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ORIGINAL PAPER

Temporal variation of methanogenic pathways in rice fields under three different cropping systems

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Abstract

Taking fresh soil samples from a rice–wheat rotation field (RW), a permanently flooded rice field (PF), and a double-rice cropping field (DR: DRE for the early rice; DRL for the late rice), we measured the CH_4 production potential (MPP), the relative contribution of acetate-dependent methanogenesis (f_{ac}), soil properties, and methanogenic archaeal communities mainly in order to reveal the temporal variation and corresponding influencing factors of methanogenic pathways in paddy soils. Consistent with the change in dissolved organic C (DOC) content, the MPP generally decreased with rice growth in RW and DRL while increasing in PF and DRE. Based on the measurements of stable carbon isotopes, the estimated f_{ac}-value in PF dropped sharply from 54%-61% at the tillering stage to 30%-35% at the booting stage and rose again at the ripening stage. This variation pattern was positively correlated with that of acetate content, perhaps resulting from the activation of acetoclastic *Methanosarcina*. In contrast, the f_{ac}-value in RW and DR rose from 20%-44% at the tillering stage to 49%-59% at the ripening stage, possibly owing to the increase in the relative abundance of acetoclastic *Methanothrix*. The relative abundance of *Methanosarcina* in PF was 3%-4% higher than those in RW and DR, whereas that of *Methanothrix* was 3%-7% lower ($P < 0.05$). Soil acetate, DOC, and moisture contents significantly affected the methanogenic community composition. Our results demonstrate that the temporal variation of methanogenic pathways was influenced by the relative abundance of acetoclastic methanogens depending on the acetate level.

Keywords Rice cropping systems · Acetate-dependent methanogenesis · Methanogenic archaeal communities · Paddy soil · Stable carbon isotopes

Introduction

Methane (CH_4) is a short-lived and powerful greenhouse gas with a global warming potential 27–29.8 times that of carbon dioxide on a centennial scale (IPCC 2021). Human-induced global warming is estimated to be 1.07 °C in 2010–2019 compared to 1850–1900, of which about 0.5 °C is due to CH_4 emissions, the second largest contributor to global warming (IPCC 2021). In 2021, the global average atmospheric CH_4 concentration had reached 1.908 μL·L^{-1}, an increase of 162% compared with the pre-industrial level (WMO 2022). Therefore, deep CH_4 emissions reduction is required to rapidly slow the rate of global warming and achieve the target of a 1.5 °C warming limit (UNEP and CCAC 2021; IPCC 2022). As a result, more than 120 countries have joined the Global Methane Pledge announced at the COP26 climate summit, aiming to reduce anthropogenic CH_4 emissions by at least 30% by 2030 (UNEP 2022). Rice fields are an important anthropogenic source of atmospheric CH_4. The global rice fields emit about 30 Tg CH_4 per year, contributing 8% of the total anthropogenic emissions (Saunois et al. 2020), thus leaving a higher mitigation potential (Wang et al. 2023).

China is one of the major rice producers in the world, with the rice field area accounting for about 18% of the whole world (FAOSTAT 2023). In China, there are various

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rice cropping systems, consisting of different crop cultivation patterns and supporting management measures. In terms of crop cultivation, rice cropping systems typically include rice–wheat rotation fields (RW), rice-fallow rotation fields (RF), and double-rice cropping fields (DR). RW is a popular cropping system applied to increase the use intensity and productivity of farmland. It adopts the rotation system of summer rice-winter wheat, with an annual CH_4 emission of 33–662 kg·ha^{-1} (Gao et al. 2018). RF grows rice in summer and fallows in winter, among which permanently flooded rice fields (PF) are particularly special ones for year-round flooding. PF is mainly distributed in hilly and mountainous areas with poor irrigation conditions, resulting in water storage during the fallow season to prevent the next spring drought and finally a higher CH_4 emission than RW (Jiang et al. 2006; Zhang et al. 2017). DR grows two crops of rice a year and thereby is mainly distributed in hot and rainy areas to meet the hydrothermal demand for rice growth. However, sufficient water and heat are also favorable for CH_4 production and emission (Wang and Li 2002). It is estimated that the CH_4 emissions from DR contribute about 50% of the total CH_4 emissions from rice fields in China (Zhang et al. 2011; Chen et al. 2013). There is a big difference in CH_4 emission within the three rice-based ecosystems as a result of different growing environments, cultivation modes, and water and fertilizer management (Wang and Shangguan 1996; Cai et al. 2000; Qin et al. 2010; Hao et al. 2016; Wu et al. 2019; Qian et al. 2022).

In the rice fields, CH_4 production is achieved through two major pathways: acetate fermentation by acetoclastic methanogens (*Methanosarcina* and *Methanothrix*) and CO_2/H_2 reduction by hydrogenotrophic methanogens (e.g., *Methanobacteriales*, *Methanomicrobiales*, *Methanocellales*) (Conrad 2007, 2020a). The relative contribution of the two pathways in rice fields presents obvious temporal variations (Krüger et al. 2001, 2002) and is affected by water management (Zhang et al. 2012, 2013a), straw application (Conrad et al. 2012; Ji et al. 2018), rice cultivation (Tyler et al. 1997; Bilek et al. 1999), soil type (Yao and Conrad 2000; Nakagawa et al. 2002), and temperature (Fey et al. 2004; Liu et al. 2018) owing to the changes in the availability of methanogenic substrates and the composition of microbial communities (Ji et al. 2018; Liu et al. 2018). Therefore, the temporal variation of CH_4 production in Chinese rice fields, especially the methanogenic pathways, may vary in different cropping systems due to their regional differences in water and fertilizer management, rice cultivation, climate conditions (e.g., temperature, precipitation), etc. However, former reports only focused on the methanogenic pathways of RW in China (Zhang et al. 2012, 2013b) without considering the involved microorganisms.

Here, we hypothesized that different rice cropping systems differ in soil properties (e.g., moisture, methanogenic substrates, available N) and thus in the composition of methanogenic archaeal communities, leading to the different temporal variations of methanogenic pathways. Soil samples were collected at four critical rice growth stages and then incubated to measure the CH_4 production potential and the relative contribution of acetate-dependent methanogenesis in RW, PF, and DR. We also investigated the soil properties [dissolved organic C (DOC), acetate, moisture, NH_4^+-N, and NO_3^--N] and methanogenic archaeal communities to explore the possible relationship between them and CH_4 production. The main objective of this study was to reveal the temporal variation of methanogenic pathways in paddy soils and the corresponding influencing factors by analyzing the three different cropping systems.

Materials and methods

Field description

The tested rice–wheat rotation field (RW) is located in Xingxiang Village, Baitu Town, Jurong City, Jiangsu Province (31°58′N, 119°18′E) (Fig. S1a). The soil is a clay loam (36.9% sand, 23.3% clay, 39.8% silt), with an initial organic C of 12.8 g·kg^{-1}, total N of 1.25 g·kg^{-1}, pH of 5.59, and cation exchange capacity (CEC) of 12.2 cmol·kg^{-1}. The mean annual temperature in this area is 15.1 °C, and the mean annual precipitation is 1018.6 mm (Zhang et al. 2017). The cropping system is rice–wheat rotation, consisting of summer rice and winter wheat. The water management method during the rice season is intermittent irrigation, that is, continuous flooding in the early season, aeration in the middle season, dry–wet alternation in the late season, and final drainage before rice harvest.

