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Methane Emission and Acetate-dependent Methanogenesis in Rice-based Cropping Systems with Urea Addition

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Abstract

Urea application is a fundamental practice to increase rice grain yields, but it also plays a significant role in CH_4 emission from rice paddy fields. To quantify the effect on CH_4 emission, we measured soil dissolved organic carbon (DOC) content, CH_4 production potentials of paddy soil and rice roots, CH_4 concentration in soil pore water, and their corresponding stable carbon isotopes ($\delta^{13}C$) under four urea application rates (0, 120, 240 and 300 kg N ha^{-1}) in 2010–2015 based on incubation, pot and field experiments. In addition, the abundance of methanogenic archaea in paddy soils was quantified by quantitative PCR targeting mcrA genes. Field and pot experiments show that urea addition decreased seasonal CH_4 emissions by 20–35% and enhanced $\delta^{13}C$-values of emitted CH_4 by 3‰ on average. The decreased CH_4 production potentials in paddy soil and on rice roots (by 16–30%) were found to be the major reason for the reduction of CH_4 emission with urea addition. In contrast, applying urea increased the content of soil DOC and the abundance of methanogenic mcrA genes. The contribution of acetoclastic methanogenesis was also increased by urea addition both in the soil (by 10–12%) and on the roots (by 5%), which might have positive effects on the $\delta^{13}C$-values of emitted CH_4 and porewater CH_4. The findings demonstrate that the decrease of CH_4 emission was attributed to CH_4 production reduced with urea addition, and the promotion of acetate-dependent methanogenesis was possibly ascribed to related methanogenic substrates and archaea increased.

Keywords: N addition; CH_4 production; Stable carbon isotopes; Methanogenic archaea; Methanogenic pathway; Rice-based cropping system

Introduction

Atmospheric methane (CH_4) is an important greenhouse gas, and the latest report shows that the concentration of atmospheric CH_4 reached a new high (1845 ppb) in 2015 (WMO, 2016). Paddy fields are considered to be a major source of anthropogenic CH_4, contributing approximately 5–6% (33–40 $Tgyr^{-1}$) to global CH_4 emissions (Ciais, 2013) [9]. CH_4 emission from paddy fields is a net product of CH_4 production and oxidation, and both of which are strongly impacted by synthetic nitrogen (N) fertilization (Conrad, 2007; Zhang et al., 2010) [11, 52]. As a consequence, CH_4 emission will be changed significantly by using N fertilizers whose application rates were increasing in the past 50 years (Peng et al., 2002; Wu et al., 2016) [35, 44]. Urea is the most common N fertilizer in rice cultivation in China. Accompanied with an obvious increase in crop productivity, the issues on responses of CH_4 production, oxidation and emission

from paddy fields to urea application are more and more focused on globally (Krüger and Frenzel, 2003; Banger et al., 2012; Pittelkow et al., 2014) [28, 1, 36].

Considerable measurements have been made in the effects of urea application on CH_4 emissions and the results are contradictory (Cai et al., 2007; Banger et al., 2012; Linquist et al., 2012) [5, 1, 30]. In rice cultivation, applying urea plays a critical role in promoting crop yields and possibly stimulates CH_4 production due to the increase of plant growth and carbon precursors for methanogenesis (Lu et al., 2000b; Schimel, 2000) [34, 37]. Pot and long-term field experiments have shown that urea increases CH_4 emissions (Banik et al., 1996; Shang et al., 2011) [2, 39], and seasonal CH_4 emission is significantly correlated with rice aboveground biomass and soil organic carbon (Shang et al., 2011) [39]. On the contrary, it was found that urea application could reduce CH_4 production and emission (Zhang et al., 2010) [52], and the inhibition in the abundance of methanogenic archaea was considered to be the possible reason (Hu et al., 2015; Fan et al., 2016) [25, 19]. As few reports are available about the effect on CH_4 production and methanogens during the rice season, CH_4 emission as affected by urea addition is not well understood.

The pathway of CH_4 production in paddy fields mainly contributes to two parts, acetate-dependent methanogenesis and CO_2/H_2-dependent methanogenesis (Conrad, 1999; Conrad et al., 2002) [10, 13]. No doubt that the methanogenic pathway is influenced by urea application owning to the methanogenic substrates and relevant methanogens changes. It is reported that there was an increasing trend in rhizospheric DOC with the addition of urea (Lu et al., 2000a) [33]. Nevertheless, the effect on methanogenic pathway and methanogenic community is poorly reported. In addition, urea application will significantly increase plant's biomass regardless of above- or belowground, and thereby methanogenic pathway on fresh rice roots is possibly differentiated by different substrates. Previous investigations have shown different pathways of methanogenesis between the soil and rice roots (Conrad et al., 2002) [13], and there are opposite responses to the straw application (Zhang et al., 2013a) [49] and water management (Zhang et al., 2013b) [51]. However, the response of methanogenic pathway on fresh rice roots to urea application is still unknown.

Therefore, field and pot observations were carried out from 2010 to 2015 to investigate the effect of urea application on CH_4 emission. In addition, CH_4 concentration in soil pore water, soil dissolved organic carbon (DOC) content, CH_4 production potentials both in the soil and on rice roots, and the abundance of *mcr*A genes of paddy soils were measured to clarify the mechanism of urea application controlling CH_4 emission. More importantly, corresponding $\delta^{13}C$-values of CH_4 and CO_2 produced in anaerobic incubations were analyzed to estimate the response of methanogenic pathway to urea application.

Materials and methods

Experimental design

The experimental field is located at Baitu Town, Jurong City, Jiangsu Province, China. The main characteristics of the soil from Jurong paddy field have been described clearly before (Zhang et al., 2011b) [56]. Two treatments, CK and N300 (urea addition at a rate of 0 and 300 Kg N ha^{-1}, respectively), were laid out with three replicates during the 2010 and 2011 rice seasons. In the 2014 and 2015 seasons, three treatments (CK, N120, and N240) were prepared with urea application at a rate of 0, 120, and 240 Kg N ha^{-1}, respectively. During the rice season, urea was fertilized three times, 50% as basal fertilizer, 25% as tillering fertilizer, and 25% as panicle fertilizer. Both $Ca(H_2PO_4)_2$ (450 kg ha^{-1}) and KCL (225 kg ha^{-1}) were applied as basal fertilizer together with urea. Rice seedlings (*Oryza sativaL.* Huajing 3) were transplanted into the field at their 3- to 4-leaf stage.

