^{13}\text{C}$  by about 5% compared with the control. The  $\delta^{13}\text{C}$  of rice plants was consistently higher in treatment II than in treatment I, but the difference was not significant.

## 2. Rates and $\delta^{13}\text{C}$ of $\text{CH}_4$ emitted from planted microcosms

In the rice microcosms without addition of RS,  $\text{CH}_4$  emission rates increased from the tillering stage (day 41) to the booting stage (day 55) and peaked at the flowering stage (day 70), then decreased again till the ripening stage (day 90) (Fig. 2A). Application of rice straw increased  $\text{CH}_4$  emission rates throughout the growth period, but particularly during tillering and booting stage (Fig. 2A). The  $\delta^{13}\text{C}$  of the emitted  $\text{CH}_4$  became gradually more negative during the cultivation period in all the treatments (Fig. 2B). The  $\delta^{13}\text{C}$  of  $\text{CH}_4$  was substantially higher in RS treatment II > RS treatment I > control, especially during the tillering stage (Fig. 2B).

## 3. Concentrations and $\delta^{13}\text{C}$ values of $\text{CH}_4$ and $\text{CO}_2$ dissolved in pore water

Concentrations and  $\delta^{13}\text{C}$  values of dissolved  $\text{CH}_4$  and  $\text{CO}_2$  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. 3, 4A and B). In the planted microcosms,  $\text{CH}_4$  concentrations increased steadily from the beginning until the ripening stage. Application of rice straw resulted in elevated  $\text{CH}_4$  concentrations in the beginning but subsequently became similar to the control (Fig. 3A). The  $\delta^{13}\text{C}$  values of the  $\text{CH}_4$  dissolved in planted and unplanted microcosms were similar with each other in both RS treatments at tillering stage (Fig. 4A). However, while  $\delta^{13}\text{C}$  values decreased with time in the planted microcosms, they did not decrease much in the unplanted microcosms. The  $\delta^{13}\text{C}$  of the dissolved  $\text{CH}_4$  was consistently higher (less negative) in RS treatment II > RS treatment I > control for both planted and unplanted microcosms (Fig. 4A). The  $\delta^{13}\text{C}$  values of the dissolved  $\text{CH}_4$  in planted microcosms (Fig. 4A) were similar to those of the emitted  $\text{CH}_4$  (Fig. 2B).

In the planted microcosms, dissolved  $\text{CO}_2$  concentrations were between 4.0 and 5.5 mM independently of the treatment and the vegetation period (Fig. 3B). The  $\delta^{13}\text{C}$  of the dissolved  $\text{CO}_2$  exhibited a temporal pattern similar to that of  $\text{CH}_4$  and was again consistently higher (less negative) in RS treatment II > RS treatment I > control (Fig. 4B). However,  $\delta^{13}\text{C}$  of dissolved  $\text{CO}_2$  was in general higher (less negative) than that of  $\text{CH}_4$ .

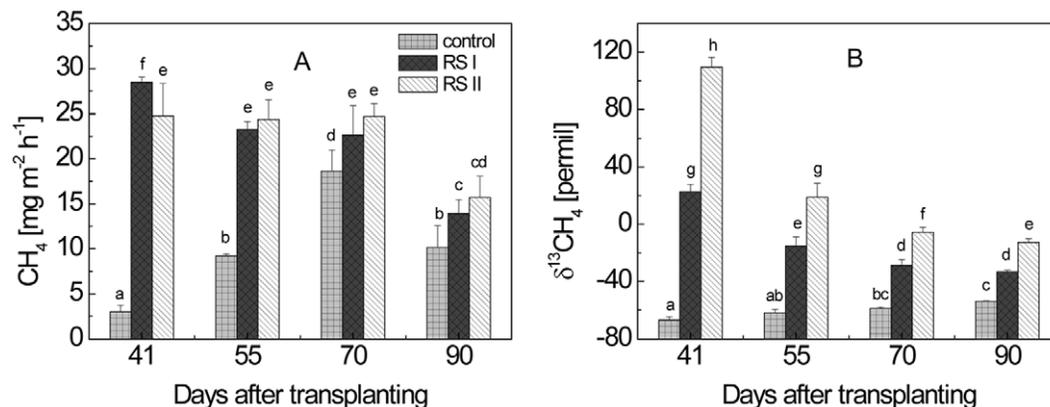
## 4. Rates and $\delta^{13}\text{C}$ of $\text{CH}_4$ and $\text{CO}_2$ produced in planted and unplanted microcosms

