

SCIENTIFIC REPORTS

OPEN

Carbon isotope fractionation reveals distinct process of CH₄ emission from different compartments of paddy ecosystem

Received: 25 January 2016

Accepted: 29 April 2016

Published: 02 June 2016

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Carbon isotopic fractionations in the processes of CH₄ emission from paddy field remain poorly understood. The δ¹³C-values of CH₄ in association with production, oxidation and transport of CH₄ in different pools of a paddy field were determined, and the stable carbon isotope fractionations were calibrated to assess relative contribution of acetate to CH₄ production (f_{ac}) and fraction of CH₄ oxidized (f_{ox}) by different pathways. The apparent isotope fractionation for CO₂ conversion to CH₄ (α_{app}) 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 CH₄ oxidation (α_{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‰ ~ –11.1‰. Rhizospheric f_{ox} 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 (CH₄) is the second most important greenhouse gas after carbon dioxide (CO₂). On a 100-year horizon, CH₄ has 25 times the global warming potential of CO₂. Paddy fields are one of the largest anthropogenic sources of atmospheric CH₄, contributing to 33–40 Tg yr⁻¹ during the 2000–2009¹. The global CH₄ 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^{2–4}. Paddy CH₄ emission is an integrated effect of the production, oxidation and transport of CH₄ in the field. A better knowledge of these processes affecting CH₄ emission may provide more information for effectively mitigating CH₄ emission in agricultural ecosystems.

The technique of stable carbon isotopes has been proved to be a useful tool in studying the processes of CH₄ emission^{5–9}. Isotope fractionation happens in all the major processes CH₄ emission, namely, ¹²C-substrate is preferentially utilized by methanogens for CH₄ production, and once formed, ¹²CH₄ is consumed faster than ¹³CH₄ by methanotrophs, and ¹²CH₄ is transported faster than ¹³CH₄ as well^{10–12}. Thereby, measurements of the δ¹³C in production, oxidation and transport of the CH₄ from different pools of the field are beneficial to support a process-based model for CH₄ emission^{8,13,14}. Moreover, the relative contribution of acetate to CH₄ production (f_{ac}) and the fraction of CH₄ oxidized (f_{ox}) can be quantitatively estimated^{5–7} using mass balance equations based on the measurements of δ¹³C in CH₄, CO₂ and acetate, and of the isotope fractionation factors (α_{CO_2/CH_4} , $\epsilon_{acetate/CH_4}$, α_{ox} and $\epsilon_{transport}$). Investigations on α_{CO_2/CH_4} , $\epsilon_{acetate/CH_4}$ and $\epsilon_{transport}$ of paddy fields were carried out greatly^{5,15–18}, however, few data are available on α_{ox} for CH₄ oxidation by methanotrophs in paddy soils, in particular α_{ox} on rice roots¹⁹.

Some uncertainties also exist in the δ¹³CH₄ that are used as newly produced δ¹³CH₄ (δ¹³CH₄_(original)) and finally oxidized δ¹³CH₄ (δ¹³CH₄_(oxidized)) in different studies for quantifying f_{ac} and f_{ox} . For example, former reports in USA using porewater δ¹³CH₄ as δ¹³CH₄_(original)^{6,7} whereas anaerobically produced δ¹³CH₄ was used in Italy^{5,20} and China^{19,21}. They believed that porewater CH₄ was a poor proxy for δ¹³CH₄_(original) as it was potentially affected by CH₄ oxidization and transport in field conditions^{11,22}. Similarly, various δ¹³CH₄, such as rhizospheric δ¹³CH₄,

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Days after rice transplanting (d)	Production		$\delta^{13}\text{CH}_4$		$\delta^{13}\text{CO}_2$		α_{app}	
	Soil	Root	Soil	Root	Soil	Root	Soil	Root
20	0.13 ± 0.18	3.4 ± 0.7	-71.1 ± 2.4	-69.4 ± 2.8	-18.8 ± 2.9	-15.0 ± 2.3	1.056 ± 0.005	1.058 ± 0.002
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	1.080 ± 0.003
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	1.046 ± 0.005
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	1.053 ± 0.001