A pot experiment was simultaneously prepared in 2011 with paddy soils from Jurong and Ziyang paddy fields, respectively, and the experiment was carried out in the greenhouse of Institute of Soil Science, Chinese Academy of Sciences. The soil parent material from Ziyang rice field is purple shale and the soil properties are as follows: initial pH 8.2, total N 0.19%, total C 3.0%, and $\delta^{13}C$-value of soil organic carbon –21.7‰. For each paddy soil, two treatments (CK and N300: urea application rate was equivalent to 0 and 300 Kg N ha^{-1}, respectively) were also laid out with six replicates. Plastic pots (height 35 cm, diameter 25 cm) were filled with 16 kg dry soil. Information about fertilization and rice transplanting was the same as the field experiment. The pots with Jurong paddy soil were drain-

ing in the winter fallow season whereas with Ziyang paddy soil was continuously flooded. Water management during the rice-growing seasons was intermittent irrigation except for the field experiment in 2011 (Table 1).

Year	*Experiment*	*WM in winter season*	*WM in rice season*	*CK*	*Urea application*	*Reduction*
2010	In situ observation in Jurong paddy field	Drainage	Intermittent irrigation	2.79 ± 0.68	2.21 ± 0.40 (N300)	21%
2011	In situ observation in Jurong paddy field	Drainage	Continuous flooding	6.41 ± 1.34	4.88 ± 1.33 (N300)	24%
2011	Pot experiment with Jurong paddy soil	Drainage	Intermittent irrigation	3.89 ± 0.51	2.57 ± 0.76 (N300)	34%
2011	Pot experiment with Ziyang paddy soil	Continuous flooding	Intermittent irrigation	117.8 ± 12.6	85.2 ± 11.7 (N300)	28%
2014	In situ observation in Jurong paddy field	Drainage	Intermittent irrigation	3.00 ± 0.97	2.32 ± 0.89 (N120) 1.96 ± 0.45 (N240)	23% 35%
2015	In situ observation in Jurong paddy field	Drainage	Intermittent irrigation	2.77 ± 0.28	2.08 ± 0.56 (N120) 2.22 ± 0.23 (N240)	25% 20%

Note: WM indicates water management; CK, N120, N240 and N300 indicate urea application at a rate of 0, 120, 240 and 300 kg N ha^{-1}, respectively; Reduction = (CK – urea application) / CK × 100%.

Table 1: The effect of urea application on CH_4 emissions (g m^{-2}) from rice-based cropping systems.

Field sampling

The CH_4 flux was measured in the static chamber made of plexiglass with 0.5 × 0.5 × 1 m, covering six hills of rice plants in the field. In contrast to the pot experiment, the chamber was 0.3 × 0.3 × 1 m with two hills of rice plants per pot. To measure CH_4 flux, gas samples were usually collected once every 3–5 days. Four gas samples from each chamber were collected using 20 mL vacuum vials at 15 min intervals between 09:00 and 11:00 in the morning on each sampling day. To determine the isotopic signature of the emitted CH_4 ($\delta^{13}CH_{4(emission)}$), samples were taken at 15–30 day intervals. Two gas samples were collected using 0.5 L bags (aluminum foil compound membrane, Delin gas packing Co., Ltd, Dalian, China) with a small battery-driven pump, and $\delta^{13}CH_{4(emission)}$ was calculated based on the CH_4 concentration in the samples the beginning and the end, respectively, and the corresponding $\delta^{13}CH_4$-values of gas samples (Zhang et al., 2011a) [55].

Soil pore water samples (at a depth of 10 cm) in paddy field were collected during the 2011 rice season using a Rhizon soil moisture sampler (10 RHIZON SMSMOM, EijkelkampAgrisearch Equipment, Giesbeek, Netherlands) (Zhang et al., 2011a) [55]. Samples (about 5 mL) were firstly extracted using 20 mL vacuum vials to flush and purge the sampler before sampling. Then approximately 10 mL of soil solution was drawn into another vial for further analysis. Subsequently, all sampling vials were equilibrated by filling in pure N_2 gas. After vigorous shaking by hand, gas samples were taken from the headspace of the vials for analyses of CH_4 and CO_2 concentrations and corresponding $\delta^{13}C$-values.

Soil cores (0–15 cm) were collected using a stainless steel corer, and samples of the same plot were first mixed (Zhang et al., 2011b)

[56]. Soil samples from the mixture, about 50 g each (dry weight), were then taken and transferred promptly into the 250-mL Erlenmeyer flasks separately. Samples in the flasks were prepared into slurries with N_2-flushed de-ionized sterile water at a soil/water ratio of 1:1. A small portion of soil mixture was kept at 4°C for determining soil DOC according to the method of (Jones and Willett, 2006) [26] with 0.5 M K_2SO_4 as the extraction solvent. A subsample was prepared at –80°C for soil DNA extraction.

Simultaneously, rice plants together with roots were carefully collected from the plots (Zhang et al., 2011b) [56]. The roots were washed clean with N_2-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 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 N_2 at 4°C and transported back to the lab as soon as possible for further analysis.

Anaerobic incubation

CH_4 production potentials in the soil and on the roots were measured under anaerobic incubation (Zhang et al., 2011b) [56]. The flasks were flushed with N_2 consecutively six times through double-ended needles connecting a vacuum pump to purge the air in the flasks of residual CH_4 and O_2. They were incubated in darkness at a temperature the same as the measurement of the field for 50 h. The concentrations of CH_4 and CO_2 and corresponding $\delta^{13}C$-values in gas samples were analyzed 1 h and 50 h later after heavily shaking the flasks by hand. CH_4 production rates were calculated using the linear regression of CH_4 increasing with the incubation time.

Measurements and analyses

The CH_4 concentrations were analyzed with a gas chromatograph (Shimadzu GC-12A, Kyoto, Japan) equipped with a flame ionization detector (FID). For analyses of $\delta^{13}C$, the continuous flow technique and a Finnigan MAT 253 isotope ratio mass spectrometer were used (Thermo Finnigan, Bremen, Germany) (Cao et al., 2008; Zhang et al., 2011a) [7, 55]. CO_2 in gas samples was directly analyzed while CH_4 in gas samples was converted into CO_2 and separated primarily on a PreCon (pre-concentration device). Then, the gas was piped into a GC at 25°C under 2.0×10^5 Pa for further separation. The separated gases were finally transferred into the mass spectrometer for $\delta^{13}C$ determination. The reference and carrier gases used were CO_2 (99.999% in purity and –23.7‰ in $\delta^{13}C_{PDB}$-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} CH_4 was injected.