At each time of sampling, soil cores were collected from microcosms with and without rice plants, in order to determine the rates and the  $\delta^{13}\text{C}$  of the  $\text{CH}_4$  and  $\text{CO}_2$  produced. Depending on the microcosm tested,  $\text{CH}_4$  and  $\text{CO}_2$  were produced from ROC (planted microcosms), SOM (all microcosms) and RS (RS-treated microcosms). In the planted control without RS treatment,  $\text{CH}_4$  production rates increased steadily during the vegetation period (Fig. 5A). However, treatment with RS resulted in further increase of  $\text{CH}_4$  production rates. In the unplanted microcosms,  $\text{CH}_4$  production rates were also enhanced by RS treatments but were lower than in the planted microcosms with RS treatment. The  $\delta^{13}\text{C}$  of produced  $\text{CH}_4$  was similar in the planted and unplanted control microcosms without RS (Fig. 4C). Treatment with RS resulted in increase of  $\delta^{13}\text{C}$  values of produced  $\text{CH}_4$ , which was higher in treatment II than treatment I. However, the increase was less in the planted than in the unplanted microcosms (Fig. 4C).

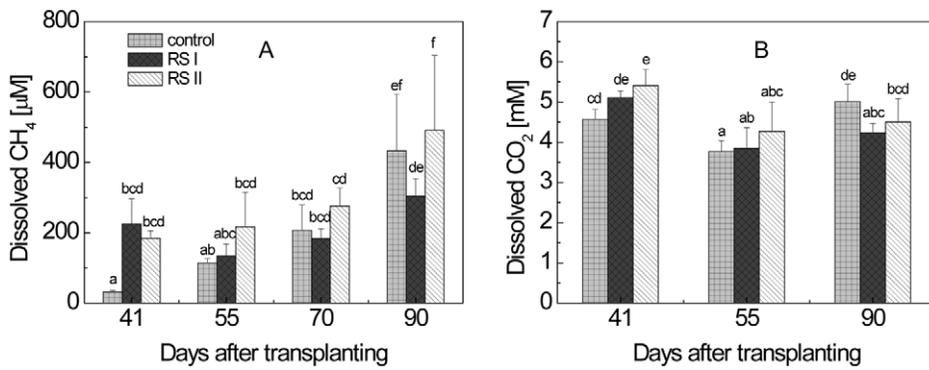
The rates of  $\text{CO}_2$  production were constant over the vegetation period in the planted microcosms and were similar for the treatments with and without RS, but were at least twice as high in planted as in unplanted microcosms (Fig. 5B). The  $\delta^{13}\text{C}$  values of  $\text{CO}_2$  exhibited a similar pattern with respect to vegetation period and treatment as that of  $\text{CH}_4$ , but the values were generally higher (Fig. 4D).

## 5. Partitioning $\text{CH}_4$ and $\text{CO}_2$ produced in rice microcosms

For calculation of  $f_{\text{ROC}}$ , first of all the  $\delta^{13}\text{C}$  of the  $\text{CH}_4$  and  $\text{CO}_2$  produced from ROC had to be determined. The data, which were calculated using eq. (4), are shown in Table 1. The  $\delta^{13}\text{C}$  of  $\text{CH}_4$  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  $\delta^{13}\text{C}$  values of  $\text{CO}_2$  produced from ROC were about -31‰ at tillering stage and increased to around -11‰ to -4‰ subsequently (Table 1). Values of  $f_{\text{ROC}}$  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  $\text{CH}_4$  production over the entire vegetation period (Fig. 6A). For  $\text{CO}_2$  production, ROC had even a higher importance (43–76%) (Fig. 7A).