The tested double-rice cropping field (DR) is located in Dengjiabu rice seed field, Yujiang District, Yingtan City, Jiangxi Province (28°15'N, 116°55'E) (Fig. S1b). The soil is a clay loam (50.5% sand, 18.2% clay, 31.3% silt), with an initial organic C of 19.8 g·kg^{-1}, total N of 1.80 g·kg^{-1}, pH of 4.89, and CEC of 7.48 cmol·kg^{-1}. The mean annual temperature and precipitation in this region are 17.6 °C and 1789 mm, respectively (Zhang et al. 2017). The cropping system consists of early rice (DRE) and late rice (DRL). The water management method in each rice season is intermittent irrigation, as mentioned above.

The permanently flooded rice field (PF) for testing is located in Xiangshui Village, Yanjiang Town, Yanjiang District, Ziyang City, Sichuan Province (30°05′N, 104°34′E) (Fig. S1c). The soil is a loamy clay (25.6% sand, 29.9% clay, 44.5% silt), with an initial organic C of 21.7 g·kg^{-1}, total N of 1.98 g·kg^{-1}, pH of 7.90, and CEC of 19.7 cmol·kg^{-1}. The mean annual temperature and precipitation in this region are 16.8 °C and 965.8 mm, respectively (Zhang et al. 2017). The cropping system is a rice-fallow rotation, consisting of

summer rice and winter fallow. The field is under year-round flooding.

Rice cultivation and field management in the three rice fields were carried out following local practices, as shown in Table S1.

Incubation experiment

In the 2019–2020 rice seasons, fresh topsoils (0–15 cm depth) were collected from RW, PF, and DR (DRE: early-rice season; DRL: late-rice season) at the tillering stage (TS) (RW: July 9; PF: May 30; DRE: May 12; DRL: August 7), booting stage (BS) (RW: August 14; PF: June 27; DRE: May 30; DRL: September 2), heading stage (HS) (RW: August 29; PF: August 5; DRE: June 16; DRL: September 19), and ripening stage (RS) (RW: October 3; PF: August 19; DRE: July 3; DRL: October 25). At each sampling time, about 20 soil samples were taken at random locations in the field and then mixed into a composite sample. Part of the composite soil was used for the determination of soil physicochemical properties and molecular analysis of methanogens, and the remaining soil was used for anaerobic incubation experiments.

For each anaerobic incubation, 20 g of fresh soil was first weighed into a 100 mL flask, and sterile anaerobic deionized water was added into the flask to make a slurry (water/soil mass ratio 1:1), and then the flask was sealed. All the flasks containing the slurry were evacuated and flushed with high-purity nitrogen. This process was repeated at least six times to remove the residual CH_4 and O_2 in the flasks. Subsequently, 2% CH_3F, an inhibitor of acetate-dependent methanogenesis (Conrad et al. 2012; Ji et al. 2018), was injected into some flasks to establish CH_3F treatment, and others without CH_3F injection were CK treatments. Five replications were conducted for each treatment. All flasks were sealed and incubated at 25 °C in the dark. The gas in the flasks was collected at 1 h and 50 h after incubation to measure the CH_4 and CO_2 concentrations, thus calculating the CH_4 production potential (Zhang et al. 2015). The stable C isotopic compositions of CH_4 and CO_2 ($\delta^{13}CH_4$ and $\delta^{13}CO_2$) were determined at the end of incubation (144 h) to calculate the relative contribution of acetate-dependent methanogenesis (f_{ac}). The flasks were resealed at the end of each gas extraction.

Chemical analyses

The concentrations of CH_4 and CO_2 were determined by gas chromatography (GC) (Agilent 7890B, USA) equipped with a hydrogen flame ionization detector (FID) and a nickel catalyst converter. CH_4 was directly detected in FID, while CO_2 was detected after converting into CH_4. The temperatures of the oven and the detectors were 60 °C and 300 °C, respectively. Nitrogen with a flow rate of 25 mL·min^{-1} was used as the carrier gas. Hydrogen with a flow rate of 45 mL·min^{-1} was used as the fuel gas and the reductant for the conversion of CO_2 to CH_4. And air with a flow rate of 400 mL·min^{-1} was used as the combustion-supporting gas. The C isotopes of CH_4 and CO_2 were analyzed by an isotope ratio mass spectrometer (Thermo Fisher Scientific., Germany) equipped with a fully automated pre-GC enrichment interface (PreCon) (Cao et al. 2008; Zhang et al. 2016). CO_2 was directly analyzed, while CH_4 was converted into CO_2 in the combustion reactor of PreCon. After separating from other components in the GC column, the CO_2 was transported into the mass spectrometer for measurement of $\delta^{13}C$. High-purity CO_2 with a $\delta^{13}C_{PDB}$-value of -23.7‰ was used as the reference gas. High-purity helium with a flow rate of 20 mL·min^{-1} was used as the carrier gas.

The DOC was extracted with 0.5 mol·L^{-1} K_2SO_4 solution at a water/soil ratio of 4:1. The extract was filtered through a 0.45 μm water-system syringe filter. After appropriate dilution of the extract, the soil DOC content was measured by a total organic C/total N analyzer (Analytik Jena, Germany). The soil acetate was extracted with ultra-pure water at a water/soil ratio of 4:1. The extract was filtered through a 0.22 μm organic-system syringe filter and then analyzed by high-performance liquid chromatography (Agilent 1260, USA) equipped with a variable wavelength UV detector (VWD) to determine the soil acetate content. Specifically, the mixture of phosphate buffer (0.02 mol·L^{-1}; pH 2.45) and methanol, mixed by a quaternary pump, was used as the mobile phase at a flow rate of 0.8 mL·min^{-1}. The temperature of the column oven was 20 °C, and 214 nm was set as the detection wavelength. The soil moisture content (water/dry soil mass ratio) was measured by drying the fresh soil at 105 °C until its weight remained constant. The soil NH_4^+-N、NO_3^--N was extracted with 2 mol·L^{-1} KCl solution at a water/soil ratio of 5:1. The extract was filtered and then analyzed by a flow analyzer (Skalar, Netherlands). Each analytical procedure was replicated three times.

Molecular analyses

Total soil DNA was extracted from unincubated soil in triplicate with the FastDNA® SPIN Kit for soil (MP Bio). The specific operation was carried out according to the instructions. The quality and length of the extracted DNA were analyzed by 1% agarose gel electrophoresis, and the concentration and purity of the DNA were determined by an ultra-micro UV spectrophotometer (NanoDrop® ND-2000). The extracted DNA was appropriately diluted before downstream experiments.

The absolute abundance of the methanogenic archaeal functional gene *mcrA* was analyzed by fluorescence quantitative PCR (qPCR) technology using the ABI7300 Real-Time

PCR System (ABI7300, Applied Biosystems, USA). SYBR Green staining method was used for qPCR analysis. The specific primer set used for amplification was mlas-mod-F/mcrA-rev-R (Angel et al. 2012).

The composition of methanogenic archaeal communities was analyzed by high-throughput sequencing on an Illumina MiSeq PE250 System (Illumina, San Diego, CA, USA). First, the specific primer set MLfR/MLrR was used to amplify the 460–490 bps fragment of the *mcrA* gene (Zhu et al. 2011). The amplified products were purified and mixed equimolarly and then sequenced on the Illumina MiSeq PE250 System. The high-throughput sequencing experiment was entrusted to Shanghai Majorbio Bio-Pharm Technology Co. Ltd.

