Carbon isotope fractionation reveals distinct process of CH$_4$ emission from different compartments of paddy ecosystem

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Carbon isotopic fractionations in the processes of CH$_4$ emission from paddy field remain poorly understood. The $\delta^{13}$C-values of CH$_4$ in association with production, oxidation and transport of CH$_4$ in different pools of a paddy field were determined, and the stable carbon isotope fractionations were calibrated to assess relative contribution of acetate to CH$_4$ production ($f_{ac}$) and fraction of CH$_4$ oxidized ($f_{ox}$) by different pathways. The apparent isotope fractionation for CO$_2$ conversion to CH$_4$ ($\epsilon_{ac}$) was 1.041–1.056 in the soil and 1.046–1.080 on the roots, indicating that $f_{ac}$ was 10–60% and 0–50%, respectively. Isotope fractionation associated with CH$_4$ oxidation ($\epsilon_{ox}$) was 1.021 ± 0.007 in the soil and 1.013 ± 0.005 on the roots, and the transport fractionation ($\epsilon_{transport}$) by rice plants was estimated to be −16.7‰ to −11.1‰. Rhizospheric $f_{ac}$ was about 30–100%, and it was more important at the beginning but decreased fast towards the end of season. Large value of $f_{ox}$ was also observed at the soil-water interface and soil and roots surfaces, respectively. The results demonstrate that carbon isotopic fractionations which might be different in different conditions were sensitive to the estimations of $f_{ac}$ and $f_{ox}$ in paddy field.

Methane (CH$_4$) is the second most important greenhouse gas after carbon dioxide (CO$_2$). On a 100-year horizon, CH$_4$ has 25 times the global warming potential of CO$_2$. Paddy fields are one of the largest anthropogenic sources of atmospheric CH$_4$, contributing to 33–40 Tg yr$^{-1}$ during the 2000–2009. The global CH$_4$ emission from paddy fields will continually increase by intensification of rice cultivation and expansion of planting area to meet the demands of the growing populations. Paddy CH$_4$ emission is an integrated effect of the production, oxidation and transport of CH$_4$ in the field. A better knowledge of these processes affecting CH$_4$ emission may provide more information for effectively mitigating CH$_4$ emission in agricultural ecosystems.

The technique of stable carbon isotopes has been proved to be a useful tool in studying the processes of CH$_4$ emission. Isotope fractionation happens in all the major processes CH$_4$ emission, namely, $^{13}$C-substrate is preferentially utilized by methanogens for CH$_4$ production, and once formed, $^{12}$CH$_4$ is consumed faster than $^{13}$CH$_4$ by methanotrophs, and $^{12}$CH$_4$ is transported faster than $^{13}$CH$_4$ as well[10–12]. Thereby, measurements of the $\delta^{13}$C in production, oxidation and transport of the CH$_4$ from different pools of the field are beneficial to support a process-based model for CH$_4$ emission. Moreover, the relative contribution of acetate to CH$_4$ production ($f_{ac}$) and the fraction of CH$_4$ oxidized ($f_{ox}$) can be quantitatively estimated using mass balance equations based on the measurements of $\delta^{13}$C in CH$_4$, CO$_2$ and acetate, and of the isotope fractionation factors ($\epsilon_{ac}$, $\epsilon_{ox}$ and $\epsilon_{transport}$). Investigations on $\alpha_{ac}$, $\epsilon_{ac}$, $\epsilon_{ox}$ and $\epsilon_{transport}$ of paddy fields were carried out greatly[6,15–18], however, few data are available on $\alpha_{ox}$ for CH$_4$ oxidation by methanotrophs in paddy soils, in particular $\alpha_{ox}$ on rice roots[19].

Some uncertainties also exist in the $\delta^{13}$CH$_4$ that are used as newly produced $\delta^{13}$CH$_4$ ($\delta^{13}$CH$_4$(original)) and finally oxidized $\delta^{13}$CH$_4$ ($\delta^{13}$CH$_4$(oxidized)) in different studies for quantifying $f_{ac}$ and $f_{ox}$. For example, former reports in USA using porewater $\delta^{13}$CH$_4$ as $\delta^{13}$CH$_4$(original) whereas anaerobically produced $\delta^{13}$CH$_4$ was used in Italy[20] and China[21]. They believed that porewater CH$_4$ was a poor proxy for $\delta^{13}$CH$_4$(original) as it was potentially affected by CH$_4$ oxidation and transport in field conditions[1,2]. Similarly, various $\delta^{13}$CH$_4$, such as rhizospheric $\delta^{13}$CH$_4$,

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Results

CH$_4$ production and $\delta^{13}$C of CH$_4$ and CO$_2$. In anaerobic incubation, both CH$_4$ production potentials in the soil and on the roots were relatively low on 20 days after rice transplanting (D20), peaked (2.2 $\mu$g CH$_4$ g soil$^{-1}$ d$^{-1}$ and 11.1 $\mu$g CH$_4$ g root$^{-1}$ d$^{-1}$) on D50, and then turned downwards gradually to the bottom on D108 (Table 1). For $\delta^{13}$C-value of the produced CH$_4$, it was more and more positive in the soil during the whole observational period, being from $-71.1\%$ to $-53.9\%$ (Table 1). For CH$_4$ produced in aerobic incubation, both CH$_4$ production potentials in the soil and on the roots, and the corresponding apparent fractionation ($\alpha_{app}$) between CO$_2$ and CH$_4$ calculated by the ratio of $(\delta^{13}CO_2 + 1000)/(\delta^{13}CH_4 + 1000)$. 