**Figure 2. Seasonal change of (A)  $\text{CH}_4$  emission rates and (B)  $\delta^{13}\text{C}$  of  $\text{CH}_4$  emitted in planted microcosms with and without treatment with  $^{13}\text{C}$ -labeled RS; 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. doi:10.1371/journal.pone.0049073.g002



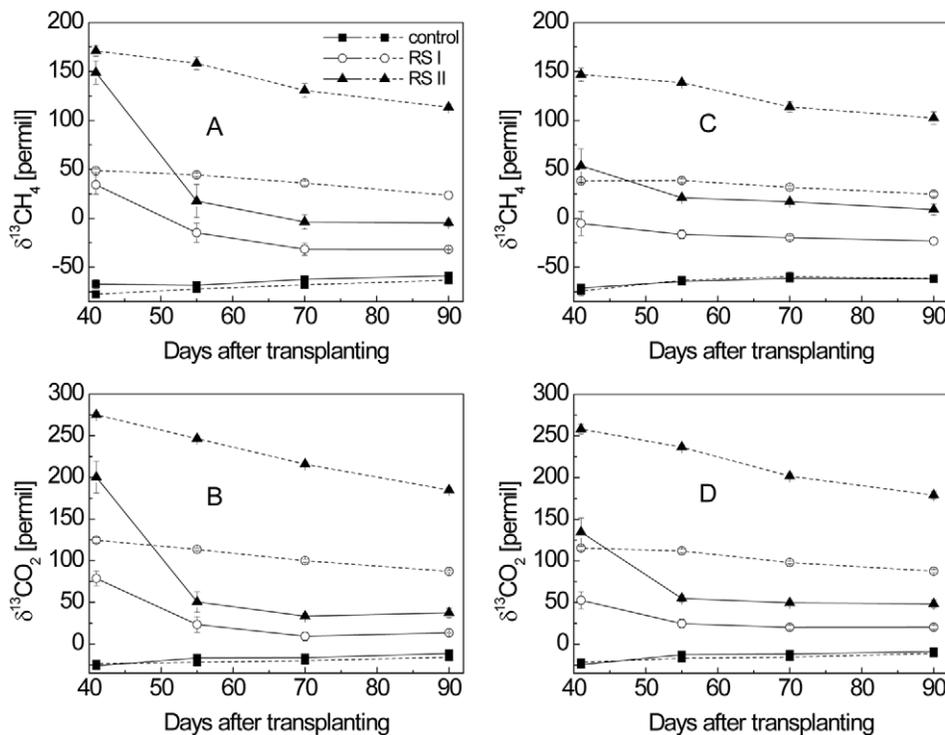
**Figure 3. Temporal change of the concentrations of dissolved (A) CH<sub>4</sub> and (B) CO<sub>2</sub> in planted microcosms with and without addition of <sup>13</sup>C-labeled RS; means ± SD (n = 4).** The differences between the treatments over time were examined using Duncan *post hoc* test of a one-way ANOVA. Different letters on the top of bars indicate significant difference ( $P < 0.05$ ) between the data. doi:10.1371/journal.pone.0049073.g003

The fractions of CH<sub>4</sub> and CO<sub>2</sub> produced from RS ( $f_{RS}$ ) were calculated using eq. (7). Values of  $\delta^{13}C$  were obtained from the CH<sub>4</sub> (Fig. 4C) and CO<sub>2</sub> (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<sub>4</sub> production (Fig. 6B) and 11–31% for CO<sub>2</sub> production (Fig. 7B).