To determine the abundance of methanogenic *mcr*A gene copies, the genomic DNA was extracted from 0.5 g soil using a FastDNA spin kit for soil (MP Biomedicals LLC, Ohio, USA) according to the manufacturer's instructions. The extracted soil DNA was dissolved in 50 μL of elution buffer, checked by electrophoresis on 1% agarose, and then quantified using a spectrophotometer (NanoDrop Technologies, Wilmington, DE, USA)(Fan et al., 2016) [19]. Fragments of the *mcr*A genes, encoding the methyl coenzyme-M reductase, were amplified using primers according to (Hales et al., 1996) [23]. Real-time quantitative PCR was performed on a CFX96 Optical Real-Time Detection System (Bio-Rad Laboratories, Inc. Hercules, USA).

Calculations

The relative contribution of acetate to total CH_4 production (F_{ac}) was calculated by the mass balance, assuming that both acetate and CO_2/H_2 are the substrates (Tyler et al., 1997; Bilek et al., 1999) [42, 3]:

$$F_{ac} = CH_{4\,(acetate)} / (CH_{4(acetate)} + CH_{4(H2/CO2)}) \quad (1)$$

$$\delta^{13}CH_4 = F_{ac} \times \delta^{13}CH_{4(acetate)} + (1 - F_{ac}) \times \delta^{13}CH_{4(H2/CO2)} \quad (2)$$

Where $\delta^{13}CH_{4(acetate)}$ and $\delta^{13}CH_{4(H2/CO2)}$ are $\delta^{13}C$ of CH_4 produced from acetate and CO_2/H_2, respectively, and $\delta^{13}CH_4$ is $\delta^{13}C$ of CH_4 originally formed.

Isotope fractionation factor for CO_2/H_2 producing CH_4 is defined by (Hayes, 1993) [24]:

$$\alpha_{CO2/CH4} = (\delta^{13}CO_2 + 1000) / (\delta^{13}CH_{4\,(H2/CO2)} + 1000) \quad (3)$$

Statistical analysis

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

Results

CH_4 emission and corresponding $\delta^{13}CH_4$

The CH_4 emissions from paddy fields during the 2010 and 2011 rice-growing seasons were reported by (Zhang et al., 2017) [53], which showed that applying 300 kg N ha^{-1} generally decreased CH_4 emission, with a reduction of 21–24% relative to CK (Table 1). For the pot experiment, there was a flux peak (about 4–6 mg m^{-2} h^{-1}) after the mid-aeration in Jurong paddy soil, and it was relatively lower with urea addition than CK over the whole observational period (Fig. 1a). A very large CH_4 flux peak was observed (over 200–350 mg m^{-2} h^{-1}) as soon as the rice was transplanted in Ziyang paddy soil. Subsequently, it decreased step by step till the end of the season (Fig. 1b). Although CH_4 emissions from Jurong paddy soil were significantly lower than those of Ziyang, they were cut down by 28–34% with urea addition (Table 1). During the 2014 and 2015 rice seasons, a similar variation pattern in CH_4 emissions from paddy fields was observed, which increased to highest before mid-aeration and then sharply decreased after drainage (Fig. 1c and d). Compared to CK, applying urea at a rate of 120 and 240 kg N ha^{-1} decreased CH_4 emissions by 23–25% and 20–35%, respectively (Table 1). The $\delta^{13}CH_{4(emission)}$ during the 2010 and 2011 rice seasons ranged from –59.5‰ to –50.8‰ on average, and CH_4 emission was generally enriched in ^{13}C by urea application, with $\delta^{13}CH_{4\,(emission)}$ higher by approximately 3‰ relative to CK (Zhang et al., 2017) [53].

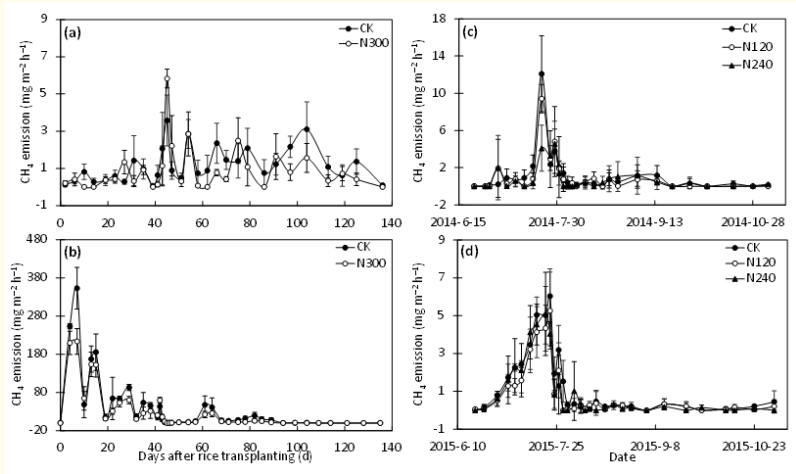

Figure 1: Seasonal variation of CH_4 emissions from greenhouse pots in 2011 (a: Jurong paddy soil and b: Ziyang paddy soil) and paddy fields in 2014 (c) and 2015 (d) rice seasons. CK, N120, N240, and N300 indicate urea application at a rate of 0, 120, 240 and 300 kg N ha^{-1}, respectively.

Soil dissolved organic carbon (DOC)

Soil DOC content in Jurong paddy soil was highest (~700–800 mg kg^{-1}) at the beginning of the season and then decreased sharply to approximately 300 mg kg^{-1} at the end (Fig. 2a). In general, it was greater for N300 than CK, particularly on D36 (36 days after rice transplanting) and D55 ($P < 0.05$). For Ziyang paddy soil, a similar variation pattern was observed in both treatments, that is, it was greatest on D26 (~1300–1400 mg kg^{-1}) and decreased to the lowest on D56 (~250 mg kg^{-1}), and then it increased again towards the end of the season. Compared to CK, soil DOC content was relatively higher in N300 throughout the whole season (Fig. 2b).

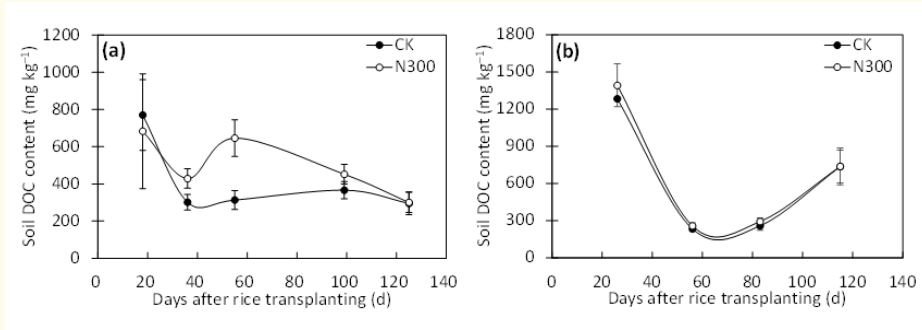

Figure 2: Temporal variation of soil DOC content from greenhouse pots with Jurong (a) and Ziyang (b) paddy soils during the 2011 rice season. CK and N300 indicate urea application at a rate of 0 and 300 kg N ha^{-1}, respectively.