![Temporal variations of CH$_4$ production rates in the soil and on the roots under aerobic (a,b) incubation, and corresponding $\delta^{13}$CH$_4$.](image)

Table 1. CH$_4$ production potentials ($\mu$g CH$_4$ g$^{-1}$ d$^{-1}$), $\delta^{13}$C-values (%o) of CH$_4$ and CO$_2$ in the soil and on the roots under anaerobic incubation, and the corresponding apparent fractionation ($\alpha_{app}$) between CO$_2$ and CH$_4$ calculated by the ratio of $(\delta^{13}CO_2 + 1000)/(\delta^{13}CH_4 + 1000)$.

| Days after rice transplanting (d) | Production $\mu$g CH$_4$ g$^{-1}$ d$^{-1}$ | $\delta^{13}$CH$_4$ | $\delta^{13}$CO$_2$ | $\alpha_{app}$ | $\delta^{13}$CH$_4$ | $\delta^{13}$CO$_2$ | $\alpha_{app}$ |
|----------------------------------|------------------------------------------|----------------------|----------------------|-----------------|----------------------|----------------------|-----------------|
|                                  | Soil                                      | Root                 | Soil                 | Root            | Soil                 | Root                 | Soil             |
| 20                               | 0.13 ± 0.21                              | 3.4 ± 0.7            | -71.1 ± 2.4          | -69.4 ± 2.8     | -18.8 ± 2.9          | -15.0 ± 2.3          | 1.056 ± 0.005    |
| 50                               | 2.15 ± 0.21                              | 11.1 ± 2.2           | -64.4 ± 0.4          | -86.9 ± 3.5     | -17.0 ± 1.8          | -14.1 ± 1.7          | 1.051 ± 0.002    |
| 88                               | 0.38 ± 0.12                              | 4.5 ± 0.6            | -57.5 ± 1.1          | -66.6 ± 2.7     | -15.1 ± 0.5          | -24.0 ± 2.9          | 1.045 ± 0.001    |
| 108                              | 0.22 ± 0.03                              | 3.2 ± 0.9            | -53.9 ± 0.2          | -72.5 ± 2.9     | -14.9 ± 0.4          | -23.3 ± 2.8          | 1.041 ± 0.000    |

CH$_4$ concentration and $\delta^{13}$Cof CH$_4$ and CO$_2$. CH$_4$ concentration in soil pore water was more than 100 $\mu$M L$^{-1}$ in most part of the season (Fig. 2), and it was highest (120 $\mu$M L$^{-1}$) on D50. CH$_4$ concentration in floodwater was in the range of 0.21–2.6 $\mu$M L$^{-1}$, being significantly lower than that of soil pore water over the season ($P < 0.01$). The $\delta^{13}$C-values of CH$_4$ in soil pore water and floodwater appeared to increase simultaneously (Fig. 2), from $\sim$70% to $\sim$60% and from $\sim$50% to $\sim$40%, respectively. Obviously, CH$_4$ in soil pore water was more depleted in $^{13}$C than that of floodwater CH$_4$ ($P < 0.05$), indicating that porewater CH$_4$ was intensively affected by CH$_4$ oxidation at the soil-water interface when it released into the atmosphere. CO$_2$ in soil pore water tended to $^{13}$C-enriched gradually during the rice season, with $\delta^{13}$C-values ranging from $-20.0\%$ to $-14.5\%$ (Fig. 2).
**Discussion**

**CH₄ production.** The processes of CH₄ production, oxidation, transport and emission from paddy field were well presented by the measurements of stable carbon isotopes in CH₄ from different pools of the field (Fig. 4). The decomposition of plants debris and root exudates, besides soil organic matters in the bulk soil, is very important to methanogenesis in paddy field24. As a key precursor for methanogens, it was slight ¹³C-depletion on the roots (Fig. 3b). Soil temperature ranged from 17.2 °C to 30.5 °C during the rice season, with a value of 24.5 °C on average.

**CH₄ oxidation and δ¹³C of CH₄.** CH₄ oxidation potential in the soil peaked on D50 ($6.9 \mu g CH_4 g^{-1} d^{-1}$), and then it dropped gradually to the lowest on D108 (Table 2). In contrast, it was highest on the roots ($850 \mu g CH_4 g root^{-1} d^{-1}$) on D20 and decreased sharply on D50. After an increase on D88, it decreased again to the lowest on D108 (Table 2). The δ¹³C-values of CH₄ before oxidation were $-41.0‰$ to $-35.8‰$ in the soil and $-40.5‰$ to $-36.0‰$ on the roots. After CH₄ oxidation, the CH₄ both in the soil and on the roots were more enriched in ¹³C, with δ¹³C-values of $-35.6‰$ to $-34.0‰$ and $-35.6‰$ to $-26.5‰$, respectively (Table 2).

**Plants emitted and aerenchymatic CH₄.** On the three sampling days (D37, D62 and D98) during the season, CH₄ emitted via the plants was relatively stable with δ¹³C-values of $-63.9‰$, $-62.6‰$ and $-63.5‰$, respectively. For δ¹³C-values of aerenchymatic CH₄, they were $-49.2‰$, $-45.9‰$ and $-52.4‰$, respectively, being significantly higher in comparison of the emitted CH₄ ($P < 0.05$). As a result, the isotope fractionations due to CH₄ transport (εtransport) were measured to be $-14.7‰$, $-16.7‰$ and $-11.1‰$, respectively, with a mean value of $-14.2‰$.

**CH₄ emission and δ¹³C of CH₄.** The CH₄ flux varied significantly, with the highest value appeared on D50 and the lowest on D108, ranging from 0.4 to 11.5 mg CH₄ m⁻² h⁻¹ during the observational period (Fig. 3a). The δ¹³CH₄(εmission) varied between $-68.7‰$ and $-61.5‰$ with the variation pattern just opposite to that of CH₄ flux (Fig. 3a). It is noteworthy that a significant negative relationship between CH₄ flux and corresponding δ¹³CH₄(εmission) was observed (Fig. 3b). Soil temperature ranged from 17.2 °C to 30.5 °C during the rice season, with a value of 24.5 °C on average.