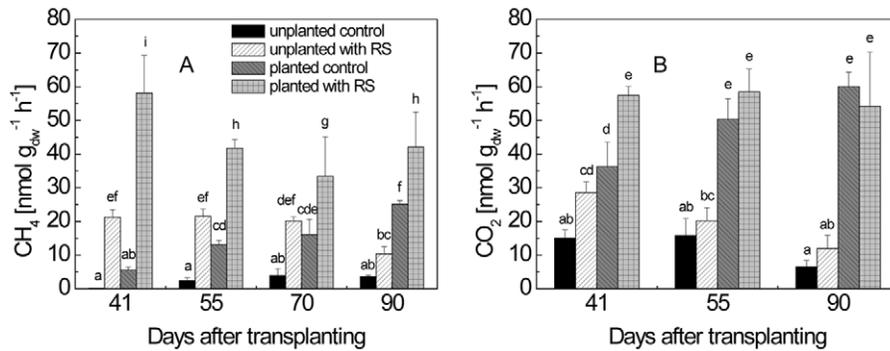
Finally,  $f_{SOM}$  was calculated by difference to  $f_{ROC}$  and  $f_{RS}$ , being in a range of 23–35% of CH<sub>4</sub> (Fig. 6C) and 13–26% of CO<sub>2</sub> production in soil from planted and straw-treated microcosms (Fig. 7C).

## 6. Partitioning CH<sub>4</sub> and CO<sub>2</sub> dissolved in rice microcosms

Similarly as for the production of CH<sub>4</sub> and CO<sub>2</sub> (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<sub>4</sub> and CO<sub>2</sub> dissolved in pore water of planted and unplanted microcosms (Fig. 4A and B). The  $\delta^{13}C$  of CH<sub>4</sub> derived from ROC was  $-30\%$  at tillering stage when calculated with  $\delta^{13}C$  of CH<sub>4</sub> in pore water (Table 2), substantially more positive than that calculated with  $\delta^{13}C$  of produced CH<sub>4</sub> (Table 1). The resulting  $f_{ROC}$  for CH<sub>4</sub> was only 13% (Fig. 6A). In



**Figure 4.  $\delta^{13}C$  of (A) CH<sub>4</sub> and (B) CO<sub>2</sub> dissolved in microcosms with and without RS application;  $\delta^{13}C$  of (C) CH<sub>4</sub> and (D) CO<sub>2</sub> produced in microcosms with and without RS application.** Solid line indicated planted microcosms, dashed lines unplanted microcosms; means ± SD (n = 4). doi:10.1371/journal.pone.0049073.g004



**Figure 5. Production rates of (A) CH<sub>4</sub> and (B) CO<sub>2</sub> 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. doi:10.1371/journal.pone.0049073.g005

contrast, the relative contribution of RS ( $f_{RS}$ ) to CH<sub>4</sub> dissolved was significantly higher than that for CH<sub>4</sub> produced at the tillering stage (Fig. 6B). However, the relative contributions of each carbon source to dissolved and produced CH<sub>4</sub> were nearly the same at later season (Fig. 6). For CO<sub>2</sub>, the  $\delta^{13}C$  of CO<sub>2</sub> derived from ROC was  $-49\%$  at tillering stage, more negative than that calculated with  $\delta^{13}C$  of produced CO<sub>2</sub> ( $-31\%$ ), but there was no significant difference between the relative contributions of each carbon source to dissolved and produced CO<sub>2</sub> (Fig. 7).