CH_4 production and corresponding $\delta^{13}CH_4$

The CH_4 production potential in soil slurry increased up to the highest on D41, with values of 0.45 and 0.25 μg g^{-1} d^{-1} for Treatment CK and N300, respectively. It then decreased gradually to almost 0 μg g^{-1} d^{-1} at the end of the season (Fig. 3a). In general, urea addition decreased CH_4 production relative to CK with the production potential lower by 30% on average. The $\delta^{13}C$-values of produced CH_4 firstly decreased from –56‰ to –70‰, and then increased to around –60‰ ~ –55‰ in the end (Fig. 3b). In contrast to the CH_4 production, the produced CH_4 was relatively enriched in ^{13}C for N300 than CK (–61‰), with $\delta^{13}C$-values higher by approximately 4‰ on average. The produced CO_2 was generally depleted in ^{13}C in the whole rice season, with $\delta^{13}C$-values decreasing from –13‰ to –15‰ (Fig. 3c).

As high as 3.60–5.56 μg g^{-1} d^{-1} of CH_4 production on rice roots was observed in the beginning, and it reached the highest peak (about 9–12 μg g^{-1} d^{-1}) on D41 (Fig. 3d). After a second producing peak on D73, the CH_4 production potential decreased to as low as 1 μg g^{-1} d^{-1} during the end of the season. Compared to CK, CH_4 production potential for urea addition was lower by 16% on average. The $\delta^{13}C$-values of CH_4 produced on rice roots decreased from around –65‰ to the lowest (–85‰) on D41, and then increased to around –65 ~ –60‰ finally (Fig. 3e). On average, the $\delta^{13}C$-values of produced CH_4 were higher by 3‰ for urea addition than CK (–69‰). The $\delta^{13}C$-values of produced CO_2 were firstly decreased from –13‰ to –18‰, and after an increase to –13‰, the $\delta^{13}C$-values decreased again to around –20‰ in the end (Fig. 3f).

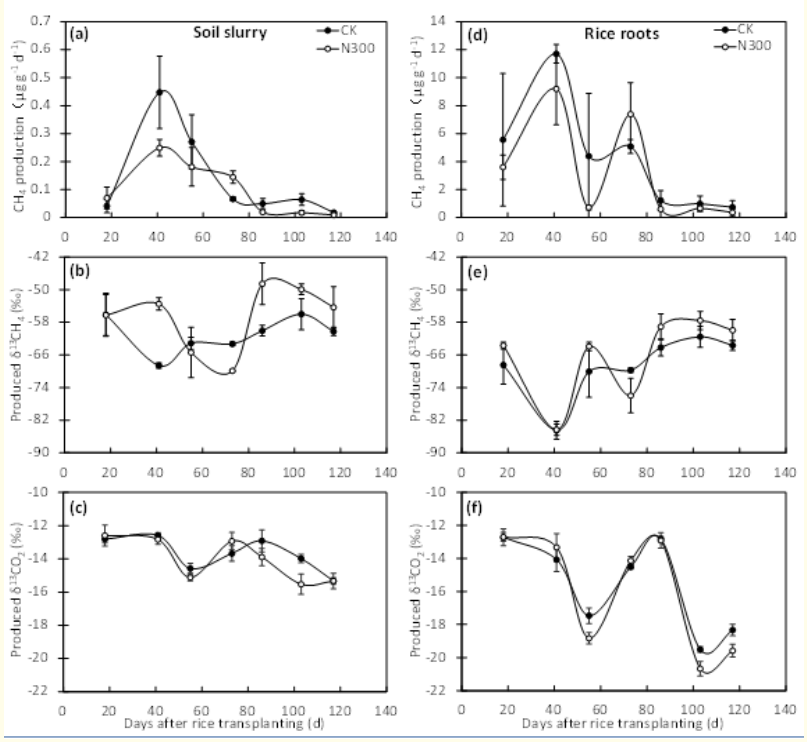

Figure 3: Temporal variation of CH_4 production potential and corresponding $\delta^{13}CH_4$ and $\delta^{13}CO_2$ in anaerobic incubation of soil slurry (a, b and c) and rice roots (d, e, and f) from paddy field during the 2011 rice season. CK and N300 indicate urea application at a rate of 0 and 300 kg N ha^{-1}, respectively.

CH_4 concentration and corresponding $\delta^{13}CH_4$

The concentration of CH_4 in soil pore water kept a relatively low level (< 10 μ mol L^{-1}) during most part of the rice season (Fig. 4a). It increased to around 30 μ mol L^{-1} on D82, in particular for CK the concentration was up to 77 μ mol L^{-1} in the end. Compared to CK, urea addition decreased the concentration at the end of the season (on D87–108). The $\delta^{13}C$-values of porewater CH_4 for N300 fluctuated greatly, decreasing from –46‰ to –62‰ firstly, and after a significant increase to –46‰, the $\delta^{13}C$-values decreased to –59‰ in the end (Fig. 4b). For CK however, the $\delta^{13}C$-values firstly increased from –53‰ to –45‰, and finally decreased to –65‰. On average, urea addition increased the $\delta^{13}C$-values by 2‰ relative to CK (–54‰), particularly on D82–108 by 6‰. The CO_2 concentration ranged from approximately 1000 μ mol L^{-1} to 4000 μ mol L^{-1} (Fig. 4c), and the $\delta^{13}C$-values of CO_2 were between around –16‰ to –9‰ (Fig. 4d). No significant difference was observed between the two Treatments in CO_2 concentration and $\delta^{13}C$-values.

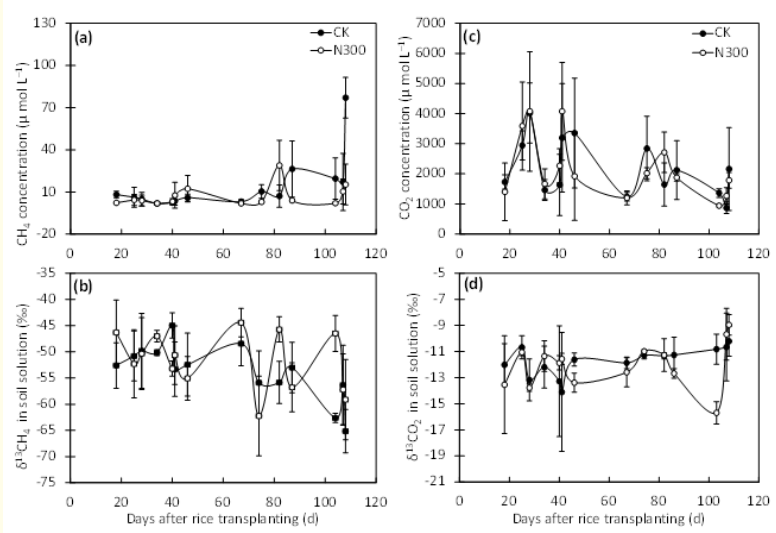

Figure 4: Seasonal variation of CH_4 (a) and CO_2 (c) concentrations and corresponding $\delta^{13}CH_4$ (b) and $\delta^{13}CO_2$ (d) in soil pore water from paddy fields during the 2011 rice season. CK and N300 indicate urea application at a rate of 0 and 300 kg N ha^{-1}, respectively.