The paired-end sequences obtained by high-throughput sequencing were merged according to the overlapping relationship, using Flash 1.2.11 software. Low-quality sequences with quality scores < 20 were removed using QIIME 1.9.1 software. After removing the singletons and chimeras, OTU clustering was performed on non-repetitive sequences according to 97% similarity, using Uparse 7.0.1090 software. All the sequences were subsampled according to the minimum number of sample sequences. Finally, the representative sequence of each OTU after subsampling was compared with the fgr/mcrA_202012 species taxonomy database using the RDP Classifier 2.11 software (the species classification confidence threshold was 0.7) to obtain the species annotation information. Raw sequence data are available in the NCBI Sequence Read Archive (SRA) under the accession number PRJNA901686.

Calculations

The CH_4 production potential was calculated using the linear regression of CH_4 increasing with the incubation time (Zhang et al. 2013b):

$$P = \mathrm{d}c/\mathrm{d}t \times V/MV \times MW/W \times 273/T \quad (1)$$

where P is the CH_4 production potential in soil with a unit of $\mu g \cdot g^{-1} \cdot d^{-1}$, dc / dt is the variation of CH_4 concentration in the flask in unit time with a unit of $\mu L \cdot L^{-1} \cdot d^{-1}$ (1 h and 50 h after incubation were taken as the starting and ending time points in this study), V is the volume of the gas phase in the flask with a unit of L, MV is the molar volume of CH_4 in standard condition with a unit of $L \cdot mol^{-1}$, MW is the molar mass of CH_4 with a unit of $g \cdot mol^{-1}$, W is the dry weight of soil with a unit of g, and T is the incubated temperature with a unit of K.

The $\alpha_{(CO_2/CH_4)}$-values can be obtained by using the CH_3F suppression method, which can be estimated by the $\delta^{13}C$-values of CO_2 and CH_4 produced in soil anaerobic incubation with the addition of 2% CH_3F (Conrad et al. 2012; Ji et al. 2018):

$$\alpha_{(CO_2/CH_4)} = (\delta^{13}CO_2 + 1000)/(\delta^{13}CH_{4(CO_2)} + 1000) \quad (2)$$

The quantification of the relative contribution of methanogenic pathways is based on precise observations of its stable C isotopic composition. It is assumed that total CH_4 production ($CH_{4(total)}$) is the sum of CH_4 from acetate fermentation ($CH_{4(ac)}$) and CH_4 from CO_2/H_2 reduction ($CH_{4(CO_2)}$), i.e.:

$$CH_{4(total)} = CH_{4(ac)} + CH_{4(CO_2)} \quad (3)$$

Then, the relative contribution of acetate-dependent methanogenesis (f_{ac}) in paddy soil is (Tyler et al. 1997):

$$f_{ac} = CH_{4(ac)}/(CH_{4(ac)} + CH_{4(CO_2)}) \times 100\% \quad (4)$$

According to the conservation of C isotope mass, we can get (Tyler et al. 1997):

$$\delta^{13}CH_{4(total)} = \delta^{13}CH_{4(ac)} \times f_{ac} + \delta^{13}CH_{4(CO_2)} \times (1 - f_{ac}) \quad (5)$$

where $\delta^{13}CH_{4(total)}$ is the $\delta^{13}C$-value of total CH_4 produced in soil, $\delta^{13}CH_{4(ac)}$ is the $\delta^{13}C$-value of CH_4 produced by acetate fermentation, $\delta^{13}CH_{4(CO_2)}$ is the $\delta^{13}C$-value of CH_4 produced by CO_2/H_2 reduction.

Among them, the $\delta^{13}CH_{4(total)}$-values can be obtained by measuring the $\delta^{13}CH_4$-values produced in soil anaerobic incubation. The $\delta^{13}CH_{4(ac)}$-values have not been determined in this study. Referring to previous results, we assumed the $\delta^{13}CH_{4(ac)}$-values to be -43‰ and -37‰ (Conrad et al. 2002; Krüger et al. 2002). The $\delta^{13}CH_{4(CO_2)}$-values can be estimated by the $\delta^{13}C$-values of CO_2 ($\delta^{13}CO_2$) produced in soil anaerobic incubation (Conrad et al. 2002; Krüger et al. 2002):

$$\delta^{13}CH_{4(CO_2)} = (\delta^{13}CO_2 + 1000)/\alpha_{(CO_2/CH_4)} - 1000 \quad (6)$$

Statistical analyses

The calculations of mean value and standard deviation were done with Microsoft Excel 2013. SPSS 20 software was used for variance analysis and correlation analysis of data, and the Tukey HSD test was used for pairwise comparison of differences in CH_4 production potential, $\delta^{13}C$-values, $\alpha_{(CO_2/CH_4)}$-values, f_{ac}-values, soil properties, *mcrA* gene abundance, and the relative abundance of methanogens. The non-metric multidimensional scaling (NMDS) analysis and analysis of similarities (Anosim) based on Bray–Curtis distance were done to determine the effects of rice growth stage and rice cropping system on the difference in composition of methanogenic communities.

The redundancy analysis (RDA) was performed to determine the effects of soil properties on the composition of methanogenic communities. And hierarchical partitioning (HP) analysis was performed to partition the individual effect of each factor in the RDA model, using the *rdacca.hp* R package (Lai et al. 2022). The Mantel test was conducted to analyze the Spearman correlation between the matrix of soil properties based on Euclidean distance and the matrix of methanogenic communities based on the Bray–Curtis distance. And Spearman correlation analysis was further done to determine the relationship between soil properties and each methanogenic genus.

Results

CH_4 production potential

The CH_4 production potential in RW and DRL generally decreased from the tillering stage to the heading and ripening stages (Fig. 1a), within the range of 0.008–0.024 $\mu g \cdot g^{-1} \cdot d^{-1}$ and 0.003–0.025 $\mu g \cdot g^{-1} \cdot d^{-1}$, respectively. On the contrary, it showed an increasing trend in DRE and PF and peaked at the heading and ripening stages, ranging from 0.006 to 0.015 $\mu g \cdot g^{-1} \cdot d^{-1}$ and from 3.22 to12.7 $\mu g \cdot g^{-1} \cdot d^{-1}$, respectively (Fig. 1a). Compared with DRE, it was much higher in RW and DRL at the tillering stage while far lower at the heading stage (Fig. 1a). On average, the CH_4 production potential in PF was 5.72 $\mu g \cdot g^{-1} \cdot d^{-1}$, which was significantly higher than that in RW and DR (0.009–0.013 $\mu g \cdot g^{-1} \cdot d^{-1}$) ($P < 0.05$, Fig. 1b).

Stable C isotopes and methanogenic pathways

As a whole, the $\delta^{13}CH_4$-values in the rice cropping systems ranged from -75.6‰ to -57.2‰ (Table 1). The CH_4 produced in RW was relatively ^{13}C-depleted at the tillering stage, and it became slightly ^{13}C-enriched from the booting stage to the ripening stage. The produced CH_4 in PF was enriched in ^{13}C at the tillering and ripening stages while depleted in ^{13}C at the booting and heading stages, although no significance was observed in the $\delta^{13}C$-values. In DR, the $\delta^{13}CH_4$-values of DRE first decreased and then increased, while it became more positive in DRL with the rice growth. The $\delta^{13}C$-values of produced CO_2 in RW and PF varied from -20.4‰ to -17.5‰ and -21.5‰ to -17.1‰ (Table 1), while it had a smaller variation range in DR (-19.1‰ to -17.7‰). With the addition of CH_3F, both the $\delta^{13}CH_4$-values and $\delta^{13}CO_2$-values in each rice field became more negative at each rice growth stage, ranging from -93.1‰ to -74.9‰ and -23.1‰ to -18.1‰, respectively (Table 1).