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

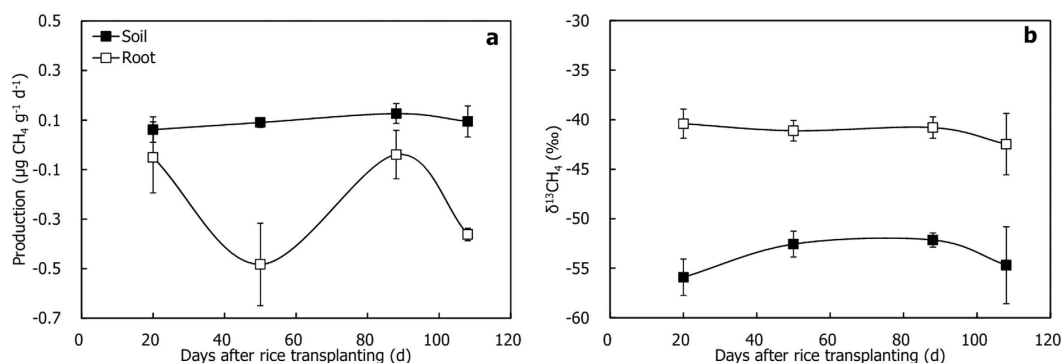


Figure 1. Temporal variations of CH_4 production rates in the soil and on the roots under aerobic (a,b) incubation, and corresponding $\delta^{13}\text{CH}_4$.

aerobically produced $\delta^{13}\text{CH}_4$, porewater $\delta^{13}\text{CH}_4$ or floodwater $\delta^{13}\text{CH}_4$ sometimes, have been regarded as $\delta^{13}\text{CH}_4$ (oxidized) for estimation of f_{ox} in the rhizosphere or at the soil-water interface^{5-7,9,19,23}. More importantly, large differences were observed in the estimated f_{ac} and f_{ox} if different $\delta^{13}\text{CH}_4$ (original) and $\delta^{13}\text{CH}_4$ (oxidized) was assumed in the same study^{5,7}. Therefore, more comparable observations in different conditions with corresponding isotope fractionation factors are needed to discuss $\delta^{13}\text{CH}_4$ (original) and $\delta^{13}\text{CH}_4$ (oxidized) in accurate estimations of f_{ac} and f_{ox} .

In this study, field and incubation experiments were conducted to observe the process of CH_4 emission closely related to the production, oxidation and transport of CH_4 , including CH_4 fluxes emitted from the field and via the plants, CH_4 concentrations in the aerenchyma of the plants, and in soil pore water and floodwater, CH_4 production and oxidation rates in the soil and on the roots, and all the corresponding $\delta^{13}\text{CH}_4$. The objectives of the present study were (1) to improve our understanding of the processes in CH_4 emission by measurements of the stable carbon isotopes, (2) to investigate the isotope fractionation factor α_{ox} in the soil and on the roots, and (3) to discuss the availabilities in the estimation of f_{ac} and f_{ox} associated with different pools of $\delta^{13}\text{CH}_4$ in the field.

Results

CH_4 production and $\delta^{13}\text{C}$ 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 ($2.2 \mu\text{g CH}_4 \text{g soil}^{-1} \text{d}^{-1}$ and $11.1 \mu\text{g CH}_4 \text{g root}^{-1} \text{d}^{-1}$) on D50, and then turned downwards gradually to the bottom on D108 (Table 1). For $\delta^{13}\text{C}$ -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 on the roots however, it was most ^{13}C -depleted on D50 and then ^{13}C -enriched again on D88, with $\delta^{13}\text{C}$ -value ranging between -86.9‰ and -66.6‰ (Table 1). Throughout the whole season, $\delta^{13}\text{C}$ -value of produced CO_2 increased from -18.8‰ to -14.9‰ in the soil while decreased from -15.0‰ to -23.3‰ on the roots (Table 1). CH_4 production under aerobic incubation was hardly observed in the soil (0.06 to $0.13 \mu\text{g CH}_4 \text{g soil}^{-1} \text{d}^{-1}$), particularly on the roots, which was lower than $0 \mu\text{g CH}_4 \text{g root}^{-1} \text{d}^{-1}$ over the whole season (Fig. 1a). The $\delta^{13}\text{C}$ -value of CH_4 produced in the soil was relatively stable around -55‰ while on the roots it was about -40‰ (Fig. 1b). Apparently, the $\delta^{13}\text{C}$ -values of CH_4 produced in aerobic incubation were significantly higher ($P < 0.05$) than those of the CH_4 that was produced in anaerobic incubation (Table 1 and Fig. 1).