Reactive contribution of acetate to total CH_4 production (F_{ac})

For calculating F_{ac}, we assumed $\delta^{13}CH_{4(acetate)}$ to be –43‰ ~ –37‰ and $\alpha_{CO2/CH4}$ to be 1.060–1.070 for soil slurry and 1.070–1.080 for rice roots, respectively, owing to the lack of measurements (The detailed reasons for this please refer to ***Discussion*** section). As F_{ac}-values were negative sometimes when using $\alpha_{CO2/CH4}$ = 1.060 and 1.070 for soil and roots, respectively, the estimations of F_{ac} appeared to be more reasonable and reliable if $\alpha_{CO2/CH4}$ was equivalent to 1.070 in the soil and 1.080 on the roots (Tables 2 and 3).

The value of F_{ac} in the soil was 50–60% at the beginning of the season, and then it decreased to the lowest to 20–25% on D41 for CK and about 20% on D73 for N300 (Table 2). Subsequently, it increased again to as high as 55–60% for CK and 70–80% for N300 towards the end of the rice season. As a whole, seasonal mean F_{ac}-value was 42–49% in CK and 52–61% in N300, and therefore it was higher by 10–12% in N300 than CK on average (Table 2).

Days after rice transplanting (d)	***$\delta^{13}CH_{4\ (acetate)}$ = –37‰***				***$\delta^{13}CH_{4\ (acetate)}$ = –43‰***			
	$\alpha_{CO2/CH4}$ = 1.060		***$\alpha_{CO2/CH4}$ = 1.070***		***$\alpha_{CO2/CH4}$ = 1.060***		***$\alpha_{CO2/CH4}$ = 1.070***	
	CK	***N300***	***CK***	***N300***	***CK***	***N300***	***CK***	***N300***
18	40 ± 9	39 ± 8	53 ± 13	52 ± 13	49 ± 10	48 ± 10	62 ± 16	61 ± 15
41	0 ± 3	48 ± 5	21 ± 2	59 ± 4	0 ± 3	59 ± 6	25 ± 2	70 ± 5
55	22 ± 4	16 ± 9	38 ± 3	33 ± 7	26 ± 5	20 ± 11	44 ± 4	39 ± 8
73	19 ± 2	–3 ± 2	36 ± 2	19 ± 1	23 ± 3	–4 ± 2	42 ± 2	22 ± 1
86	27 ± 6	65 ± 8	43 ± 4	72 ± 13	34 ± 7	79 ± 10	51 ± 5	84 ± 15
103	42 ± 1	62 ± 4	54 ± 1	70 ± 3	52 ± 2	75 ± 5	63 ± 1	81 ± 4
117	32 ± 3	49 ± 7	46 ± 3	59 ± 12	39 ± 4	60 ± 9	53 ± 3	69 ± 14

Note: CK and N300 indicate urea application at a rate of 0 and 300 kg N ha^{-1}, respectively.

Table 2: Reactive contribution of acetate to total methanogenesis (F_{ac}, %) in fresh soil slurry during the 2011 rice season using Equations (1)-(3).

The F_{ac}-value on the roots was about 40–50% on D18 and then decreased sharply to the lowest to 5% on D41 both for CK and N300 (Table 3). After that, it tended to increase up to 50–60% for CK at the end of the rice season. For N300 however, after a significant increase (50–60% on D55), it decreased again to 20–25% on D73. In the end, the value of F_{ac} increased to approximately 60–65%. Compared with CK (38–43%), the seasonal mean F_{ac}-value in N300 was greater by 5% (Table 3).

Days after rice transplanting (d)	$\delta^{13}CH_{4\ (acetate)}$ = −37‰				$\delta^{13}CH_{4\ (acetate)}$ = −43‰			
	$\alpha_{CO2/CH4}$ = 1.070		$\alpha_{CO2/CH4}$ = 1.080		$\alpha_{CO2/CH4}$ = 1.070		$\alpha_{CO2/CH4}$ = 1.080	
	CK	N300	CK	N300	CK	N300	CK	N300
18	22 ± 8	34 ± 2	36 ± 7	45 ± 1	26 ± 10	40 ± 5	40 ± 8	52 ± 2
41	−14 ± 6	−16 ± 2	5 ± 5	4 ± 2	−17 ± 7	−19 ± 2	6 ± 6	5 ± 2
55	26 ± 15	41 ± 2	38 ± 12	51 ± 2	30 ± 17	48 ± 2	43 ± 14	57 ± 2
73	22 ± 1	6 ± 10	35 ± 1	22 ± 9	26 ± 1	7 ± 12	40 ± 1	25 ± 10
86	33 ± 5	45 ± 8	44 ± 4	55 ± 7	38 ± 6	53 ± 9	51 ± 5	63 ± 8
103	47 ± 4	57 ± 5	55 ± 3	63 ± 4	54 ± 4	65 ± 6	62 ± 4	71 ± 5
117	41 ± 3	51 ± 5	51 ± 2	58 ± 5	48 ± 3	58 ± 6	57 ± 3	66 ± 5

Note: CK and N300 indicate urea application at a rate of 0 and 300 kg N ha^{-1}, respectively.

Table 3: Reactive contribution of acetate to total methanogenesis (F_{ac}, %) on fresh rice roots during the 2011 rice season using Equations (1)-(3).

Abundance of the methanogen populations

The *mcr*A copy numbers were determined on D18, D36, and D55, respectively. The abundance was decreased gradually during the pot experiment (Fig. 5), and it was generally greater for N300 (2.30–3.87×10^8 g^{-1} soil) than that of CK (1.34–3.27x10^8 g^{-1} soil), in particular on D55 there was a significant difference ($P < 0.001$).