The calculated $\alpha_{(CO_2/CH_4)}$-values ranged from 1.058 to 1.080 (Table 1). It generally increased with the rice growth in RW. However, it showed a variation pattern of decrease and then increase in PF ($P < 0.05$). In DR, it generally decreased in DRE while remaining relatively constant after a significant decrease in DRL ($P < 0.05$). The $\delta^{13}C$-values of CH_4 from CO_2/H_2 were more negative than that of total CH_4,

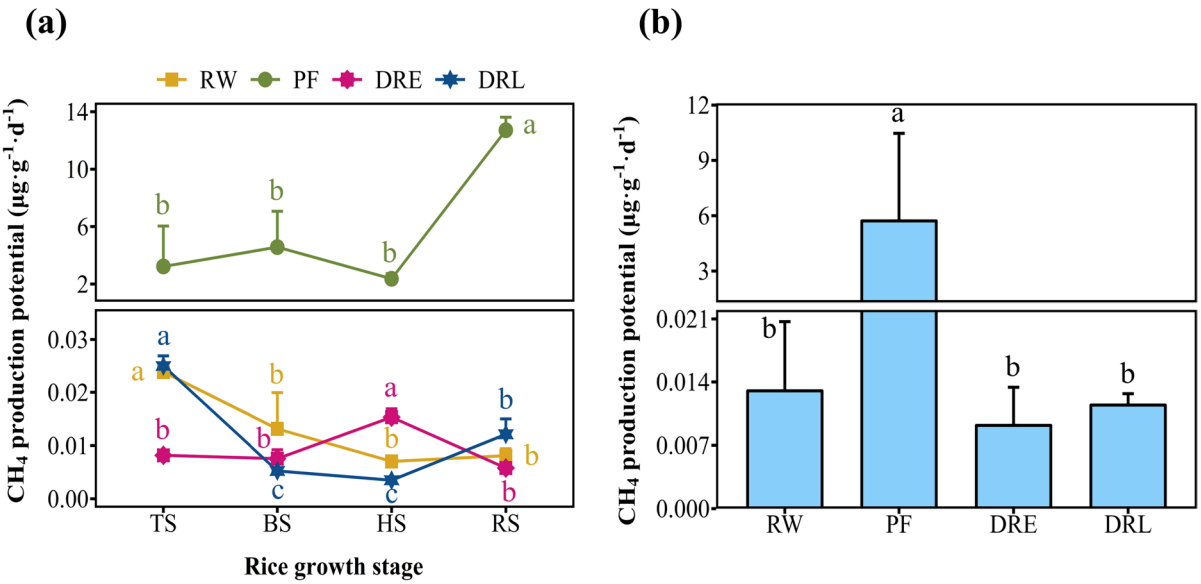

Fig. 1 Temporal variation (**a**) and mean value (**b**) of CH_4 production potential in the three rice cropping systems. RW, PF, DRE, and DRL stand for the rice–wheat rotation field, permanently flooded rice field, and double-rice cropping field in the early-rice season and the late-rice season, respectively. TS, BS, HS, and RS stand for the tillering stage, booting stage, heading stage, and ripening stage, respectively. Different letters in the different rice growth stages represent significant differences ($P < 0.05$)

Table 1 Carbon isotopic fractionation factor for the conversion of CO_2 to CH_4 ($\alpha_{(CO_2/CH_4)}$) and relative contribution of acetate-dependent methanogenesis (f_{ac}) in the three rice cropping systems at the four rice growth stages; mean ± SD, $n = 5$

Rice growth stage	δ^{13}C-values of produced CH_4 (‰)		δ^{13}C-values of produced CO_2 (‰)		$\alpha_{(CO_2/CH_4)}$ [a]	δ^{13}C-values of CH_4 from CO_2/H_2 (‰) [b]	f_{ac} [c] (%)	f_{ac} [d] (%)
	CK	CH_3F	CK	CH_3F				
Rice–wheat rotation field:								
TS	−67.2 ± 3.3a	−75.3 ± 5.6a	−17.5 ± 0.2a	−18.1 ± 0.3a	1.062 ± 0.006b	−74.7 ± 0.2a	20.0 ± 8.7b	23.8 ± 10.4b
BS	−62.7 ± 1.6a	−78.7 ± 6.9ab	−19.5 ± 0.4b	−20.1 ± 0.8b	1.064 ± 0.007ab	−78.2 ± 0.4b	37.6 ± 4.3a	44.0 ± 4.9a
HS	−61.4 ± 1.9a	−74.9 ± 3.7a	−20.4 ± 0.2c	−20.8 ± 0.5b	1.058 ± 0.004b	−74.5 ± 0.1a	34.9 ± 5.3a	41.5 ± 6.2a
RS	−62.3 ± 6.4a	−87.6 ± 4.7b	−19.3 ± 0.3b	−20.0 ± 0.2b	1.074 ± 0.005a	−87.0 ± 0.3c	49.3 ± 12.9a	56.1 ± 14.6a
Permanently flooded rice field:								
TS	−61.3 ± 7.2a	−90.8 ± 1.8b	−21.5 ± 0.7b	−22.1 ± 0.5b	1.076 ± 0.002a	−90.3 ± 0.7c	54.2 ± 14.1a	61.1 ± 15.8a
BS	−66.8 ± 0.6a	−82.3 ± 5.1a	−20.3 ± 0.7b	−23.2 ± 0.5b	1.064 ± 0.006b	−79.6 ± 0.7a	29.9 ± 2.0b	34.8 ± 2.3b
HS	−63.9 ± 0.9a	−87.6 ± 3.7ab	−17.1 ± 0.3a	−19.6 ± 2.1a	1.075 ± 0.003a	−85.4 ± 0.3b	44.4 ± 1.7ab	50.7 ± 2.0ab
RS	−59.6 ± 7.6a	−87.5 ± 1.8ab	−21.3 ± 2.2b	−22.4 ± 1.1b	1.071 ± 0.003a	−86.5 ± 2.0b	54.0 ± 16.5a	61.4 ± 18.6a
Double-rice cropping field in the early-rice season:								
TS	−69.7 ± 3.1bc	−80.2 ± 5.4a	−17.7 ± 0.3a	−18.8 ± 0.4a	1.067 ± 0.006ab	−79.2 ± 0.2a	22.5 ± 7.5c	26.3 ± 8.7c
BS	−75.6 ± 1.9c	−90.5 ± 8.6b	−18.8 ± 0.8a	−20.3 ± 1.1a	1.077 ± 0.010a	−89.1 ± 0.7c	26.0 ± 3.1bc	29.4 ± 3.6bc
HS	−63.3 ± 1.9ab	−85.8 ± 0.5ab	−17.8 ± 0.9a	−19.7 ± 1.0a	1.072 ± 0.001ab	−84.1 ± 0.9b	44.3 ± 3.6ab	50.8 ± 4.2ab
RS	−57.6 ± 9.0a	−79.1 ± 5.0a	−19.1 ± 1.0a	−20.2 ± 0.9a	1.064 ± 0.006b	−78.1 ± 1.0a	50.4 ± 20.6a	59.1 ± 24.3a
Double-rice cropping field in the late-rice season:								
TS	−70.3 ± 1.7b	−93.1 ± 0.6b	−18.6 ± 0.5a	−20.3 ± 0.4b	1.080 ± 0.001a	−91.5 ± 0.5d	39.0 ± 2.9ab	43.8 ± 3.3ab
BS	−62.2 ± 2.1a	−76.5 ± 2.3a	−18.7 ± 0.1a	−19.8 ± 0.1ab	1.061 ± 0.003b	−75.4 ± 0.1b	34.5 ± 5.6b	40.8 ± 6.6b
HS	−60.6 ± 4.9a	−76.1 ± 2.4a	−19.0 ± 0.1a	−20.3 ± 0.1b	1.060 ± 0.003b	−74.9 ± 0.1a	37.7 ± 13.0ab	44.7 ± 15.4ab
RS	−57.2 ± 2.3a	−77.7 ± 2.2a	−18.6 ± 0.2a	−19.4 ± 0.5a	1.063 ± 0.002b	−76.9 ± 0.2c	49.4 ± 5.6a	58.2 ± 6.6a