**δ¹³C of organic carbon in soil and plant samples.** The values of δ¹³C in soil organic carbon did not show much variation during the rice season, being $-26.84‰$ on D37 and $-27.66‰$ on D108, respectively. The organic carbon in the plant samples also remained relatively stable over the season, with δ¹³C-values being $-29.19‰$ on D37 and $-28.70‰$ on D108, respectively, although they were slightly lighter than those of the soil organic carbon.

**Table 2. CH₄ oxidation potentials (µg CH₄ g⁻¹ d⁻¹), δ¹³C-values (‰) of CH₄ at time 0 (δ¹³CH₄(initial)) and at time t (δ¹³CH₄(final)) in the soil and on the roots under aerobic incubation with high CH₄ concentration supplemented, and the corresponding CH₄ oxidation fractionation factor (αox) calculated by the Equation (5).**

| Days after rice transplanting (d) | Oxidation | δ¹³CH₄(initial) | δ¹³CH₄(final) | αox  
|----------------------------------|-----------|----------------|---------------|-------
|                                  | Soil      | Root           | Soil          | Root  |
| 20                               | 4.4 ± 1.4 | 580 ± 116      | -38.4 ± 1.6   | -38.7 ± 1.9 |
| 50                               | 6.9 ± 1.1 | 335 ± 84       | -41.0 ± 0.4   | -40.4 ± 0.7 |
| 88                               | 5.1 ± 1.9 | 454 ± 68       | -38.7 ± 1.1   | -40.5 ± 2.6 |
| 108                              | 2.3 ± 1.3 | 258 ± 78       | -40.3 ± 0.0   | -36.0 ± 0.2 |

\[
\delta^{13}CH_4 = f_{ac} \times \delta^{13}CH_4(acetate) + (1 - f_{ac}) \times \delta^{13}CH_4(H_2/CO_2)
\]
During the process of acetate fermentation forming CH4, isotopic fractionation occurs and the fractionation factor is generally expressed as $\varepsilon_{\text{acetate}/\text{CH}_4}$. It was found to be $-21\%$ in pure cultures of acetoclastic Methanosarcina barkeri and $-18\%$ for acetoclastic Methanosaeta concilii. Using $\varepsilon_{\text{acetate}/\text{CH}_4} = -21\%$, Krüger et al. estimated $\delta^{13}C$ of CH4 produced from acetate ($\delta^{13}C_{\text{CH}_4(\text{acetate})}$) between $-43\%$ and $-37\%$ according to the measurements of $\delta^{13}C_{\text{acetate}}$ ($-22\%$ to $-16\%$) in soil pore water of an Italian rice field. Meanwhile, both values of $-43\%$ and $-37\%$ have well been applied in many studies.

Due to a lack of knowledge on $\varepsilon_{\text{acetate}/\text{CH}_4}$ and in order to compare the data interpretation with those of above mentioned, both $\delta^{13}C_{\text{CH}_4(\text{acetate})}$ of $-43\%$ and $-37\%$ were used in the present study (Table 3).

When H2/CO2 reduction produces CH4, isotopic fractionation factor $\alpha_{\text{CO}_2/\text{CH}_4}$ is defined by Hayes:

$$\alpha_{\text{CO}_2/\text{CH}_4} = \frac{\delta^{13}\text{CO}_2}{\delta^{13}\text{CH}_4} = \frac{(\delta^{13}\text{CO}_2 + 1,000)}{(\delta^{13}\text{CH}_4 + 1,000)}$$

where $\delta^{13}\text{CH}_4(\text{H}_2/\text{CO}_2)$ is $\delta^{13}C$ of the CH4 produced from H2/CO2 reduction. In addition, based on the ratio of $\delta^{13}\text{CO}_2$ to $\delta^{13}\text{CH}_4$ in anaerobic incubation (Table 1), an approximation of apparent fractionation ($\alpha_{\text{app}}$) between CO2 and CH4 can be calculated by using $\alpha_{\text{app}} = \frac{(\delta^{13}\text{CO}_2 + 1,000)}{(\delta^{13}\text{CH}_4 + 1,000)}$. The $\alpha_{\text{app}}$ is calculated from the isotopic signatures of total CH4 produced from H2/CO2 reduction and acetate cleavage, and theoretically, it is lower than $\alpha_{\text{CO}_2/\text{CH}_4}$. Results of 16 different lake sediments from tropical freshwater wetlands in Brazil have well

Table 3. Relative contribution of acetate to total CH4 production (%) in the soil ($f_{\text{ac}}^a$) and on the roots ($f_{\text{ac}}^b$). $f_{\text{ac}}^a$ and $f_{\text{ac}}^b$ was calculated with Equation (2) using $\delta^{13}C$-values of CH4 anaerobically produced in the soil and on the roots (Table 1) as originally produced $\delta^{13}\text{CH}_4$, respectively.
demonstrated that $\alpha_{\text{CO}_2/\text{CH}_4} (1.075 \pm 0.008)$ is much higher than $\alpha_{\text{app}} (1.059 \pm 0.009)$. In this study (Table 1), the $\alpha_{\text{app}}$ decreased gradually from 1.056 on D20 to 1.041 on D108 for the soil. In contrast, it increased sharply from 1.058 on D20 to 1.080 on D50, and then decreased again to 1.053 for the roots. Totally, $\alpha_{\text{app}}$ was relatively lower in the soil (1.041–1.056) than on the roots (1.046–1.080). The $\alpha_{\text{CO}_2/\text{CH}_4}$ was hence assumed to be 1.050–1.060 in the soil and 1.070–1.080 on the roots (Table 3). Incubating three different soils, Conrad et al. also found that $\alpha_{\text{CO}_2/\text{CH}_4}$ was between 1.050 and 1.060 for two paddy soils. Additionally, previous studies approved the relatively larger $\alpha_{\text{CO}_2/\text{CH}_4} (\geq 1.070)$ on the roots than in the soil due to their methanogenic populations were different.