## Discussion

### 1. Partitioning of methane production

Our study comprehensively determined the partitioning of CH<sub>4</sub> and CO<sub>2</sub> 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<sub>4</sub> 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<sub>4</sub> production. The same was the case for CO<sub>2</sub> production. The results are consistent with the observation that CH<sub>4</sub> and CO<sub>2</sub> 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<sub>4</sub> and CO<sub>2</sub> produced in planted versus unplanted microcosms also indicated that ROC-derived CH<sub>4</sub> and CO<sub>2</sub> production diluted the CH<sub>4</sub> and CO<sub>2</sub> produced from labeled rice straw (Fig. 4C and 4D). Our results are consistent with two earlier experiments reporting 40–60% of the CH<sub>4</sub> 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<sub>4</sub> and CO<sub>2</sub> was rather similar despite the different approaches. Hence, the results that plant-derived carbon is the most important carbon source for CH<sub>4</sub> production in flooded rice fields is a rather robust finding.

In contrast to ROC, straw contributed only about 20% to CH<sub>4</sub> 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<sub>4</sub> 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<sub>4</sub> 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<sub>4</sub> 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<sub>2</sub> (Fig. 4D), although isotope discrimination is much smaller for production of CO<sub>2</sub> than of CH<sub>4</sub>. (3) Still after day 40,  $\delta^{13}C$  of the produced CH<sub>4</sub> and CO<sub>2</sub> tended to decrease with vegetation time. Hence, we conclude that contribution of decomposition of straw to CH<sub>4</sub> 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<sub>4</sub> emission rates from rice fields by process-based modeling.

### 2. Contribution of different carbon sources to the dissolved CH<sub>4</sub> and CO<sub>2</sub>

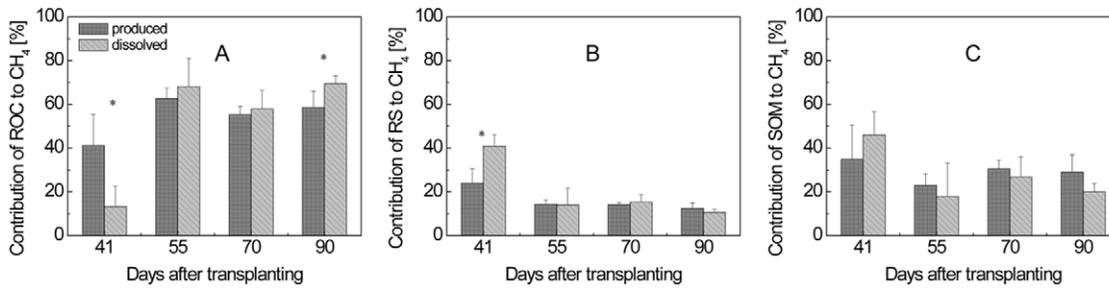
Previous studies reported that  $\delta^{13}C$  values of pore water CH<sub>4</sub> and emitted CH<sub>4</sub> were relatively poor proxies for those of produced CH<sub>4</sub> [32,33]. This assessment is plausible, since in rice field soil pore water CH<sub>4</sub> and emitted CH<sub>4</sub> are not only affected by CH<sub>4</sub> production, but also by CH<sub>4</sub> oxidation [34–36] and CH<sub>4</sub> transport [37–39], which all undergo carbon isotopic fractionation. Therefore, we primarily used the CH<sub>4</sub> produced in soil samples for determining flux partitioning. However, we found that not only the data of the produced CH<sub>4</sub> but also of the dissolved CH<sub>4</sub> allowed determination of flux partitioning and resulted in similar values. Thus, more than 60% of the CH<sub>4</sub> and CO<sub>2</sub>

**Table 1.**  $\delta^{13}C$  values of CH<sub>4</sub> and CO<sub>2</sub> derived from ROC in planted rice microcosms with RS application.

Days after transplanting	41	55	70	90
$\delta^{13}C_{CH_4-ROC}$	$-67.4 \pm 66.7$	$-49.4 \pm 14.2$	$-61.3 \pm 10.2$	$-57.2 \pm 17.4$
$\delta^{13}C_{CO_2-ROC}$	$-31.3 \pm 65.1$	$-3.6 \pm 14.6$	$-10.7 \pm 8.8$	$-9.7 \pm 10.6$

The values were calculated using  $\delta^{13}C$  of CH<sub>4</sub> and CO<sub>2</sub> produced in rice field soil; means  $\pm$  SD (n = 4).

doi:10.1371/journal.pone.0049073.t001



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

dissolved in soil pore water were derived from root organic carbon after tillering stage, nearly the same as for produced CH<sub>4</sub> and CO<sub>2</sub> (Fig. 6 and 7).