CH_3F addition of CH_3F, *CK* without the addition of CH_3F, *TS* tillering stage, *BS* booting stage, *HS* heading stage, *RS* ripening stage. Values with different letters in the same column of each rice cropping system differ significantly ($P < 0.05$)

[a] Calculated with Eq. (2) using the δ^{13}C-values of CH_4 and CO_2 produced in soil anaerobic incubation with the addition of CH_3F. [b] Calculated with Eq. (6) using the δ^{13}C-values of CO_2 produced in soil anaerobic incubation without the addition of CH_3F and $\alpha_{(CO_2/CH_4)}$ [a]. [c] Calculated with Eq. (5) using $\delta^{13}CH_{4(ac)}$-values of -37‰ for CH_4 from acetate. [d] Calculated with Eq. (5) using $\delta^{13}CH_{4(ac)}$-values of -43‰ for CH_4 from acetate

with values of -91.5‰ to -74.5‰ (Table 1). The f_{ac}-values in RW, DRE, and DRL all showed an upward trend with the growth of rice (Table 1), with various ranges of 20%-56%, 23%-59%, 34%-58%, respectively, and reached a maximum value at the ripening stage. In contrast to these two fields, the f_{ac}-values in PF showed a temporal variation that first decreased and then increased (Table 1); that is, it dropped sharply from 54%-61% at the tillering stage to 30%-35% at the booting stage and rose to 54%-61% again at the ripening stage.

Soil properties

At each rice growth stage (Fig. 2a), the DOC contents in RW and DR (54.4–205 mg·kg^{-1}) were always lower than those in PF (224–316 mg·kg^{-1}). It showed a decreasing trend with the rice growth in RW and DRL, as opposed to in PF and DRE. The acetate contents in RW and DR (0–21.5 mg·kg^{-1}) generally increased from the tillering stage to the heading stage and then dropped a lot to the lowest value at the ripening stage (Fig. 2b). In PF, it decreased from the highest value of 161 mg·kg^{-1} at the tillering stage to 56.4 mg·kg^{-1} at the booting stage, and then rose to a higher value again (128 mg·kg^{-1}) at the ripening stage (Fig. 2b). The moisture contents varied from 38.9% to 63.9% in RW and DR and sharply increased in PF with the values of 106%-147% (Fig. 2c). The NH_4^+-N contents in RW, PF, DRE, and DRL ranged from 10.0 mg·kg^{-1} to 68.3 mg·kg^{-1}, showing a decreasing temporal trend but no significant difference between their mean values (Fig. 2d, 2f). Almost no NO_3^--N could be observed in RW, PF, and DRE, whereas it kept around 0.5 mg·kg^{-1} in DRL (Fig. 2e).

The results of correlation analysis showed that CH_4 production potential was significantly correlated with DOC content in RW, PF, and DRL and with acetate content in DRE (Table S2). For all samples from the three rice cropping systems, CH_4 production potential was positively correlated with acetate and moisture contents (Table S2). The f_{ac} was positively related to acetate and moisture contents in PF but negatively related to NH_4^+-N and NO_3^--N contents in RW and DR (Table S3).

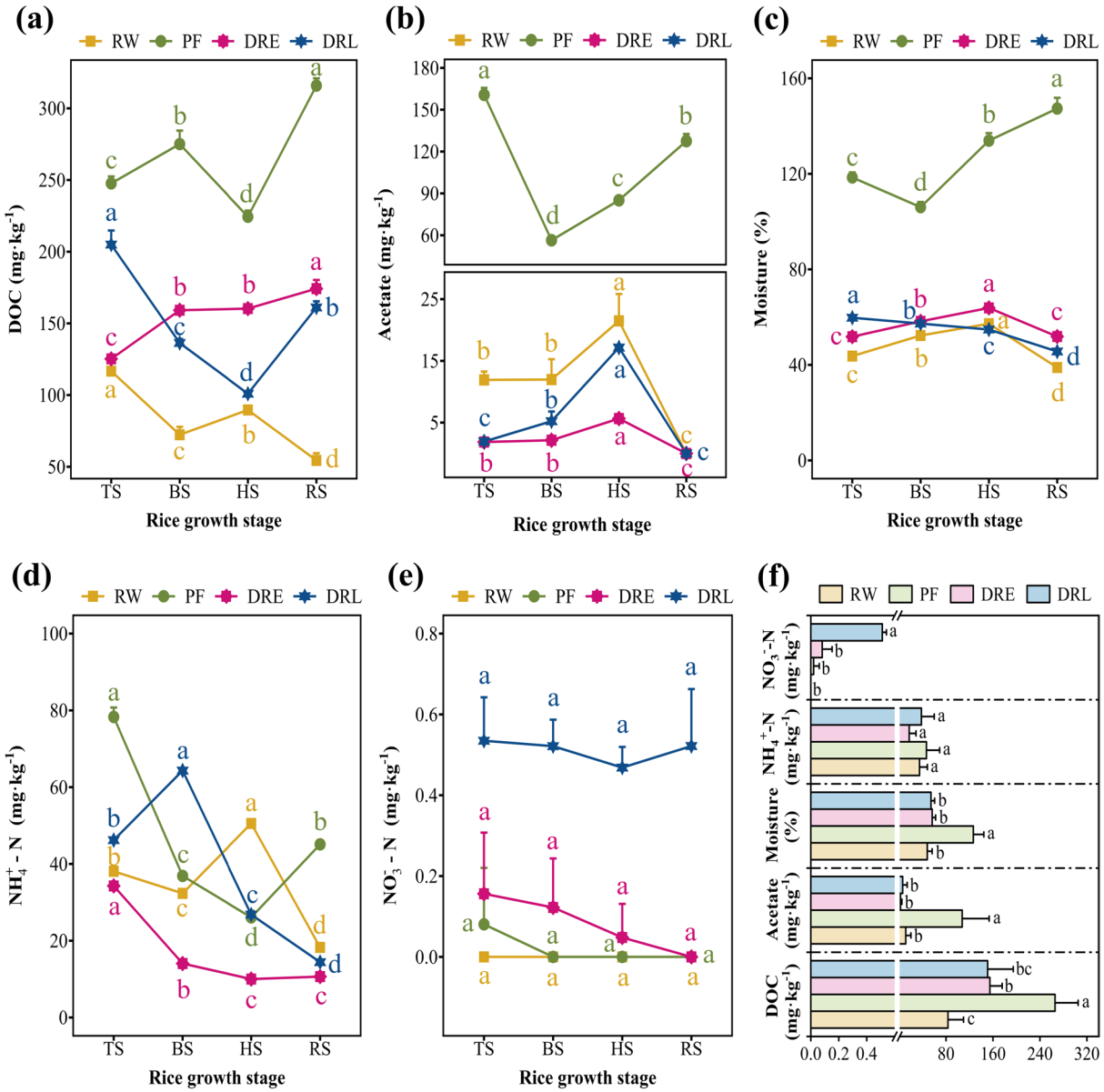

Fig. 2 Temporal variation of soil DOC (**a**), acetate (**b**), moisture (percentage considering dry soil mass) (**c**), NH_4^+-N (**d**), and NO_3^--N (**e**) contents, and mean value of them (**f**) in the three rice cropping systems. RW, PF, DRE, and DRL stand for the rice–wheat rotation field, permanently flooded rice field, and double-rice cropping field in the early-rice season and the late-rice season, respectively. TS, BS, HS, and RS stand for the tillering stage, booting stage, heading stage, and ripening stage, respectively. Different letters in the different rice growth stages (**a**-**e**) or mean values (**f**) represent significant differences ($P < 0.05$)