CH_4 concentration and $\delta^{13}\text{C}$ of CH_4 and CO_2 . CH_4 concentration in soil pore water was more than $100 \mu\text{M L}^{-1}$ in most part of the season (Fig. 2), and it was highest ($\sim 120 \mu\text{M L}^{-1}$) on D50. CH_4 concentration in floodwater was in the range of 0.21 – $2.6 \mu\text{M L}^{-1}$, being significantly lower than that of soil pore water over the season ($P < 0.01$). The $\delta^{13}\text{C}$ -values of CH_4 in soil pore water and floodwater appeared to increase simultaneously (Fig. 2), from $\sim -70\text{‰}$ to -60‰ and from $\sim -50\text{‰}$ to -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 $\delta^{13}\text{C}$ -values ranging from -20.0‰ to -14.5‰ (Fig. 2).

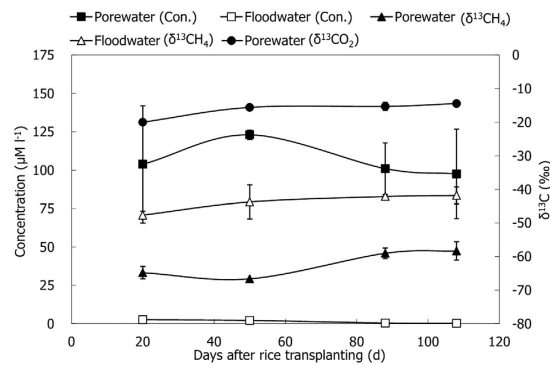


Figure 2. Temporal variations of CH₄ concentrations in soil pore water and floodwater, and corresponding δ¹³C-values of CH₄ and CO₂.

Days after rice transplanting (d)	Oxidation		δ ¹³ CH ₄ (initial)		δ ¹³ CH ₄ (final)		α _{ox}	
	Soil	Root	Soil	Root	Soil	Root	Soil	Root
20	4.4 ± 1.4	580 ± 116	-38.4 ± 1.6	-38.7 ± 1.9	-35.6 ± 2.2	-34.0 ± 2.8	1.014 ± 0.002	1.019 ± 0.005
50	6.9 ± 1.1	335 ± 84	-41.0 ± 0.4	-40.4 ± 0.7	-35.0 ± 1.7	-26.5 ± 1.9	1.020 ± 0.002	1.012 ± 0.007
88	5.1 ± 1.9	454 ± 68	-38.7 ± 1.1	-40.5 ± 2.6	-32.5 ± 1.0	-30.6 ± 2.2	1.030 ± 0.004	1.015 ± 0.003
108	2.3 ± 1.3	258 ± 78	-40.3 ± 0.0	-36.0 ± 0.2	-38.4 ± 0.2	-31.2 ± 3.4	1.021 ± 0.009	1.008 ± 0.009

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 (α_{ox}) calculated by the Equation (5).

CH₄ oxidation and δ¹³C of CH₄. CH₄ oxidation potential in the soil peaked on D50 (6.9 µg CH₄ g soil⁻¹ d⁻¹), and then it dropped gradually to the lowest on D108 (Table 2). In contrast, it was highest on the roots (580 µg CH₄ 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‰ ~ -38.4‰ in the soil and -40.5‰ ~ -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 -38.4‰ ~ -32.5‰ and -34.0‰ ~ -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 (ε_{transport}) 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 (emission) 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 (emission) 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.