The CH$_4$ produced in anaerobic incubation changed significantly during the rice season, and it was much more $^{13}$C-enriched in the soil than on the roots (Table 1). It indicates that methanogenic pathway was changed with rice growing, and also demonstrates that acetate-dependant methanogenesis was more important in the soil. In this study, $f_{\text{ac}}$ in the soil was initially very low (<10%) on D20, but it increased obviously with the rice growing and reached over 60% on D108. In contrast, $f_{\text{ac}}$ on the roots was relatively high (~30–40%) on D20. It decreased sharply in the middle of the season (near 0%) and then increased again to about 50% on D108. As a whole, $f_{\text{ac}}$ was relatively higher in the soil than that on the roots (Table 3). Previous study in Italian paddy soil has also demonstrated that acetoclastic methanogenesis was higher than 60% at the end of the season. High contribution of H$_2$/CO$_2$-dependent methanogenesis to total CH$_4$ production on rice roots was considerably reported, and the major reasons were supposed to be the methanogens population on rice roots dominated by Rice Cluster I archaea. Methanogenic substrates of organic carbon in the plant appeared to be slightly $^{13}$C-depleted relative to those of the bulk soil (Fig. 4), which might be a potential reason for the lower $f_{\text{ac}}$ in the soil.

On the other hand, Belik et al. and Tyler et al. estimated $f_{\text{ac}}$ of the USA paddy fields by using porewater $\delta^{13}$C-value of the produced CH$_4$, and they found that it was as high as 80% when $\alpha_{\text{CO}_2/\text{CH}_4} = 1.045 - 1.060$. However, Canadian field data have showed that porewater CH$_4$ is possibly influenced by CH$_4$ oxidation and transport, and in an Italian paddy field Krüger et al. also considered that porewater CH$_4$ was a poor proxy for produced CH$_4$ due to the potential CH$_4$ oxidation therein. Recently, a pot experiment in Germany suggested that porewater CH$_4$ could be used as newly produced CH$_4$ after tillering stage since they were similar in $^{13}$C-value. In this study, both porewater $^{13}$CH$_4$ and produced $^{13}$CH$_4$ generally tended to be enhanced during the rice season (Table 1 and Fig. 2), and on average they were similar with each other (Fig. 4). According to $^{13}$C-values of porewater CH$_4$ and CO$_2$ (Fig. 2), it was found that the $\alpha_{\text{app}}$ in soil pore water was from 1.047 to 1.054. Therefore, $\alpha_{\text{CO}_2/\text{CH}_4} = 1.050 - 1.060$ was assumed for comparing with former reports and present data of the paddy soil. Hydrogenotrophic methanogenesis was estimated to be dominated over the season (~60–80%), which differed much from the field data in USA. More importantly, the methanogenic pathway in soil pore water was different from that in paddy soil (Table 3). Although reasons for the difference in $f_{\text{ac}}$ between paddy soil and porewater were not clear, it is not recommended here that porewater CH$_4$ was absolutely regarded as newly produced CH$_4$.

**CH$_4$ oxidation.** The produced CH$_4$ in paddy field is mainly oxidized in the rhizosphere and at the soil-water interface, and accurate estimation of the CH$_4$ oxidation is one of the major aims of this study. Compared to $^{13}$C of newly produced CH$_4$ ($\delta^{13}$CH$_4$(original)), $^{13}$C of remaining CH$_4$ after it has undergone oxidation ($\delta^{13}$CH$_4$(oxidized)) was significant $^{13}$C-enriched (Fig. 4). Therefore, fraction of the CH$_4$ that is oxidized ($f_{\text{ox}}$) in the field can be estimated by the mass balance equation:

$$f_{\text{ox}} = \frac{\delta^{13}\text{CH}_4(\text{original}) - \delta^{13}\text{CH}_4(\text{oxidized})}{(1/\alpha_{\text{ac}} - 1) \times (\delta^{13}\text{CH}_4(\text{oxidized}) + 1.000)}$$  \hspace{1cm} (3)

In general, anaerobically produced $^{13}$CH$_4$ is regarded as $\delta^{13}$CH$_4$(original) and $\delta^{13}$CH$_4$(oxidized) is estimated by the measurements of $\delta^{13}$CH$_4$(emission) corrected with transport fractionation factor ($\varepsilon_{\text{transport}}$) using a semi-empirical equation:

$$\delta^{13}\text{CH}_4(\text{oxidized}) = \delta^{13}\text{CH}_4(\text{emission}) - \varepsilon_{\text{transport}}$$  \hspace{1cm} (4)

In the closed-system incubation, CH$_4$ oxidation fractionation factor $\alpha_{\text{ac}}$ is known to be calculated according to the Rayleigh equation:

$$\alpha_{\text{ac}} = 1 + \left[ \log(\delta^{13}\text{CH}_4(\text{initial})) + 1.000 \right] / \log f$$  \hspace{1cm} (5)

where $\delta^{13}$CH$_4$(initial) stands for $\delta^{13}$C-value of CH$_4$ at time 0, $\delta^{13}$CH$_4$(final) for $\delta^{13}$C-value of CH$_4$ at time t, and f (%) for the percentage of CH$_4$ remaining at time t.