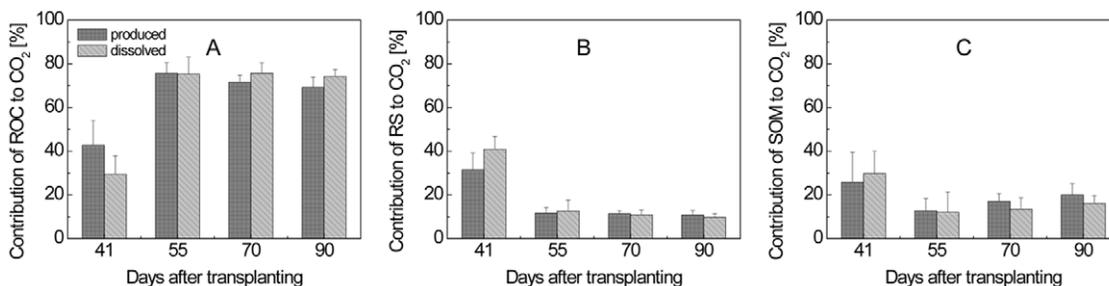
At tillering stage, however, the relative contribution of ROC to the dissolved CH<sub>4</sub> was significantly lower and that of RS significantly higher when compared to the contribution to the produced CH<sub>4</sub>. The difference was probably due to the gas transport limitation of rice plants at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

In contrast, at later growth season, the residence time of CH<sub>4</sub> in pore water of planted soil was much shorter (several hours) [32], this was consistent with the rapid decrease of  $\delta^{13}\text{C}$  values of dissolved CH<sub>4</sub> and CO<sub>2</sub> after tillering stage. Furthermore, the  $\delta^{13}\text{C}$  values of dissolved and produced CH<sub>4</sub> 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 CH<sub>4</sub> were similar to each other (Fig. 6). This suggested that pore water CH<sub>4</sub> could be used as a proxy for produced CH<sub>4</sub> and could be suitable for partitioning the CH<sub>4</sub> production after tillering stage.

### 3. Stable carbon isotope fractionation during CH<sub>4</sub> production from ROC

The  $\delta^{13}\text{C}$  of the CH<sub>4</sub> produced from ROC ( $\delta^{13}\text{C}_{\text{CH}_4\text{-ROC}}$ ) were in a range of  $-67\%$  to  $-49\%$ . These values are similar to  $\delta^{13}\text{C}_{\text{CH}_4}$  values observed in rice field soil or in incubations of soil slurries [23,33]. Theoretically the value of  $\delta^{13}\text{C}_{\text{CH}_4\text{-ROC}}$  should be equal to the  $\delta^{13}\text{C}$  of ROC plus the overall isotopic enrichment factor ( $\epsilon_{\text{ROC,CH}_4}$ ) for the conversion of ROC to CH<sub>4</sub>. The  $\delta^{13}\text{C}_{\text{ROC}}$  should be similar to the  $\delta^{13}\text{C}$  of the rice plant biomass (Fig. 1). Using these values and the  $\delta^{13}\text{C}_{\text{CH}_4\text{-ROC}}$ , the overall enrichment factor  $\epsilon_{\text{ROC,CH}_4}$  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  $\epsilon_{\text{ROC,CH}_4}$  is composed of (1) the enrichment factors involved in the conversion of ROC to the methanogenic substrates (i.e., acetate and H<sub>2</sub>/CO<sub>2</sub>) and (2) in the enrichment factors involved in the conversion of the methanogenic substrates to CH<sub>4</sub>. The latter enrichment factors are the larger ones, in particular those involved in the production of CH<sub>4</sub> from H<sub>2</sub>/CO<sub>2</sub> [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 CH<sub>4</sub> production from ROC is dominated by hydrogenotrophic methanogenesis, which is consistent with earlier observations studying CH<sub>4</sub> production on rice roots [42,44,45].