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 relative to soil organic carbon (Fig. 4). Previous studies also showed δ¹³C-value of organic carbon relatively negative in the plant than in the soil^{5,19}. Paddy field CH₄ is mainly produced out of either cleavage of acetate (*f*_{ac}) or reduction of H₂/CO₂ (1 - *f*_{ac}), and the δ¹³C-value of produced CH₄ primarily depends on relative contribution of the two main methanogenic pathways^{10,25}. The *f*_{ac} was calculated by the following mass balance^{6,7}:

$$\delta^{13}\text{CH}_4 = f_{ac} \times \delta^{13}\text{CH}_4(\text{acetate}) + (1 - f_{ac}) \times \delta^{13}\text{CH}_4(\text{H}_2/\text{CO}_2) \quad (1)$$

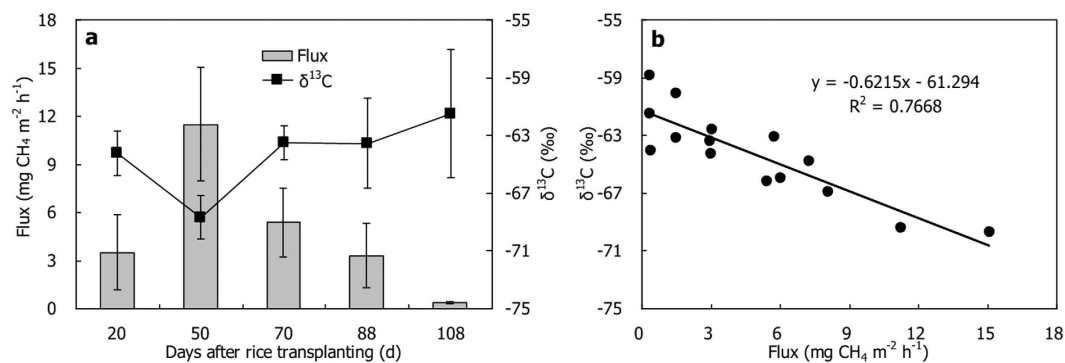


Figure 3. Temporal variations of CH₄ flux and δ¹³CH₄ (a) and their relationship (b).

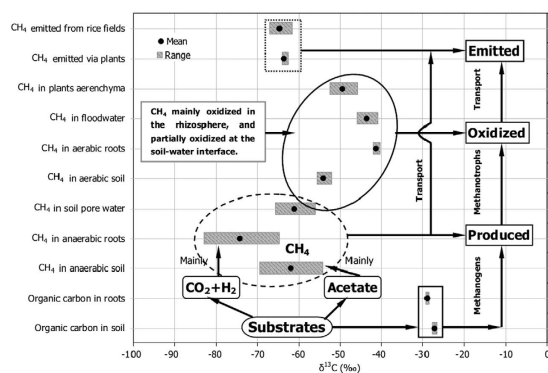


Figure 4. Stable carbon isotopes in the processes of CH₄ emission from the paddy field. Note: each δ¹³C-value was given in arithmetic mean of the rice season.

Days after rice transplanting (d)	f_{ac}^a				f_{ac}^b			
	$\delta^{13}CH_4(\text{acetate}) = -37\text{‰}$		$\delta^{13}CH_4(\text{acetate}) = -43\text{‰}$		$\delta^{13}CH_4(\text{acetate}) = -37\text{‰}$		$\delta^{13}CH_4(\text{acetate}) = -43\text{‰}$	
	$\alpha_{CO_2/CH_4} = 1.050$	$\alpha_{CO_2/CH_4} = 1.060$	$\alpha_{CO_2/CH_4} = 1.050$	$\alpha_{CO_2/CH_4} = 1.060$	$\alpha_{CO_2/CH_4} = 1.070$	$\alpha_{CO_2/CH_4} = 1.080$	$\alpha_{CO_2/CH_4} = 1.070$	$\alpha_{CO_2/CH_4} = 1.080$
20	-21 ± 4	8 ± 3	-27 ± 4	9 ± 3	24 ± 6	37 ± 7	28 ± 7	41 ± 8
50	-2 ± 6	23 ± 3	-3 ± 7	28 ± 3	-20 ± 1	0 ± 0	-23 ± 2	1 ± 0
88	18 ± 3	39 ± 2	24 ± 4	48 ± 3	42 ± 2	50 ± 15	47 ± 2	56 ± 17
108	32 ± 0	50 ± 0	42 ± 0	61 ± 0	29 ± 0	39 ± 11	33 ± 0	44 ± 12

Table 3. Relative contribution of acetate to total CH₄ production (%) in the soil (f_{ac}^a) and on the roots (f_{ac}^b). f_{ac}^a and f_{ac}^b was calculated with Equation (2) using δ¹³C-values of CH₄ anaerobically produced in the soil and on the roots (Table 1) as originally produced δ¹³CH₄, respectively.