To our knowledge, $\alpha_{\text{ac}} = 1.025 - 1.038$ at a temperature of 12–35 °C is initially measured in methanotrophs-enriched cultures and then widely in landfill cover soils and has been used in the studies of paddy soil and rice field. Recently, $\alpha_{\text{ac}} = 1.025 - 1.033$ was found in a Chinese paddy soil at 28.3 °C. By far, reports on $\alpha_{\text{ac}}$ in paddy soil, in particular on rice roots, are very few available. In the present study (Table 2), $\alpha_{\text{ac}}$ in the soil firstly increased from 1.014 on D20 to the highest value of 1.030 on D88, and then it decreased again to 1.021 on D108. In contrast, $\alpha_{\text{ac}}$ on the roots generally declined from 1.019 on D20 to the lowest 1.008 on D108. As a whole, it was higher in the soil (1.021 ± 0.007) than on the roots (1.013 ± 0.005) at 24.5 °C, being much lower than those of measured or used in previous studies under a similar temperature as above mentioned. In addition to $\alpha_{\text{ac}}$-value of 1.021 ± 0.007 in the soil and 1.013 ± 0.005 on the roots was used, we made an alternative calculation using $\alpha_{\text{ac}} = 1.038$ for better comparable to the previous studies (Table 4). Reasons for the difference in $\alpha_{\text{ac}}$ between paddy soils and rice roots are not understood, but Jahneke et al. found that there were complex factors influencing the isotopic fractionation in CH$_4$ oxidation and carbon assimilation.
in various methanotrophs. Besides, main groups of methanotrophs in rice microcosm (Methyllococccaceae and Methylocalystaceae) are active, but their dominance may change depending upon substrate supply and nutrient status. Therefore, it is no wonder that our measurement of $\alpha_{\text{rin}}$ in paddy soil was different from that on rice roots, and that both of them differed much from that found in different environments and habitats as above mentioned. On the other hand, $\varepsilon_{\text{transport}}$ is equivalent to the difference between emitted and aerenchymatic $\delta^{13}$CH$_4$ at the soil-water interface, and it was estimated to be $-14.2\%$ on average (Detailed descriptions please see below).

Rhzospheric CH$_4$ oxidation was the most important on D20 (Table 4). At that time, almost the produced CH$_4$ was oxidized before it was emitted into the atmosphere. With the rice growing, the $f_{\text{ox}}$ decreased fast to ~30% in the end. Both CH$_4$ oxidation potentials in the soil and on the roots were highest between D20 and D50, and decreased gradually towards the end of the season (Table 2), which might be the important reason. In situ inhibitor experiments, Krüger et al. also found that $f_{\text{ox}}$ was highest just at the beginning of the season with a peak of ~40%, and then it became negligible at the end of the season. Soon later, it was reported that $f_{\text{ox}}$ was no more than 50% over the season and it decreased rapidly from the beginning till the end of the season. They further concluded the possible reason was that activities of the methanotrophs were limited by nitrogen consumption with the rice growing under field conditions.

When porewater CH$_4$ released into the floodwater of the paddy fields, it was strongly oxidized at the soil-water interface since floodwater CH$_4$ was much more $^{13}$C-enriched than porewater CH$_4$ (Fig. 2). So, $f_{\text{ox}}$ in this oxidizing area, in principle, can be estimated using porewater $^{13}$CH$_4$ as $^{13}$CH$_4$ (original) and floodwater $^{13}$CH$_4$ as $^{13}$CH$_4$ (oxidized). Value of $f_{\text{ox}}$ was found to be over 80% throughout the whole season, which was generally higher than that of $f_{\text{ox}}$ in the rhizosphere (Table 4). Although $f_{\text{ox}}$ at the soil-water interface appeared to be much high, the amount of the CH$_4$ oxidized must be significantly lower than that in the rhizosphere. Because produced CH$_4$ is mostly oxidized in the rhizosphere during the rice-growing season as over 90% of the CH$_4$ emits to the atmosphere through the aerenchyma of the plants while less than 0.1% releases via ebullition and diffusion. In addition, it was reported that the absolute CH$_4$ oxidation rate at the soil-water interface was much lower than that in the rhizosphere.

Compared to methanogenesis in anaerobic soil, that was in aerobic soil significantly lower in CH$_4$ production rate but more positive in $^{13}$C (Fig. 4). The findings demonstrate that intensive CH$_4$ oxidation happened at the soil surface in lab conditions. As a result, $f_{\text{ox}}$ at the soil surface (Table 4) was estimated using anaerobically produced $^{13}$CH$_4$ as $^{13}$CH$_4$ (original) and aerobically produced $^{13}$CH$_4$ as $^{13}$CH$_4$ (oxidized). It was the highest (~80%) at the beginning of the season and decreased rapidly later (<0%). In field conditions, CH$_4$ oxidation in paddy field without rice plants occurs mainly at the soil-water interface, which is similar to CH$_4$ oxidation under aerobic incubation in lab conditions. Therefore, it is feasible to quantitatively estimate $f_{\text{ox}}$ in paddy fields during the non-rice-growing season or at the soil-water interface during the rice-growing season based on the difference in $^{13}$CH$_4$ between anaerobic and aerobic incubations. What is more, $f_{\text{ox}}$ at the root surface was also estimated by comparing $^{13}$C-value of the CH$_4$ produced under aerobic conditions with those under anaerobic conditions (Table 4).

### Table 4. Fraction of CH$_4$ oxidized (% in the rhizosphere ($f_{\text{ox}}$) and at the soil-water interface ($f_{\text{ox}}$) in field conditions, and at the surfaces of soil ($f_{\text{ox}}$) and rice roots ($f_{\text{ox}}$) in lab conditions.

| Days after rice transplanting (d) | $f_{\text{ox}}$ (α = 1.021) | $f_{\text{ox}}$ (α = 1.013) | $f_{\text{ox}}$ (α = 1.038) |
|---------------------------------|-----------------|-----------------|-----------------|
| 20                             | 108 ± 16        | 88 ± 16         | 78 ± 18         |
| 50                             | 51 ± 6          | 116 ± 25        | 61 ± 6          |
| 88                             | 42 ± 16         | 86 ± 22         | 27 ± 3          |
| 108                            | 33 ± 22         | 84 ± 12         | −4 ± 13         |