Methanogenic archaeal communities

Except for DRE, there were obvious temporal variations in the absolute abundance of the *mcrA* gene in the three rice cropping systems (Fig. 3a). Among these rice fields, DR had the highest *mcrA* gene abundance (3.5×10^7–4.4×10^7 copies·g^{-1}), which was much higher than that in RW (1.2×10^7–2.7×10^7 copies·g^{-1}) (Fig. 3a; Fig. S3). But the *mcrA* gene abundance in PF was not significantly different from that in the other two rice fields, ranging from 1.4×10^7 to 3.9×10^7 copies·g^{-1} (Fig. 3a; Fig. S3). Correlation analysis showed that there was a positive correlation between CH_4 production potential and *mcrA* gene abundance in PF (Table S2).

RW was dominated by unclassified_f__*Methanobacteriaceae*, while PF and DR were dominated by *Methanoregula* and *Methanosarcina*, respectively (Fig. 3b). NMDS analysis indicated the composition of methanogenic communities presented significant temporal variation in each rice cropping system (Anosim: $R = 0.296$–0.483,

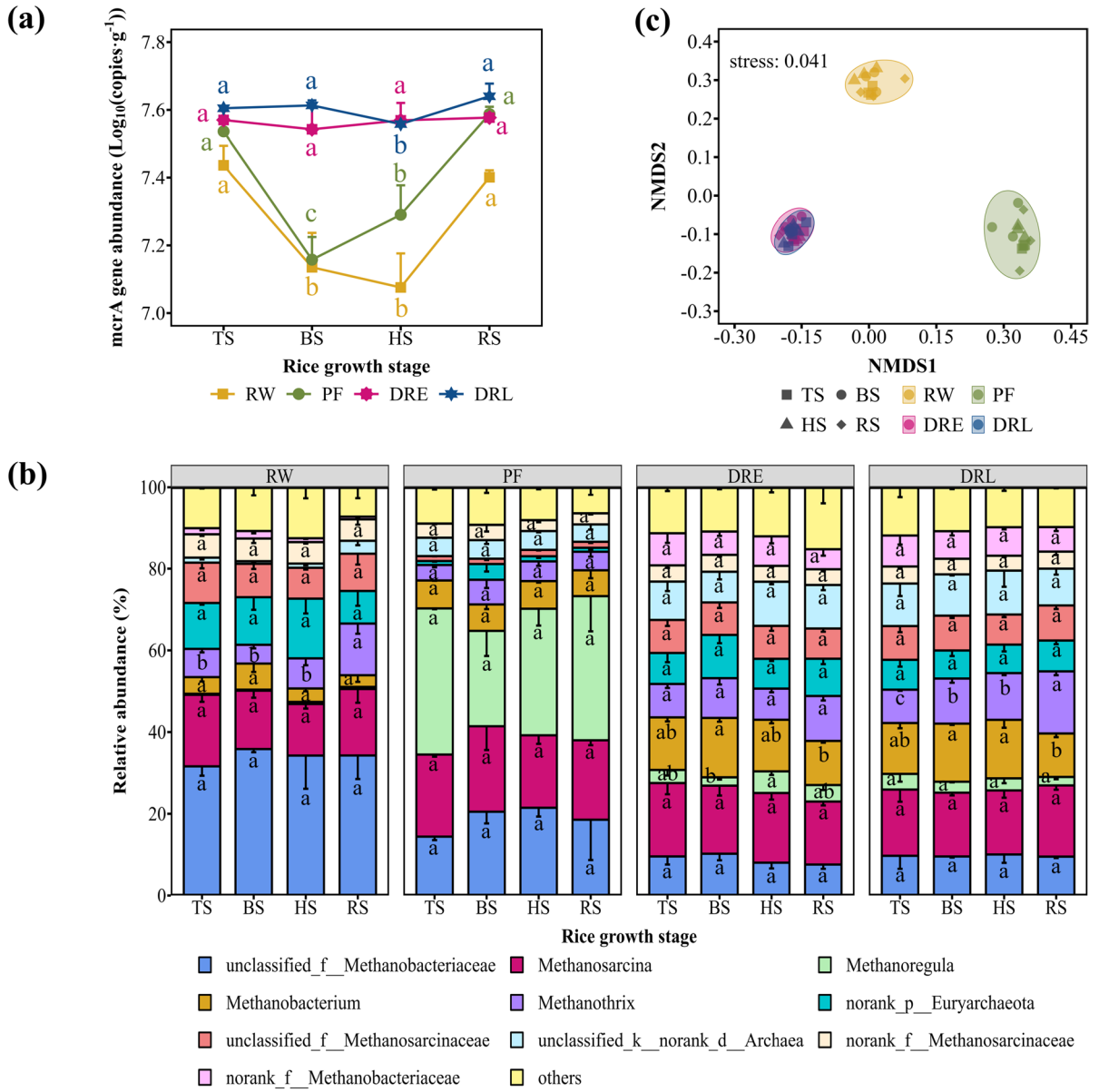

Fig. 3 The abundance of *mcrA* gene (**a**), the composition of methanogenic archaeal communities at the genus level (**b**), and NMDS analysis based on Bray–Curtis distance (**c**) in the three rice cropping systems. RW, PF, DRE, and DRL stand for the rice–wheat rotation field, permanently flooded rice field, and double-rice cropping field in the early-rice season and the late-rice season, respectively. TS, BS, HS, and RS stand for the tillering stage, booting stage, heading stage, and ripening stage, respectively. Different letters in the different rice growth stages represent significant differences ($P < 0.05$)

$P = 0.0003$–0.0274; Fig. S4, Table S4), of which differed significantly in the methanogenic community composition to a larger extent (Anosim: $R = 0.815$, $P < 0.001$; Fig. 3c, Table S5). The relative abundance of *Methanosarcina* had no significant change in all rice fields, while that of *Methanothrix* had an increasing temporal trend in RW and DR (Fig. 3b). The relative abundance of *Methanosarcina* in PF was 3%-4% higher than those in RW and DR, whereas that of *Methanothrix* was 3%-7% lower ($P < 0.05$, Fig. S5). The genus *Methanoregula* possessed the highest relative abundance in PF (23%-36%), while *Methanobacterium* had the highest relative abundance in DR (11%-15%) (Fig. 3b). Correlation analysis showed that the relative abundance of *Methanoregula* was positively correlated with the CH_4 production potential of all samples from the three rice cropping systems ($P < 0.01$, Table S2). And the f_{ac} of RW and DRL was positively related to the relative abundance of *Methanothrix* ($P < 0.05$), while that of PF was negatively related to the relative abundance of *Methanothrix* ($P < 0.01$, Table S3).

RDA and corresponding HP analysis indicated that soil DOC, acetate, NH_4^+-N, NO_3^--N, and moisture contents together explained 73.3% of the variation in methanogenic community composition, and the first two axes of the RDA model can explain 72.2% of the variation (Fig. 4a, b). Furthermore, moisture content had the biggest individual effect on the methanogenic community composition, with a variation explanation of 21.1% (Fig. 4b). DOC and acetate contents, with a variation explanation of 18.5% and 18.6%, respectively, also greatly affected the methanogenic community composition. The Mantel test also indicated soil moisture, DOC, and acetate contents had a significant correlation with methanogenic community composition (Fig. 4c, $P < 0.001$). However, soil moisture content was positively and negatively correlated with the relative abundances of *Methanosarcina* ($P < 0.05$) and *Methanothrix* ($P < 0.001$), respectively (Fig. 4c).