During the process of acetate fermentation forming CH₄, isotopic fractionation occurs and the fractionation factor is generally expressed to $\epsilon_{\text{acetate}/CH_4}$. It was found to be -21‰ in pure cultures of acetoclastic *Methanosarcina barkeri*²⁶ and -18‰ for acetoclastic *Methanosaeta concilii*^{15,27}. Using $\epsilon_{\text{acetate}/CH_4} = -21\text{‰}$, Krüger *et al.*⁵ estimated δ¹³C of CH₄ produced from acetate (δ¹³CH₄(acetate)) between -43‰ and -37‰ according to the measurements of δ¹³C_{acetate} (-22‰ ~ -16‰) in soil pore water of an Italian rice field. Meanwhile, both values of -43‰ and -37‰ have well been applied in many studies^{6,7,9,16,19,21}. Due to a lack of knowledge on $\epsilon_{\text{acetate}/CH_4}$ and in order to compare the data interpretation with those of above mentioned, both δ¹³CH₄(acetate) of -43‰ and -37‰ were used in the present study (Table 3).

When H₂/CO₂ reduction produces CH₄, isotopic fractionation factor α_{CO_2/CH_4} is defined by Hayes²⁸:

$$\alpha_{CO_2/CH_4} = (\delta^{13}CO_2 + 1,000)/(\delta^{13}CH_4(H_2/CO_2) + 1,000) \quad (2)$$

where δ¹³CH₄(H₂/CO₂) is δ¹³C of the CH₄ produced from H₂/CO₂ reduction. In addition, based on the ratio of δ¹³CO₂ to δ¹³CH₄ in anaerobic incubation (Table 1), an approximation of apparent fractionation (α_{app}) between CO₂ and CH₄ can be calculated by using $\alpha_{app} = (\delta^{13}CO_2 + 1000)/(\delta^{13}CH_4 + 1000)$. The α_{app} is calculated from the isotopic signatures of total CH₄ produced from H₂/CO₂ reduction and acetate cleavage, and theoretically, it is lower than α_{CO_2/CH_4} . Results of 16 different lake sediments from tropical freshwater wetlands in Brazil²⁹ have well

demonstrated that $\alpha_{\text{CO}_2/\text{CH}_4}$ (1.075 ± 0.008) is much higher than α_{app} (1.059 ± 0.009). In this study (Table 1), the α_{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, α_{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.*¹⁷ 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 $\alpha_{\text{CO}_2/\text{CH}_4}$ (≥ 1.070) on the roots than in the soil due to their methanogenic populations were different^{16,30}.

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_{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_{ac} on the roots was relatively high ($\sim 30\text{--}40\%$) 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_{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 season⁵. High contribution of H_2/CO_2 -dependent methanogenesis to total CH_4 production on rice roots was considerably reported^{5,9,16,19}, and the major reasons were supposed to be the methanogens population on rice roots dominated by Rice Cluster I archaea^{31–33}. 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_{ac} in the soil.

On the other hand, Belik *et al.* and Tyler *et al.*^{6,7} estimated f_{ac} of the USA paddy fields by using porewater $\delta^{13}\text{CH}_4$ as $\delta^{13}\text{C}$ -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 transport²², and in an Italian paddy field Krüger *et al.*⁵ 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 $\delta^{13}\text{C}$ ³⁴. In this study, both porewater $\delta^{13}\text{CH}_4$ and produced $\delta^{13}\text{CH}_4$ 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 $\delta^{13}\text{C}$ -values of porewater CH_4 and CO_2 (Fig. 2), it was found that the α_{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^{6,7} and present data of the paddy soil. Hydrogenotrophic methanogenesis was estimated to be dominated over the season ($\sim 60\text{--}80\%$), which differed much from the field data in USA^{6,7}. 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_{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 $\delta^{13}\text{C}$ of newly produced CH_4 ($\delta^{13}\text{CH}_4(\text{original})$), $\delta^{13}\text{C}$ of remaining CH_4 after it has undergone oxidization ($\delta^{13}\text{CH}_4(\text{oxidized})$) was significant ^{13}C -enriched (Fig. 4). Therefore, fraction of the CH_4 that is oxidized (f_{ox}) in the field can be estimated by the mass balance equation^{6,7}:

$$f_{\text{ox}} = (\delta^{13}\text{CH}_4(\text{original}) - \delta^{13}\text{CH}_4(\text{oxidized})) / [(1/\alpha_{\text{ox}} - 1) \times (\delta^{13}\text{CH}_4(\text{oxidized}) + 1,000)] \quad (3)$$