$\varepsilon_{\text{transport}}$ at the root surface was also estimated by comparing $^{13}$C-values of CH$_4$ produced under aerobic conditions with those under anaerobic conditions (Table 4) and floodwater $^{13}$CH$_4$ as $^{13}$CH$_4$ (oxidized). The findings demonstrate that intensive CH$_4$ oxidization happened at the soil-water interface since floodwater CH$_4$ was much more $^{13}$C-enriched than porewater CH$_4$ (Fig. 2). So, $f_{\text{ox}}$ in this oxidizing area, in principle, can be estimated using porewater $^{13}$CH$_4$ as $^{13}$CH$_4$ (original) and floodwater $^{13}$CH$_4$ as $^{13}$CH$_4$ (oxidized). Value of $f_{\text{ox}}$ was found to be over 80% throughout the whole season, which was generally higher than that of $f_{\text{ox}}$ in the rhizosphere (Table 4). Although $f_{\text{ox}}$ at the soil-water interface appeared to be much high, the amount of the CH$_4$ oxidized must be significantly lower than that in the rhizosphere. Because produced CH$_4$ is mostly oxidized in the rhizosphere during the rice-growing season as over 90% of the CH$_4$ emits to the atmosphere through the aerenchyma of the plants while less than 0.1% releases via ebullition and diffusion. In addition, it was reported that the absolute CH$_4$ oxidation rate at the soil-water interface was much lower than that in the rhizosphere.

Compared to methanogenesis in anaerobic soil, that was in aerobic soil significantly lower in CH$_4$ production rate but more positive in $^{13}$C (Fig. 4). The findings demonstrate that intensive CH$_4$ oxidation happened at the soil surface in lab conditions. As a result, $f_{\text{ox}}$ at the soil surface (Table 4) was estimated using anaerobically produced $^{13}$CH$_4$ as $^{13}$CH$_4$ (original) and aerobically produced $^{13}$CH$_4$ as $^{13}$CH$_4$ (oxidized). It was the highest (~80%) at the beginning of the season and decreased rapidly later (<0%). In field conditions, CH$_4$ oxidation in paddy field without rice plants occurs mainly at the soil-water interface, which is similar to CH$_4$ oxidation under aerobic incubation in lab conditions. Therefore, it is feasible to quantitatively estimate $f_{\text{ox}}$ in paddy fields during the non-rice-growing season or at the soil-water interface during the rice-growing season based on the difference in $^{13}$CH$_4$ between anaerobic and aerobic incubations. What is more, $f_{\text{ox}}$ at the root surface was also estimated by comparing $^{13}$C-value of the CH$_4$ produced under aerobic conditions with those under anaerobic conditions (Table 4). It was found that $f_{\text{ox}}$ at the root surface stayed over 100% throughout the whole season. Even if the $\alpha_{\text{rin}} = 1.021$ was used, it was still as high as 100% (Table 4), further suggesting that CH$_4$ oxidation on rice roots was extremely strong indeed. CH$_4$ oxidation rate much higher on the roots (Table 2) was supposed to be the main reason for the $f_{\text{ox}}$ was higher than that in the soil.

**CH$_4$ transport and emission.** Transporting CH$_4$ is the last step of CH$_4$ emission from paddy field to the atmosphere. Although CH$_4$ oxidation leads to the produced CH$_4$ obviously enriched in $^{13}$C, isotope fractionation in CH$_4$ transport offsets the positive effect on $^{13}$CH$_4$, causing the CH$_4$ $^{13}$C-depleted again. As a result, the $^{13}$C-values of emitted CH$_4$ were close to the produced $^{12}$CH$_4$ (Fig. 4). The isotope fractionation changes with the efficiency of CH$_4$ transport in growth of the plants. In the middle of the season, CH$_4$ transport capacity of the plants should get to highest because of full-developed rice plants and roots. Transport fractionation at that time is believed to be strongest and a value of $-16.7\%$ for $\varepsilon_{\text{transport}}$ was measured on D62. At the beginning of the season or aging in the late part of the season, transport fractionation would be relatively weak due to the undeveloped plants with low CH$_4$ transport capacity. Therefore, the $\varepsilon_{\text{transport}}$ was found to be $-14.7\%$ on D37 and $-11.1\%$ on D98. Many reports have shown a similar variation and it is generally between $-16\%$ and $-11\%$.
Similar to CH₄ emission from paddy fields, δ¹³CH₄(emission) is significantly affected by all each process of CH₄ production, oxidation and transport. At the beginning of the season, the high δ¹³CH₄(emission) was most likely ascribed to the relatively low transport fractionation and the highest fₓₐₓ. Subsequently, it became lowest, which was supposed to be the biggest transport fractionation and significantly decreased fₓₐₓ. At the late period of the season, the emitted CH₄ was ¹³C-enriched again, mainly due to an obvious increase of fₓₐₓ at this moment. The δ¹³CH₄(emission) was negatively correlated with CH₄ emission (Fig. 3b), which further indicates that the higher the CH₄ flux, the lower the fₓₐₓ, thus causing the lower the emitted δ¹³CH₄. Similar relationships were also observed in the previous studies²⁻⁷,⁸⁻¹⁰.

In conclusion, this study well showed each process of CH₄ emission by the measurements of δ¹³CH₄ from various pools of the paddy field, and found that stable carbon isotope fractionation occurred in CH₄ production, oxidation and transport. Compared to the roots (1.046–1.080 and 1.013 ± 0.005), whereas αₚ was lower (1.041–1.056) whereas αₑₓ was greater (1.021 ± 0.007) in the soil. This suggests that acetate-dependent methanogenesis was more important in paddy soil whereas CH₄ oxidation was much stronger on the roots. Rice plant-mediated CH₄ transport fractionation (εtransport) was found to be −16.7‰~−11.9‰. Temporal variation of CH₄ emission negatively correlated with δ¹³CH₄(emission) indicates the important relationships of CH₄ emission with production, oxidation and transport of the CH₄, which could be demonstrated by the changes of pathway of CH₄ production and fraction of CH₄ oxidation. Besides related newly produced δ¹³CH₄ and finally oxidized δ¹³CH₄, available carbon fractionation factors were needed to estimate relative contribution of acetate to total CH₄ production and fraction of CH₄ oxidized.