Discussion

CH_4 production potential in the three rice cropping systems

The temporal patterns of CH_4 production potential in RW and DRL were opposite to those in PF and DRE, possibly owing to the differences in methanogenic substrate DOC. In RW and DRL, straw incorporation before rice transplanting increased DOC content (Bertora et al. 2018), thus significantly promoting the CH_4 production at the tillering stage (Fig. 1a). With the rice growth, however, the residual straw C became less available, contributing less to CH_4 production (Ji et al. 2018). Moreover, dry farming and dry–wet alternation might enhance aerobic respiration, accelerating the loss of DOC (Keiluweit et al. 2017), thereby decreasing CH_4 production at the middle and late rice growth stages (Fig. 1a). In PF and DRE, rice planting was several months earlier than in RW and DRL. Therefore, the temperature in

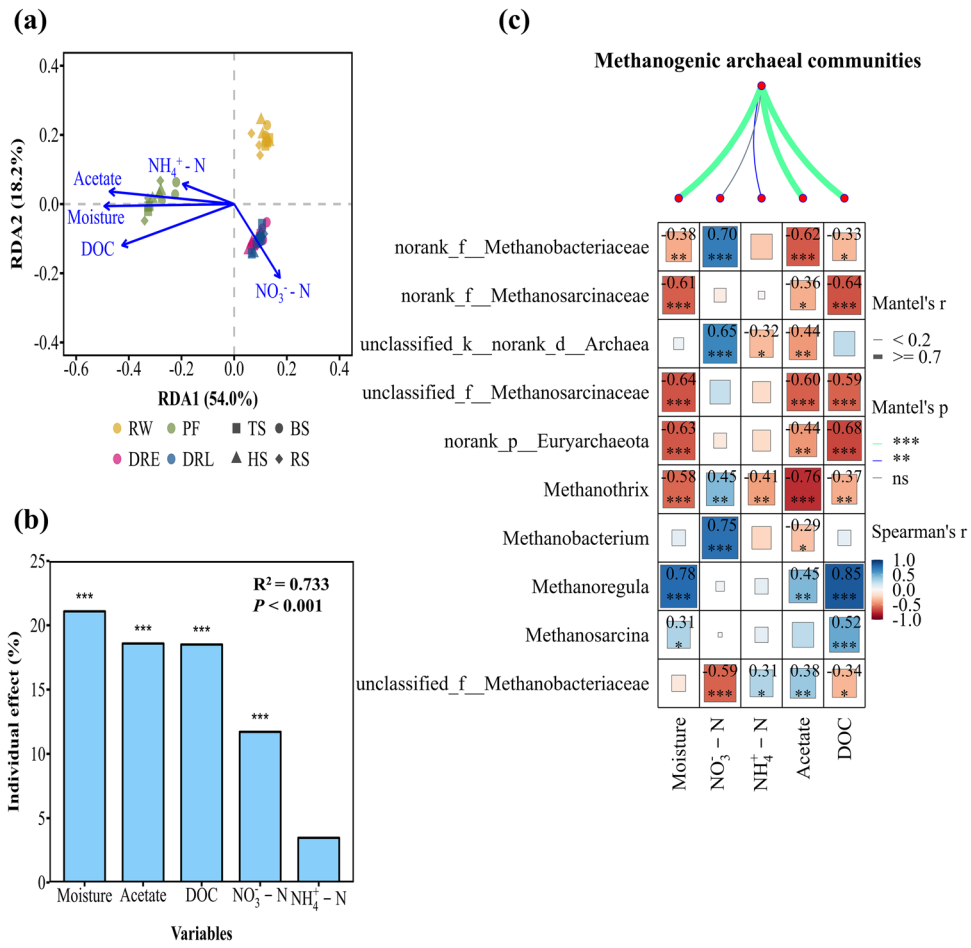

Fig. 4 The RDA (**a**), HP analysis (**b**), Mantel test, and Spearman correlation analysis (**c**) between soil properties and methanogenic archaeal communities at the genus level. RW, PF, DRE, and DRL stand for the rice–wheat rotation field, permanently flooded rice field, and double-rice cropping field in the early-rice season and the late-rice season, respectively. TS, BS, HS, and RS stand for the tillering stage, booting stage, heading stage, and ripening stage, respectively

PF and DRE was initially lower and probably not conducive to the decomposition of organic C (Wei et al. 2021), thus reducing the availability of methanogenic substrates, especially in PF without straw incorporation, causing a decrease in CH_4 production at the early stage of rice growth (Fig. 1a). However, root exudates and litter, the important sources of soil DOC (Kalbitz et al. 2000), increased in C supply for methanogenesis with rice growth (Lu et al. 2000; Jia et al. 2001). Consequently, the CH_4 production potential in PF and DRE reached a maximum value at the ripening and heading stages, respectively (Fig. 1a).

Remarkably, the CH_4 production potential in PF was hundreds of times greater than that in the other two rice fields, which was possibly ascribed to its highest moisture and acetate contents (Fig. 2b). Compared with RW and DRE, PF possessed finer soil particles with a larger proportion of clay and silt, causing a strong water retention capacity (Li et al. 2022). The high moisture level can enhance CH_4 production by decreasing soil Eh and the content of oxidants (e.g., Fe^{3+}) (Krüger et al. 2001; Ji et al. 2022). Furthermore, the good anaerobic conditions in PF were conducive to the accumulation of acetate, which is the main precursor of methanogenesis (Conrad 2007, 2020a). In addition, previous studies have found a positive correlation between CH_4 production capacity and the relative abundance of *Methanoregula* (Zhang et al. 2018), which was similar to our result (Table S2). Since the relative abundance of *Methanoregula* in PF was far higher than that in RW and DR (Fig. 3b), this methanogenic genus potentially made substantial contributions to the higher CH_4 production in PF.

Methanogenic pathways in the three rice cropping systems

Before quantifying the relative contribution of acetate-dependent methanogenesis (f_{ac}), the $\alpha_{(CO_2/CH_4)}$-value should be calculated because f_{ac} may change greatly with the variation of $\alpha_{(CO_2/CH_4)}$ in different periods and environments (Games et al. 1978; Fey et al. 2004; Valentine et al. 2004). The $\alpha_{(CO_2/CH_4)}$ currently showed a significant temporal variation ranging from 1.058 to 1.080 (Table 1), confirming previous values (Conrad et al. 2002; Krüger et al. 2002; Fey et al. 2004). Unlike $\alpha_{(CO_2/CH_4)}$, the fractionation factor for acetate to CH_4 ($\varepsilon_{(ac/CH_4)}$) was relatively stable. Krüger et al. (2002) determined that $\delta^{13}C_{acetate}$ in pore water of Italian rice fields ranged from -20.70 ± 2.31‰ to -16.17 ± 0.30‰. By assuming $\varepsilon_{(ac/CH_4)}$ = -21‰ (Gelwicks et al. 1994), they obtained $\delta^{13}CH_{4(ac)}$-values ranging from -43‰ to -37‰ that had been adopted by several investigations (Sugimoto and Wada 1993; Tyler et al. 1997; Bilek et al. 1999; Conrad et al. 2002; Nakagawa et al. 2002; Fey et al. 2004; Zhang et al. 2012, 2013a, b).