In general, anaerobically produced $\delta^{13}\text{CH}_4$ is regarded as $\delta^{13}\text{CH}_4(\text{original})$ and $\delta^{13}\text{CH}_4(\text{oxidized})$ is estimated by the measurements of $\delta^{13}\text{CH}_4(\text{emission})$ corrected with transport fractionation factor ($\varepsilon_{\text{transport}}$) using a semi-empirical equation^{5,7,16}:

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

In the closed-system incubation, CH_4 oxidation fractionation factor α_{ox} is known to be calculated according to the Rayleigh equation^{35,36}:

$$\alpha_{\text{ox}} = 1 + [\log(\delta^{13}\text{CH}_4(\text{initial}) + 1,000) - \log(\delta^{13}\text{CH}_4(\text{final}) + 1,000)] / \log f \quad (5)$$

where $\delta^{13}\text{CH}_4(\text{initial})$ stands for $\delta^{13}\text{C}$ -value of CH_4 at time 0, $\delta^{13}\text{CH}_4(\text{final})$ for $\delta^{13}\text{C}$ -value of CH_4 at time t, and f (%) for the percentage of CH_4 remaining at time t.

To our knowledge, $\alpha_{\text{ox}} = 1.025 - 1.038$ at a temperature of $12\text{--}35^\circ\text{C}$ is initially measured in methanotrophs-enriched cultures³⁵ and then widely in landfill cover soils^{36–38}, and it has substantially been used in the studies of paddy soil^{5–7,9,20,21,23}. Recently, $\alpha_{\text{ox}} = 1.025 - 1.033$ was found in a Chinese paddy soil at 28.3°C ¹⁹. By far, reports on α_{ox} in paddy soil, in particular on rice roots, are very few available. In the present study (Table 2), α_{ox} 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, α_{ox} 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 α_{ox} -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 $\alpha_{\text{ox}} = 1.038$ for better comparable to the previous studies (Table 4). Reasons for the difference in α_{ox} between paddy soils and rice roots are not understood, but Jahnke *et al.*³⁹ found that there were complex factors influencing the isotopic fractionation in CH_4 oxidation and carbon assimilation

Days after rice transplanting (d)	$\alpha_{ox} = 1.021$			$\alpha_{ox} = 1.013$	$\alpha_{ox} = 1.038$			
	f_{ox}^a	f_{ox}^b	f_{ox}^c	f_{ox}^d	f_{ox}^a	f_{ox}^b	f_{ox}^c	f_{ox}^d
20	108 ± 16	88 ± 16	78 ± 18	235 ± 26	61 ± 9	49 ± 7	44 ± 10	82 ± 9
50	51 ± 6	116 ± 25	61 ± 6	372 ± 43	29 ± 3	65 ± 14	34 ± 3	130 ± 16
88	42 ± 16	86 ± 22	27 ± 3	209 ± 27	23 ± 9	48 ± 12	15 ± 2	73 ± 10
108	33 ± 22	84 ± 12	-4 ± 13	244 ± 24	19 ± 12	47 ± 7	-2 ± 8	86 ± 9

Table 4. Fraction of CH₄ oxidized (%) in the rhizosphere (f_{ox}^a) and at the soil-water interface (f_{ox}^b) in field conditions, and at the surfaces of soil (f_{ox}^c) and rice roots (f_{ox}^d) in lab conditions. f_{ox}^a was calculated with Equation (5) using $\delta^{13}C$ -values of CH₄ anaerobically produced in the soil (Table 1) as $\delta^{13}CH_4$ (original) and $\delta^{13}CH_4$ (emission) (Fig. 3a) minus -14.2‰ as $\delta^{13}CH_4$ (oxidized); f_{ox}^b was calculated with Equation (5) using $\delta^{13}C$ -values of CH₄ in soil pore water (Fig. 2) as $\delta^{13}CH_4$ (original), and $\delta^{13}C$ -values of CH₄ in floodwater (Fig. 2) as $\delta^{13}CH_4$ (oxidized); f_{ox}^c and f_{ox}^d were calculated with Equation (5) using $\delta^{13}C$ -values of CH₄ anaerobically produced in the soil and on the roots (Table 1) as $\delta^{13}CH_4$ (original) and $\delta^{13}C$ -values of CH₄ aerobically produced in the soil and on the roots (Fig. 1) as $\delta^{13}CH_4$ (oxidized), respectively.