Methods

Experimental site. The experimental plots are located at Baitu Town, Jurong City, Jiangsu Province, China (31°58′N, 119°18′E). Soil of the field is classified as Typic Haplaquepts, with 11.1 g kg⁻¹ in total C, 1.3 g kg⁻¹ in total N and −26.8‰ in ¹³C-value of soil carbon. After wheat was harvested on June 13, 2009, wheat straw with stubble and even wild weeds were all removed from the plots. Then, the plots were flooded from June 24 to October 15 and drained on October 16 before rice harvest. Seeds of the rice ("Oryza sativa L. Huajia 3") crop were sown into the nursery bed on May 25, seedlings were transplanted into the field on June 26, and the crop was harvested on November 3. Urea was applied at a rate of 300 kg N ha⁻¹, 50% as basal fertilizer on June 26, 25% as tillering fertilizer on July 17, and 25% as panicle fertilizer on August 16. Ca(H₂PO₄)₂ (450 kg ha⁻¹) and KCL (225 kg ha⁻¹) was applied with urea just as basal fertilizer on June 26.

Field sampling. CH₄ flux was monitored within the static chamber technique. The flux chambers (0.5 × 0.5 × 1 m), made of plexiglass, covered six hills of rice plants each. Plastic bases for the chambers were installed before rice transplantation in the plots. Removable wooden boardwalks (2 m long) were set up at the beginning of the rice season to avoid soil disturbance during sampling and measuring. To measure CH₄ flux, gas samples were usually collected once every 4–7 days. Four gas samples from each chamber were collected using 18 mL vacuum vials at 15 min intervals between 09:45 and 10:30 in the morning on each sampling day. To determine carbon isotope composition of the CH₄ gas (δ¹³CH₄(emission)), samples were taken at 15–30 day intervals. Only two gas samples were collected using 0.5 L bags (aluminium foil compound membrane, Delin gas packing Co., Ltd, Dalian, China) with a small battery-driven pump⁹. The first sample was taken after the chamber was closed for 3–5 min, and the second at the end of the 2 h closure period. When CH₄ flux was monitored, soil temperature at 10 cm depth was simultaneously measured with a hand-carried digital thermometer (Yokogawa Meter and Instruments Corporation, Japan).

Soil pore water samples, 10 cm in depth, were collected using a Rhizon soil moisture sampler (10 RHIZON SMSMOM, Eijkelkamp Agrisearch Equipment, Giesbeek, Netherlands)¹⁰. The samplers were installed (in triplicate) prior to rice transplantation and then left in the soil throughout the whole season. Samples (about 5 mL) were firstly extracted using 18 mL vacuum vials to flush and purge the sampler before sampling. Then about 10 mL of soil solution was drawn into another vial. Simultaneously, 10 mL of floodwater was collected using a plastic syringe and then transferred in to an 18 mL vacuum vial. Subsequently, all sampling vials were equilibrated by filling in pure N₂ gas for further analysis with a GC-FID.

CH₄ emitted via rice plants and the aerenchymatic CH₄ was measured using specially designed PVC bottomless pots¹⁰. The pot, 30 cm in height and 17 cm in diameter, was designed to have a water-filled trough around its top, avoiding any possible gas exchange during the sampling times. A PVC plate (18 cm in diameter) with a hole adjustable in diameter to fit the growing plant in the center was placed on top of the pot, allowing the plant to grow through the hole and keeping the plant into two parts. Then, the plant in one pot was cut right above the plate while the plant in the other pot remained intact as control. Finally, chambers (0.3 × 0.3 × 1 m) were laid on the pots, and gas samples in the headspace of the chambers were collected simultaneously with a small battery-driven pump.

Soil cores of the top layer (0–15 cm) were collected at about 15–30-day intervals, and samples of the same plot were first mixed together⁴⁸. Two samples from the mixture, about 50 g each (dry weight), were then taken and transferred promptly into two 250–mL Erlenmeyer flasks separately. Samples in the flasks were prepared into slurries with N₂-flushed de-ionized sterile water at a soil/water ratio of 1:1. During the whole process, N₂ was constantly flushed through the samples to remove O₂ and CH₄. One flask was sealed for anaerobic incubation. Other flask with air headspace was sealed directly for aerobic incubation. Simultaneously, rice plants together with roots were carefully collected from the plots. The roots were washed clean with N₂-flushed demineralized water and cut off at 1–2 cm from the root with a razor blade. The fresh roots, about 20 g each portion, were put into flasks, separately, for further preparation and processing in the same way as for the soil samples. All the flasks were sealed with rubber stoppers fitted with silicon septum that allowed sampling of headspace gas. Finally, they
were stored under N₂ at 4 °C and transported back to the lab as soon as possible for further analysis. A small portion of the soil and plant samples were dried for 72 h at 60 °C for determination of isotopic composition of the organic carbon.

Lab incubations. CH₄ production potentials were measured under anaerobic incubation. The flasks were flushed with N₂ consecutively for six times through double-ended needles connecting a vacuum pump to purge the air in the flasks of residual CH₄ and O₂. Simultaneously, methanogenesis was determined aerobically using flasks with air headspace directly. They were incubated in darkness at a temperature the same as measured in the field for 50 h. Gas samples were analyzed 1 h and 50 h later after heavily shaking the flasks by hand. CH₄ production rates were calculated using the linear regression of CH₄ increasing with the incubation time.

CH₄ oxidation potentials were determined under aerobic incubation with high concentration of CH₄ supplemented, using equipment the same as described above. Firstly, pure CH₄ was injected into each flask to make a high concentration inside (~10,000 μL L⁻¹). Then, the flasks were incubated in darkness under the same temperature as measured in the field and shaken at 120 r.p.m. CH₄ depletion was measured by sampling the headspace gas in the flask after vigorous shaking for subsequent analysis. The first sample was collected generally 30 min after pure CH₄ was injected. Samples were then taken at 2–3 h intervals during the first 8 h of the experiment. The flasks were left over night and sampled the next day at 2 h intervals again. CH₄ oxidation rates were calculated by linear regression of CH₄ depletion with incubation time.