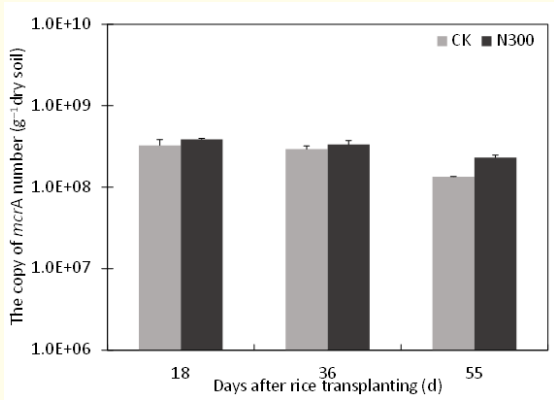

Figure 5: The abundance of *mcr*A genes in Jurong paddy soil from greenhouse pots during the 2011 rice season. CK and N300 indicate urea application at a rate of 0 and 300 kg N ha^{-1}, respectively.

Discussion

Although considerable studies have reported the effect of urea application on CH_4 emission from rice fields (Table 4), the results are controversial and related mechanisms are still poorly known. In particular, the systemic investigation is few available on the response of CH_4 emission closely associated with CH_4 production and methanogenic archaea to urea application (Table 4). Our field, pot and incubation experiments demonstrated that urea addition reduced CH_4 production and then its emission regardless of different water managements and paddy soils. In addition, both the relative contributions of acetate to CH_4 production (F_{ac}) in the soil and on the roots were increased with the addition of urea, which might have positive effects on the $\delta^{13}C$-values of emitted CH_4 and porewater CH_4. Importantly, applying urea promoted the content of soil DOC and the abundance of methanogenic archaea, indicating abundant methanogenic precursors were selectively consumed by more methanogenic archaea, thus promoting acetate fermentation to CH_4 production.

Site	*Urea addition*	*Methanogens*	*CH_4 production*	*CH_4 emission*	*Reference*
China	300 kg N ha^{-1}	+	–	–	This study
China	300 kg N ha^{-1}	–	N.m.	–	(Fan et al., 2016) [19]
China	400 mg N kg^{-1} soil	–	–	N.m.	(Hu et al., 2015) [25]
China	180 kg N ha^{-1}	+	N.m.	N.m.	(Xu, 2012) [46]
China	183 kg N ha^{-1}	N.m.	N.m.	+	(Shang et al., 2011) [39]
China	150 and 250 kg N ha^{-1}	N.m.	N.m.	–	(Dong et al., 2011) [18]
China	150 and 250 kg N ha^{-1}	N.m.	N.m.	–	(Xie et al., 2010) [45]
China	300 kg N ha^{-1}	N.m.	–	–	(Zhang et al., 2010) [52]
China	427.5 kg N ha^{-1}	N.m.	+	N.m.	(Zheng et al., 2007) [57]
China	From 150 to 250 kg N ha^{-1}	N.m.	N.m.	±	(Zheng et al., 2006) [58]
China	From 150 to 250 kg N ha^{-1}	N.m.	N.m.	–	(Xu et al., 2004) [47]
Italy	40 kg N ha^{-1}	N.m.	+	–	(Krüger and Frenzel, 2003) [28]
Italy	50 kg N ha^{-1}	N.m.	+	–	(Dan et al., 2001) [17]
Italy	200 and 400 kg N ha^{-1}	N.m.	±	–	(Bodelier et al., 2000) [4]
China	100~2000 mg N kg^{-1} soil	N.m.	–	N.m.	(Yang and Chang, 1998) [48]
China	100 and 300 kg N ha^{-1}	N.m.	N.m.	–	(Cai et al., 1997) [6]
Bengal	81.3~406.5 kg N ha^{-1}	N.m.	N.m.	+	(Banik et al., 1996) [2]

Note: "+" means increase the abundance of methanogens, promote CH_4 production and emission (positive effect); "–" means decrease the abundance of methanogens, inhibit CH_4 production and emission (negative effect); "±" means uncertain or variable response. N.m. indicates no measurement.

Table 4 Comparisons of the effect of urea addition on methanogens, CH_4 production and CH_4 emission from rice-based cropping systems among different studies.

Urea application decreased seasonal CH_4 emissions no matter what were the water managements and paddy soils (Table 1). When the water management was intermittent irrigation during the rice season, total mean CH_4 emissions from Jurong paddy soils and fields were comparable to about 2.0–4.0 g m^{-2}, which was significantly lower (7.5–27.6 g m^{-2}) than that of a similar rice cropping system in Wuxi, China (Xie et al., 2010) [45]. If the field was continuously flooded, seasonal CH_4 emissions were significantly increased to about 5.0–6.5 gm^{-2} on average. Nevertheless, urea addition always decreased CH_4 emissions by about 20–35% (Table 1). Based on the pot experiment with different water managements in the winter season, the measurements also showed an obvious decrease in CH_4 emission with urea addition, although it was significantly greater in Ziyang soil than that of Jurong soil (Table 1). These findings suggest that the negative effect of urea application on CH_4 emission was hardly influenced by water management, and they also indicate that soil properties might play a slight role in the decrease of CH_4 emission by applying urea. Across various climate zones with different soil properties in China, a four-site field experiment found that the application of ammonium-based fertilizers generally inhibited seasonal CH_4 emission, on average, by 28–30% (Xie et al., 2010) [45].

The decrease in CH_4 production should be the key reason for the reduction of CH_4 emission with urea addition in this study. Compared with CK, N300 reduced CH_4 production potentials both in the soil and on the roots by 30% and 16% on average (Fig. 3a and d), and compared to N120, N240 further decreased CH_4 production (***Supplementary material*** Figure S1). These results indicate that urea addition had a negative effect on CH_4 production, and the inhibition was strengthened by the increasing rate of urea addition from 0 to 240 kg N ha^{-1}. In addition, soil samples from greenhouse pots showed that urea addition always decreased CH_4 production, although much higher CH_4 production was observed in Ziyang soil than that of Jurong soil (***Supplementary material*** Figure S2). It suggests that both changes in field water management and soil property significantly varied CH_4 production but these variations scarcely affected the methanogenesis response to urea addition. The early investigation also found that urea application decreased CH_4 emission from paddy fields mainly attributed to the reduction of CH_4 production (Zhang et al., 2010) [52]. However, some reports indicate that urea addition stimulated methanotrophic bacteria and their activity and thereby resulted in a reduction of CH_4 emission (Dan et al., 2001; Krüger and Frenzel, 2003) [17, 28]. Unfortunately, CH_4 oxidation potential either in the soil or on the roots and the abundance of related methanotrophs were not measured in this study. More measurements are needed in future studies on CH_4 production and oxidation as affected by the combinations of urea addition, water management and soil property.