in various methanotrophs. Besides, main groups of methanotrophs in rice microcosm (*Methylococcaceae* and *Methylocystaceae*) are active, but their dominance may change depending upon substrate supply and nutrient status^{40,41}. Therefore, it is no wonder that our measurement of α_{ox} 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, $\epsilon_{transport}$ is equivalent to the difference between emitted and aerenchymatic $\delta^{13}CH_4$ ^{5,7}, and it was estimated to be -14.2‰ on average (Detailed descriptions please see below).

Rhizospheric CH₄ oxidation was the most important on D20 (Table 4). At that time, almost the produced CH₄ was oxidized before it was emitted into the atmosphere. With the rice growing, the f_{ox} decreased fast to ~30% in the end. Both CH₄ 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.*⁴² also found that f_{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_{ox} was no more than 50% over the season and it decreased rapidly from the beginning till the end of the season^{5,20}. They further concluded the possible reason was that activities of the methanotrophs were limited by nitrogen consumption with the rice growing under field conditions^{5,20,42}.

When porewater CH₄ released into the floodwater of the paddy fields, it was strongly oxidized at the soil-water interface since floodwater CH₄ was much more ¹³C-enriched than porewater CH₄ (Fig. 2). So, f_{ox} in this oxidizing area, in principle, can be estimated using porewater $\delta^{13}CH_4$ as $\delta^{13}CH_4$ (original) and floodwater $\delta^{13}CH_4$ as $\delta^{13}CH_4$ (oxidized). Value of f_{ox} was found to be over 80% throughout the whole season, which was generally higher than that of f_{ox} in the rhizosphere (Table 4). Although f_{ox} at the soil-water interface appeared to be much high, the amount of the CH₄ oxidized must be significantly lower than that in the rhizosphere. Because produced CH₄ is mostly oxidized in the rhizosphere during the rice-growing season as over 90% of the CH₄ emits to the atmosphere through the aerenchyma of the plants while less than 0.1% releases via ebullition and diffusion^{24,43,44}. In addition, it was reported that the absolute CH₄ oxidation rate at the soil-water interface was much lower than that in the rhizosphere^{24,45,46}.

Compared to methanogenesis in anaerobic soil, that was in aerobic soil significantly lower in CH₄ production rate but more positive in $\delta^{13}C$ (Fig. 4). The findings demonstrate that intensive CH₄ oxidization happened at the soil surface in lab conditions. As a result, f_{ox} at the soil surface (Table 4) was estimated using anaerobically produced $\delta^{13}CH_4$ as $\delta^{13}CH_4$ (original) and aerobically produced $\delta^{13}CH_4$ as $\delta^{13}CH_4$ (oxidized)¹⁹. It was the highest (~80%) at the beginning of the season and decreased rapidly later (<0%). In field conditions, CH₄ oxidation in paddy field without rice plants occurs mainly at the soil-water interface, which is similar to CH₄ oxidation under aerobic incubation in lab conditions. Therefore, it is feasible to quantitatively estimate f_{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 $\delta^{13}CH_4$ between anaerobic and aerobic incubations. What is more, f_{ox} at the root surface was also estimated by comparing $\delta^{13}C$ -value of the CH₄ produced under aerobic conditions with those under anaerobic conditions (Table 4). It was found that f_{ox} at the root surface stayed over 100% throughout the whole season. Even if the $\alpha_{ox} = 1.038$ was used, it was still as high as 100% (Table 4), further suggesting that CH₄ oxidation on rice roots was extremely strong indeed. CH₄ oxidation rate much higher on the roots (Table 2) was supposed to be the main reason for the f_{ox} was higher than that in the soil.