Chemical measurements. CH₄ concentrations were analyzed with a gas chromatograph (Shimadzu GC-12A, Kyoto, Japan) equipped with a flame ionization detector. For analysis of carbon isotope composition, the continuous flow technique and a Finnigan MAT 253 isotope ratio mass spectrometer was used (Thermo Finnigan, Bremen, Germany)⁴⁷,⁴⁹. CO₂ in gas samples was directly analyzed while CH₄ in gas samples was converted into CO₂ and separated primarily on a PreCon (pre-concentration device). Then, the gas was piped into a GC equipped with a Pora PLOT Q column (25 m length; 0.35 mm i.d.) at 25 °C under 2.0 mL min⁻¹. Reference and carrier gases used were CO₂ (99.999% in purity, 20 mL min⁻¹), pure CH₄ was injected. Samples were then taken at 2–3 h intervals during the first 8 h of the experiment. The flasks were left over night and sampled the next day at 2 h intervals again. CH₄ oxidation rates were calculated by linear regression of CH₄ depletion with incubation time.

Statistical analyses. Statistical analysis was done using the SPSS 18.0 software for Windows (SPSS Inc., Chicago). Differences between the four treatments were determined through one-way analysis of variance (ANOVA) and least significant difference (LSD) test. Relationships were assessed using correlation analysis. Significant differences and correlations were set at P < 0.05.

References

1. Ciais, P. et al. (ed | Stocker, T. F., Qin, D., Plattner, G.-K., Tignor, M., Allen, S. K., Boschung, J., Nauels, A., Xia, Y., Bex, V. & Midgley, P. M.) (Cambridge University Press, Cambridge, United Kingdom and New York, NY, USA, 2013).
2. Bouwman, A. F. Agronomic aspects of wetland rice cultivation and associated methane emissions. Biogeochemistry 15, 65–88 (1991).
3. Ali, M. A., Lee, C. H., Lee, Y. B. & Kim, P. J. Silicate fertilization in no-tillage rice farming for mitigation of methane emission and increasing rice productivity. Agric Ecosyst Environ 132, 16–22 (2009).
4. Linquist, B., van Groenigen, K. J., Adviento-Borbe, M. A., Pelttikow, C. & van Kessel, C. An agronomic assessment of greenhouse gas emissions from major cereal crops. Global Change Biol 18, 194–209 (2012).
5. Krüger, M., Eller, G., Conrad, R. & Frenzel, P. Seasonal variation in pathways of CH₄ production and in CH₄ oxidation in rice fields determined by stable carbon isotopes and specific inhibitors. Global Change Biol 8, 265–280 (2002).
6. Bilek, R. S., Tyler, S. C., Sass, R. L. & Fisher, F. M. Differences in CH₄ oxidation and pathways of production between rice cultivars deduced from measurements of CH₄ flux and δ¹³C of CH₄ and CO₂. Global Biogeochem Cy 13, 1029–1044 (1999).
7. Tyler, S. C., Bilek, R. S., Sass, R. L. & Fisher, F. M. Methane oxidation and pathways of production in a Texas paddy field deduced from measurements of flux, δ¹³C, and δ¹⁸O of CH₄. Global Biogeochem Cy 11, 323–348 (1997).
8. Bergamaschi, P. Seasonal variations of stable hydrogen and carbon isotope ratios in methane from a Chinese rice paddy. J Geophys Res 102, 25383–25393 (1997).
9. Zhang, G. B. et al. Methanogenic pathway and fraction of CH₄ oxidized in paddy fields: Seasonal variation and effect of water management in winter fallow season. PLoS one 8, e73982 (2013).
10. Whiticar, M. J. Carbon and hydrogen isotope systematics of bacterial formation and oxidation of methane. Chem Geol 161, 291–314 (1999).
11. Chanton, J. P. The effect of gas transport on the isotope signature of methane in wetlands. Org Geochem 36, 753–768 (2005).
12. Venkiteswaran, J. J. & Schiff, S. L. Methane oxidation: isotopic enrichment factors in freshwater boreal reservoirs. Appl Geochem 20, 683–690 (2005).
13. Chanton, J. P., Whiting, G. J., Blair, N. E., Lindau, C. W. & Bollich, P. K. Methane emission from rice: Stable isotopes, diurnal variations, and CO₂ exchange. Global Biogeochem Cy 11, 15–27 (1997).
14. Mark, T., Fischer, H., Cohen, F. & Smith, K. Seasonal variations in stable carbon and hydrogen isotope ratios in methane from rice fields. Global Biogeochem Cy 16, Artn 1094 (2002).
15. Fey, A., Claus, P. & Conrad, R. Temporal change of δ¹³C-isotope signatures and methanogenic pathways in rice field soil incubated anaerobically at different temperatures. Geochim Cosmochim Acta 68, 293–306 (2004).
16. Conrad, R., Kloke, M. & Claus, P. Pathway of CH₄ formation in anoxic rice field soil and rice roots determined by δ¹³C-stable isotope fractionation. Chemosphere 47, 797–806 (2002).
17. Conrad, R., Kloke, M., Lu, Y. & Chidthaisong, A. Methanogenic pathway and archaeal communities in three different anoxic soils amended with rice straw and maize straw. Fron microb 3, 4–4 (2012).
18. Conrad, R. & Kloke, M. Stable carbon isotope discrimination in rice field soil during acetate turnover by syntrophic acetate oxidation or acetoclastic methanogenesis. Geochim Cosmochim Acta 75, 1531–1539 (2011).
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Author Contributions
G.Z. and H.X. conceived and designed the research; G.Z., J.M., H.Y. and X.F. performed the experiment; G.Z., J.M. and H.X. analyzed data; G.Z. and H.X. wrote the main manuscript text; G.Z., J.M. and H.X. reviewed the manuscript.

Additional Information
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