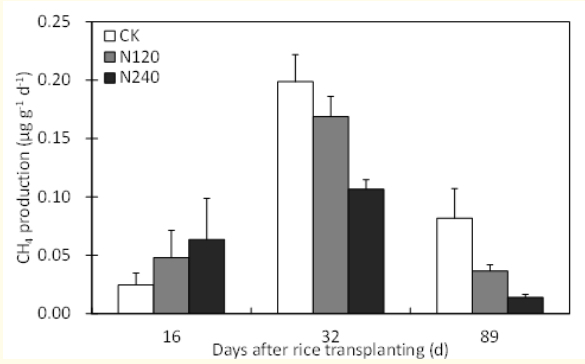

Figure S1: The CH_4 production potential in anaerobic incubation of soil slurry from Jurong paddy fields during the 2015 rice season. CK, N120 and N240 indicate urea application at a rate of 0, 120 and 240 kg N ha^{-1}, respectively.

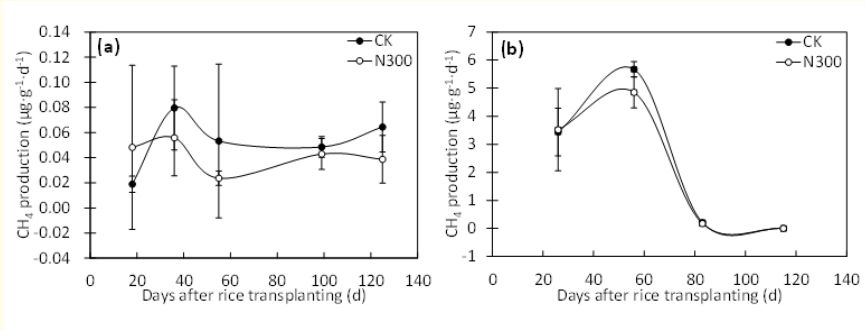

Figure S2: Temporal variation of CH_4 production potential in anaerobic incubation of soil slurry from the greenhouse pot experiment with Jurong (a) and Ziyang (b) paddy soils during the 2011 rice season. CK and N300 indicate urea application at a rate of 0 and 300 kg N ha^{-1}, respectively.

In addition to the decrease of CH_4 emission, the emitted CH_4 was more enriched in ^{13}C by applying urea relative to CK. An important reason for this phenomenon might be the increase in the relative contribution of acetate to total methanogenesis with the application of urea. CH_4 production in paddy fields is mainly from acetate fermentation and CO_2/H_2 reduction, and compared with CO_2-dependent methanogenesis, CH_4 from acetate-dependent methanogenesis is more ^{13}C-enriched (Sugimoto and Wada, 1993; Conrad et al., 2012b) [40, 15]. Based on the present study, urea application increased F_{ac}-values both in the soil and on the roots (Tables 2 and 3) regardless of different $\delta^{13}CH_{4\ (acetate)}$ and $\alpha_{CO2/H2}$. It should be noted that $\delta^{13}CH_{4\ (acetate)}$ was assumed to be –43‰ and –37‰ in this study mainly due to the $\delta^{13}C$-values of acetate were found to be –22‰ ~ –16‰ (Krüger et al., 2002) [27]with an isotopic shift of –21‰ for acetate-dependent methanogenesis (Gelwicks et al., 1994) [22]. It is a matter of fact that the $\delta^{13}CH_{4\ (acetate)}$-values of –43‰ and –37‰ have been used in many studies for estimating F_{ac} (Sugimoto and Wada, 1993; Tyler et al., 1997; Bilek et al., 1999; Krüger et al., 2002; Zhang et al., 2012) [40, 42, 3, 27, 50].

On the other hand, the ratio of $\delta^{13}CO_2$ and $\delta^{13}CH_4$ from anaerobic incubation (Fig. 3) shows an approximation of apparent fractionation (α_{app}) between CO_2 and CH_4, which can be calculated as $\alpha_{app} = (\delta^{13}CO_2 + 1000) / (\delta^{13}CH_4 + 1000)$ (Fey et al., 2004) [20]. Theoretically, α_{app} is lower than $\alpha_{CO2/H2}$ and some investigations have well demonstrated this in rice paddy soils (Fey et al., 2004) [20] and freshwater lake sediments (Conrad et al., 2011) [31]. In this study, the α_{app} in the soil was from 1.036 to 1.061 while it ranged from 1.039 to 1.078 on the roots (***Supplementary material*** Table S1). The α_{app} on the roots was relatively greater than that in the soil, indicating methanogenesis on the roots from CO_2/H_2 reduction with a stronger isotopic fractionation. Actually, early reports have found a larger value of $\alpha_{CO2/H2}$ on the roots than in the soil (Conrad et al., 2000, 2002; Conrad et al., 2012a) [12-14]. Due to a lack of measurement in this study and for a direct comparison with previous studies (Conrad et al., 2002; Krüger et al., 2002) [13, 27], we assumed $\alpha_{CO2/H2}$ to be 1.060 and 1.070 in the soil and 1.070 and 1.080 on the roots in the quantification of F_{ac}.

Days after rice transplanting (d)	$\delta^{13}CH_4$ in the soil		$\delta^{13}CH_4$ on the roots		$\delta^{13}CO_2$ in the soil		$\delta^{13}CO_2$ on the roots		α_{app} in the soil		α_{app} on the roots	
	CK	N300	CK	N300	CK	N300	CK	N300	CK	N300	CK	N300
18	−56.13	−56.23	−68.47	−63.70	−12.81	−12.60	−12.72	−12.70	1.046	1.046	1.060	1.054
41	−68.60	−53.47	−84.49	−84.34	−12.59	−12.83	−14.07	−13.33	1.060	1.043	1.077	1.078
55	−63.12	−65.40	−70.07	−63.93	−14.58	−15.12	−17.47	−18.83	1.052	1.054	1.057	1.048
73	−63.30	−69.88	−69.72	−75.97	−13.68	−12.94	−14.51	−14.13	1.053	1.061	1.059	1.067
86	−60.04	−48.55	−64.17	−59.07	−12.92	−13.89	−12.82	−12.91	1.050	1.036	1.055	1.049
103	−56.00	−49.92	−61.56	−57.57	−13.99	−15.53	−19.51	−20.67	1.045	1.036	1.045	1.039
117	−60.24	−54.34	−63.66	−59.94	−15.34	−15.33	−18.33	−19.58	1.048	1.041	1.048	1.043

Note: $\alpha_{app} = (\delta^{13}CO_2 + 1000)/(\delta^{13}CH_4 + 1000)$; CK and N300 indicates urea application at a rate of 0 and 300 kg N ha^{-1}, respectively.