CH₄ transport and emission. Transporting CH₄ is the last step of CH₄ emission from paddy field to the atmosphere. Although CH₄ oxidation leads to the produced CH₄ obviously enriched in ¹³C, isotope fractionation in CH₄ transport offsets the positive effect on $\delta^{13}CH_4$, causing the CH₄ ¹³C-depleted again^{13,22}. As a result, the $\delta^{13}C$ -values of emitted CH₄ were close to the produced $\delta^{13}CH_4$ (Fig. 4). The isotope fractionation changes with the efficiency of CH₄ transport in growth of the plants^{5,9,23}. In the middle of the season, CH₄ 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 $\epsilon_{transport}$ 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 CH₄ transport capacity. Therefore, the $\epsilon_{transport}$ 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‰^{5-7,9,13,19}.

Similar to CH₄ emission from paddy fields, $\delta^{13}\text{CH}_4$ (emission) is significantly affected by all each process of CH₄ production, oxidation and transport. At the beginning of the season, the high $\delta^{13}\text{CH}_4$ (emission) was most likely ascribed to the relatively low transport fractionation and the highest f_{ox} . Subsequently, it became lowest, which was supposed to be the biggest transport fractionation and significantly decreased f_{ox} . At the late period of the season, the emitted CH₄ was ¹³C-enriched again, mainly due to an obvious increase of f_{ac} at this moment. The $\delta^{13}\text{CH}_4$ (emission) was negatively correlated with CH₄ emission (Fig. 3b), which further indicates that the higher the CH₄ flux, the lower the f_{ox} , thus causing the lower the emitted $\delta^{13}\text{CH}_4$. Similar relationships were also observed in the previous studies^{9,21,47}.

In conclusion, this study well showed each process of CH₄ emission by the measurements of $\delta^{13}\text{CH}_4$ 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), α_{app} was lower (1.041–1.056) whereas α_{ox} 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 ($\epsilon_{\text{transport}}$) was found to be $-16.7\text{‰} \sim -11.1\text{‰}$. Temporal variation of CH₄ emission negatively correlated with $\delta^{13}\text{CH}_4$ (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 $\delta^{13}\text{CH}_4$ and finally oxidized $\delta^{13}\text{CH}_4$, 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 $\delta^{13}\text{C}$ -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 kept flooded from June 24 to October 15 and drained on October 16 before rice harvest. Seeds of the rice ("*Oryza sativa* L. Huajing 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. $\text{Ca}(\text{H}_2\text{PO}_4)_2$ (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 using the static chamber technique. The flux chambers ($0.5 \times 0.5 \times 1 \text{ m}$), 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 ($\delta^{13}\text{CH}_4$ (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 pump⁴⁷. 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)⁴⁷. The samplers were installed (in triplicate) prior to rice transplanting 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 pots¹⁹. 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 ($0.3 \times 0.3 \times 1 \text{ m}$) 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 plots⁴⁸. 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 × 10⁵ Pa for further separation. The separated gases were finally transferred into the mass spectrometer for δ¹³C determination. The reference and carrier gases used were CO₂ (99.999% in purity and −23.73‰ in δ¹³C_{PDB}-value) and He (99.999% in purity, 20 mL min⁻¹), respectively. The precision of the repeated analysis was ±0.2‰ when 2.02 μL L⁻¹ CH₄ was injected. The dried soil and plant samples were analyzed for carbon isotope composition with a Finnigan MAT-251 Isotope Ratio Mass Spectrometer (Thermo Finnigan, Bremen, Germany).

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 $P < 0.05$.

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Acknowledgements

The authors are grateful to our anonymous reviewers for their helpful comments. Many thanks are also given to Prof. Jia ZJ for his valuable suggestions. This research work was financially supported by the “Strategic Priority Research Program” of the Chinese Academy of Sciences (XDB15020103), the National Key Technology Research and Development Program (2013BAD11B02), the National Natural Sciences Foundation of China (41571232, 41271259, 41201243), and Foundation of the State Key Laboratory of Soil and Sustainable Agriculture (Y412010003).

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

Competing financial interests: The authors declare no competing financial interests.

How to cite this article: Zhang, G. *et al.* Carbon isotope fractionation reveals distinct process of CH₄ emission from different compartments of paddy ecosystem. *Sci. Rep.* **6**, 27065; doi: 10.1038/srep27065 (2016).



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