The  $\delta^{13}\text{C}$  of the CO<sub>2</sub> produced from root organic carbon was in a range of  $-31\%$  to  $-4\%$  (Table 1). The overall isotopic



**Figure 7. Percentage contribution of (A) ROC, (B) SOM and (C) RS to produced and dissolved CO<sub>2</sub> in planted microcosms with RS treatment; means  $\pm$  SD (n=4).** The differences between contributions to produced and dissolved CH<sub>4</sub> were tested by two-tailed independent t-tests, indicated by \* when  $P < 0.05$ . doi:10.1371/journal.pone.0049073.g007

**Table 2.**  $\delta^{13}\text{C}$  values of  $\text{CH}_4$  and  $\text{CO}_2$  derived from ROC in planted rice microcosms with RS application.

Days after transplanting	41	55	70	90
$\delta^{13}\text{C}_{\text{CH}_4\text{-ROC}}$	$-29.9 \pm 95.2$	$-38.7 \pm 25.4$	$-72.2 \pm 28$	$-51.0 \pm 7.6$
$\delta^{13}\text{C}_{\text{CO}_2\text{-ROC}}$	$-49.2 \pm 81.1$	$-3.8 \pm 22$	$-14.2 \pm 14.2$	$-8.5 \pm 6.1$

The values were calculated using  $\delta^{13}\text{C}$  of  $\text{CH}_4$  and  $\text{CO}_2$  dissolved in pore water; means  $\pm$  SD (n = 4).

doi:10.1371/journal.pone.0049073.t002

enrichment factors involved in  $\text{CO}_2$  production from organic matter were thus about  $-6\%$  to  $+21\%$ . These enrichment factors are much smaller than those involved in  $\text{CH}_4$  production. Nevertheless, the range is similarly large, which may be due to carbon isotopic fractionation during  $\text{CO}_2$  consumption by hydrogenotrophic methanogenesis [23] and also during reactions between gaseous  $\text{CO}_2$  and bicarbonate/carbonate [46].

#### 4. Practical considerations

Our study demonstrated the possibility to determine the partitioning of  $\text{CH}_4$  and  $\text{CO}_2$  flux from degradation of straw, soil organic matter, and plant root-derived carbon, by treating soil with  $^{13}\text{C}$ -labeled rice straw. The procedure is more practical than labeling of the rice plants with  $^{13}\text{CO}_2$  that requires cumbersome incubation techniques or expensive FACE treatment. For calculation of  $f_{\text{ROC}}$ , it was important that the  $\delta^{13}\text{C}$  of the two RS applications were sufficiently different from each other, and in addition were sufficiently different from the  $\delta^{13}\text{C}$  of both ROC and SOM. This was achieved by two RS treatments using the same amount of RS but  $^{13}\text{C}$ -labeled to different extent. As a result, the  $\delta^{13}\text{C}$  of emitted  $\text{CH}_4$  (Fig. 2B),  $\delta^{13}\text{C}$  of dissolved and produced  $\text{CH}_4$  and  $\text{CO}_2$  (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  $f_{\text{RS}}$  was simply achieved by using the  $\delta^{13}\text{C}$  values of the applied RS and the  $\text{CH}_4$  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  $^{13}\text{C}$  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  $\text{CO}_2$ ) 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  $\delta^{13}\text{C}$  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  $\text{CH}_4$  production from ROC, RS and SOM on a regional and seasonal scale. This will help improving process-based modeling of  $\text{CH}_4$  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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