Table S1: The δ^{13}C-values of CH_4 and CO_2 produced in anaerobic incubation of soil slurry (Fig. 3b and c) and rice roots (Fig. 3e and f) and corresponding α_{app} during the 2011 rice season.

However, more reasonable estimations of F_{ac} were obtained when assuming $\alpha_{CO2/H2}$ to be 1.070 in the soil and 1.080 on the roots (Tables 2 and 3). The CH_4 production in the soil was mainly from acetate-dependent methanogenesis at the beginning and in the late seasons (F_{ac}-value = 50–85%). Previous field reports from Italy and China found a different variation pattern that acetoclastic methanogenesis dominated just in the late of the season (Krüger et al., 2001; Krüger et al., 2002; Zhang et al., 2013a; Zhang et al., 2013b) [29, 27, 49, 51]. Compared to CK, urea addition increased the mean F_{ac}-value by 10–12% (Table 2), which should be the direct reason for the produced CH_4 enriched in ^{13}C (Fig. 3b). The increased F_{ac} was also considered to play a positive role in the δ^{13}C-value of emitted CH_4 that was higher by 3‰ for N300 than CK on average. Although the changes in $\delta^{13}CH_{4\ (emission)}$ were dependent on the integrated effects of CH_4 production, oxidation and transportation, our previous field measurements found that the contribution of acetoclastic methanogenesis was dominant to $\delta^{13}CH_{4\ (emission)}$ sometimes. For example, the emitted CH_4 was gradually enriched in ^{13}C in the late of the rice season, which was mainly ascribed to an obvious increase of F_{ac} at this moment (Zhang et al., 2013a; Zhang et al., 2016) [49, 54].

The promoted acetate-dependent methanogenesis by urea addition was possibly related to the increase of methanogenic precursor-acetate. However, soil organic acids, such as formic and acetic, etc., were not measured directly in this study. Soil DOC content was always higher in N300 than CK (Fig. 2), which to some extent indicated that the content of acetate in the soil was possibly increased with the addition of urea. Based on an incubation experiment, Shan et al. (2006) [38] found that there was an increasing transformation of acetate to CH_4 when the urea application rates increased from 0 to 0.4 g kg^{-1}. This demonstrates that applying urea can increase the contribution of acetate-dependent methanogenesis. In addition, the abundance of methanogenic archaea was raised by urea addition (Fig. 5), suggesting the growth of acetate-dependent methanogens might be increased much more. To our knowledge, no reports are available on the response of acetate-dependent methanogens to urea addition in paddy soils. Nevertheless, Fotidis et al. (2013) [21] found that aceticlastic*Methanosarcinaceae* spp. dominated in the thermophilic culture which was exposed to high ammonia concentrations. The result indicates that urea application might stimulate the growth of acetoclastic methanogens, thus in favor of acetate-dependent methanogenesis. A potential situation hence appeared to exist in this study that more methanogenic archaea selectively consumed plentiful methanogenic precursor, thus promoting acetate fermentation to CH_4 production.

In contrast to the soil, CO_2-dependent methanogenesis (1 – F_{ac}-value = 50–95%) was dominant on the rice roots in most of the season except acetate-dependent methanogenesis (F_{ac}-value = 60–70%) was more important in late of the rice season (Table 3). On average, the seasonal F_{ac}-value was around 40–45% which was in good agreement with previous reports (Krüger et al., 2002; Zhang et al., 2013a; Zhang et al., 2013b) [27, 49, 51]. It is found that the methanogenic microbial community on rice roots mainly was RC-I methanogens which dominated CH_4 production from CO_2/H_2 reduction (Lu and Conrad, 2005; Lu et al., 2005) [31, 32]. Compared with CK,

urea addition increased mean F_{ac}-value on the roots by 5%, which would be another potential reason for the emitted CH_4 ^{13}C-enriched. This indicates that applying urea was most likely to increase acetate content as a result of promoted roots biomass and exudates. Although root exudates were not measured in the present study, early pot experiments had well demonstrated that N fertilization stimulated root growth and increased its activity (Sun et al., 2003) [41], and the more the roots, the higher the root exudation rates and rhizospheric DOC concentrations (Lu et al., 2000b) [34].

Compared with CH_4 produced in anaerobic incubations, porewater CH_4 was significantly enriched in ^{13}C (Figs. 3 and 4, $P < 0.05$), indicating intensive CH_4 oxidization happening during the process of produced CH_4 release into the soil pore water (Whiticar, 1999; Krüger et al., 2002; Zhang et al., 2013b) [43, 51]. Porewater $\delta^{13}CH_4$ (averaged –54‰ ~ –52‰) was also significantly higher than $\delta^{13}CH_{4\ (emission)}$ (–60‰ ~ –57‰), mainly due to the negative effect of CH_4 transport fractionation on emitted CH_4. It is reported that the ^{13}C-enriched effect after CH_4 oxidation was able to be significantly offset by the transport of rice plants (Chanton, 2005; Zhang et al., 2013b) [8, 51]. Apparently, porewater $\delta^{13}CH_4$ was probably related to the CH_4 production, oxidation and transport. In comparison of CK, urea addition increased mean F_{ac}-values both in the soil (10–12%) and on the roots (5%), and meanwhile porewater CH_4 was relatively more ^{13}C-enriched over the season. This suggests that the increase of F_{ac}-values by urea addition possibly played a positive role in porewater $\delta^{13}CH_4$. Noted that mean F_{ac}-value was much higher in N300 than CK both in the soil (19–23%) and on the roots (9–10%) towards the end of the season, which might be the major reason for the porewater $\delta^{13}CH_4$ increase much more (by around 6‰) during this period (Fig. 4). Both the effect of CH_4 oxidation on porewater $\delta^{13}CH_4$ and the effect of CH_4 transport on $\delta^{13}CH_{4\ (emission)}$ were still poorly understood. More study in the future needs to be focused on the fraction of CH_4 oxidation and the isotopic fractionation in CH_4 transport as affected by urea application.

In conclusion, urea application reduced CH_4 emission as a result of the decrease in CH_4 production both in the soil and on the roots, although both soil DOC and abundance of methanogenic archaea were generally promoted with the addition of urea. Applying urea increased the relative contribution of acetate to CH_4 production both in the soil and on the roots, which was considered to have important effects on the ^{13}C-enriched in emitted CH_4 and porewater CH_4. It is speculated that abundant methanogenic precursors were selectively consumed by more methanogenic archaea, thus promoting acetate fermentation to CH_4 production. To better estimate the effect of urea addition on CH_4 emission from paddy fields, further attention should be paid to the methanogenic microbial community and CH_4 oxidation and corresponding methanotrophs, in particular to the isotopic fractionations in CH_4 oxidation and transport.

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