The acetate-dependent methanogenesis in RW and DR became more important towards the late stage of rice growth (Table 1), confirming previous studies (Krüger et al. 2001, 2002; Zhang et al. 2013b), but it was dominant at both the early and late stages of rice growth in PF (Table 1). Even if the $\alpha_{(CO_2/CH_4)}$-values of 1.06, 1.07, and 1.08 were used, the variation pattern of the estimated f_{ac}-value did not change (Fig. S2). Without measurements of $\alpha_{(CO_2/CH_4)}$, a fixed $\alpha_{(CO_2/CH_4)}$-value was used to quantify the methanogenic pathways because it not only accurately reflects the differences in methanogenic pathways between different treatments but also eliminates the numerical differences caused by different $\alpha_{(CO_2/CH_4)}$-values to have a better comparison with previous findings.

The temporal patterns of f_{ac}-value in RW and DR probably depended on the variations in the relative abundance of *Methanothrix*. Since the acetate contents in RW and DR were relatively low (Fig. 2b), the acetoclastic *Methanothrix* might play a key role in the acetate-dependent methanogenesis of these rice fields due to its high affinity for acetate (Jetten et al. 1990, 1992). However, the acetoclastic *Methanosarcina* becomes more active in the acetate-abundant environment (Conklin et al. 2006; Yuan et al. 2011), thus possibly being crucial to the acetate-dependent methanogenesis of PF.

Notably, the f_{ac}-value in PF was higher than that of RW and DR at the tillering stage (Table 1), probably due to the higher acetate content in PF at this time. At relatively low temperatures, methanogenesis is more restrained than hydrolysis and fermentation (Juottonen et al. 2008), and acetogens can outcompete methanogens for H_2/CO_2 to increase the production of acetate (Liu and Conrad 2011; Fu et al. 2019). Therefore, the accumulated acetate during the winter fallow season would increase the acetate content in the initial rice season (Fig. 2b), thus possibly stimulating the activity of *Methanosarcina* (Yuan et al. 2011) and increasing the f_{ac}-value at the tillering stage. In RW and DR, however, both the C substrate (acetate) and acetoclastic methanogen (*Methanothrix*) were at relatively lower levels at the tillering stage than at other stages (Figs. 2b and 3b), thus decreasing the f_{ac}-value.

Methanogenic archaeal communities in the three rice cropping systems

The abundance of methanogens has temporal variations (Ji et al. 2020; Pan et al. 2021), and this was observed in RW, PF, and DRL (Fig. 3a). Other studies found that the abundance of methanogens remained constant during the rice growth stages (Asakawa and Hayano 1995), and this was the case of DRE (Fig. 3a). Such a difference between these rice cropping systems may be attributed to the differences in climatic conditions, management of water and fertilizers, soil type, etc. (Dubey et al. 2013; Pan et al. 2021).

The relative abundance of acetoclastic *Methanothrix* increased with rice growth in RW and DR, while that of acetoclastic *Methanosarcina* remained relatively stable in all rice fields (Fig. 3b). This was likely because *Methanothrix* uses only acetate to produce CH_4, while *Methanosarcina*, with a faster growth rate, is capable of using diverse methanogenic substrates (e.g., acetate, H_2/CO_2, methanol) (Rosenberg et al. 2014). Therefore, *Methanothrix* seemed more sensitive to environmental changes than *Methanosarcina* (De Vrieze et al. 2012). It may be possible that *Methanosarcina* was less relevant to the acetate-dependent methanogenesis in RW and DR, but it shifted from hydrogenotrophic metabolism to acetoclastic metabolism when *Methanothrix*'s activity was decreased by environmental stress. The presence of *Methanosarcina* might thereby enhance the stability of the CH_4 production function, especially the function of acetate-dependent methanogenesis because of the much fewer types of acetoclastic methanogens than hydrogenotrophic methanogens (Conrad 2007, 2020a).

The spatial variation of methanogenic community composition was greater than its temporal variation, which was consistent with the result of Watanabe et al. (2006). Because all species of *Methanoregula* require acetate for growth (Rosenberg et al. 2014), it's probably not surprising that *Methanoregula* was dominant in acetate-abundant PF (Fig. 3b). Former study also showed the dominance of *Methanoregula* in a continuously flooded rice field (Ji et al. 2022). However, the intermittently irrigated RW and DR were dominated by unclassified_*f*__*Methanobacteriaceae* and *Methanosarcina*, respectively (Fig. 3b), because these methanogens are tolerant to oxygen exposure during soil drying and drainage (Fetzer et al. 1993; Angel et al. 2012) due to the transcription of genes encoding catalase or superoxide dismutase (Takao et al. 1991; Meile et al. 1995; Angel et al. 2011).

Soil DOC, acetate, and moisture contents showed significant effects on methanogenic community composition (Fig. 4). DOC and acetate, the important methanogenic substrates, are expected to differentiate the ecologic niches of methanogens with different utilization capacities of them (Conrad 2007). The most typical case is *Methanothrix* can metabolize acetate at a much lower concentration than *Methanosarcina* (Jetten et al. 1990, 1992; Rosenberg et al. 2014), causing a higher relative abundance of *Methanothrix* in an acetate-poor environment whereas *Methanosarcina* becoming more abundant in acetate-abundant habitat (Fey and Conrad 2000; Krüger et al. 2005; Yuan et al. 2011). Moisture content could affect methanogens through physiological water stress directly and by changing the availability of substrates and oxygen indirectly (Fetzer et al. 1993; Conrad 2020b). In other words, the different effects of moisture content on those two acetoclastic methanogens might be regulated by changes in acetate availability. PF possessed much higher moisture contents than those in RW and DR, thus resulting in stronger reduction conditions (Ji et al. 2022) and higher acetate contents. Therefore, *Methanosarcina* was relatively more abundant in PF, while *Methanothrix* increased in relative abundance in RW and DR (Fig. S5).

Conclusions

This study mainly analyzed the relative contribution of acetate-dependent methanogenesis, soil properties, and methanogenic archaeal communities in RW, PF, and DR to reveal the temporal variation and its influencing factors of methanogenic pathways in paddy soils. Based on stable C isotopes and specific inhibitors of acetate-dependent methanogenesis, an obvious temporal variation of methanogenic pathways was observed in different rice cropping systems. It is estimated that the acetate-dependent methanogenesis in RW and DR became more and more important towards the ripening stage, whereas it was dominant at both the tillering and ripening stages in PF. This temporal variation was possibly due to that the relative abundance of acetoclastic *Methanothrix* increased with the rice growth in RW and DR, while the acetate-dependent methanogenesis was positively correlated with the acetate content in PF, perhaps resulting from the activation of acetoclastic *Methanosarcina*. The findings indicate that *Methanothrix* outcompeted *Methanosarcina* for acetate in acetate-poor RW and DR, whereas *Methanosarcina* was more competitive in acetate-abundant PF. Collectively, our results suggest that the temporal variation of methanogenic pathways was influenced by the relative abundance of acetoclastic methanogens depending on the acetate level.

Supplementary Information The online version contains supplementary material available at https://doi.org/10.1007/s00374-023-01769-7.

Author contributions Xiaoli Zhu was mainly responsible for the data collection and analysis and the writing of the original manuscript. Yang Ji was primarily involved in the manuscript revision. Guangbin Zhang contributed greatly to the experimental design and manuscript revision. Qiong Huang, Wanyu Shen, and Zhijun Wei mainly gave assistance in data analysis. Jing Ma and Hua Xu were mainly responsible for the manuscript review.

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Data availability The datasets of the current study are available from the corresponding author on reasonable request.

Declarations

Conflict of interest No potential conflict of interest was reported by the authors.

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