Differences in methanogenic pathways and methanogenic communities in paddy soils under three typical cropping modes

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Key Points:

- The CH_4 production potential, methanogenic pathways, and communities differ in three cropping mode soils.
- Paddy soil properties, mainly soil pH and soil texture regulate methanogenesis by changing methanogenic communities.

Abstract

Microbial methane (CH_4) production varies among different cropping modes, which has important implications for how to reduce CH_4 emissions from paddy fields. However, little is known about the values of anaerobically produced $\delta^{13}CH_4$, methanogenic pathways, and their dominant communities in different paddy soils. Through anaerobic incubation experiments and the stable carbon isotope with fluoromethane inhibitor method, CH_4 production potential (MPP), the relative contribution of acetoclastic methanogenesis (f_{ac}), and the abundance and community composition of methanogens in paddy soils were measured under three typical cropping modes (Rice-Wheat, RW; Rice-Fallow, RF; Double-Rice, DR) in China. The results showed that MPP was 30.7 μg CH_4 g^{-1} d^{-1} in DR soil, 57% and 66% higher than that in RW and RF soils, respectively, possibly due to the lower pH and higher abundance of *mcrA* gene. Moreover, RF soil had the highest produced $\delta^{13}CH_4$ value (–43.9‰) and the lowest produced $\delta^{13}CO_2$ value (–26.3‰). Based on the carbon isotope fractionations associated with H_2/CO_2-dependent methanogenesis (1.049–1.062), the values of f_{ac} estimated in RF soil (80–98%) were much higher than that in RW (39–60%) and DR (52–75%) soils. It could be supported by that the *Methanosarcina* (acetoclastic methanogens) were dominant in RF soil while *Methanosarcina* and *Methanobacterium* (hydrogenotrophic methanogens) dominated in RW and DR soils. Redundancy analysis revealed that the community structure of methanogens was significantly affected by soil pH, indicating that the differences in methanogenic pathways under the three typical cropping modes might be caused by the changes in community composition driven by soil pH. The findings suggest that soil pH-induced methanogenic abundance and community composition drive paddy MPP and methanogenic pathways, which would provide important insights into the CH_4 reduction in paddies.

Plain Language Summary

In paddy soils, the microbial methanogenesis and its mediated CH_4 production potential are various due to their various rice-based cropping modes. However, the methanogenic pathways, microbial mechanisms, and their responses to key influencing factors under different cropping modes in China are still poorly documented. We investigated the differences in pathways of CH_4 production,

methanogenic communities, and their responses to the corresponding soil properties under three typical cropping modes, i.e. Rice-Wheat, Rice-Fallow, and Double-Rice rotation systems. Our results demonstrated that there were significant differences in acetoclastic methanogenesis and dominant methanogenic communities in the three cropping mode paddy soils, which could mainly be caused by soil pH. This study provides a new perspective and further understanding of the methanogenic pathways and their microbial mechanisms in different cropping modes.

Keywords

Rice-based cropping modes; Methanogenic pathway; Methanogenic microbial community; Soil pH; Carbon isotopic fractionation

1. Introduction

As the second-most powerful greenhouse gas after carbon dioxide (CO_2), methane (CH_4) has received great global concern to address climate change (Liu et al., 2022). Rice fields are important anthropogenic sources of worldwide CH_4 emissions and are estimated at 24–39 Tg yr^{-1}, accounting for 12–21% of the global agriculture emission per year (Saunois et al., 2020). Expect for India (about 7.4 Tg yr^{-1}), the largest CH_4 emissions are found in China (about 6.2 Tg yr^{-1}), which contributes to about 21% of the total global budget from paddy fields (Carlson et al., 2016). Therefore, paddy fields in China play a critical role in the global carbon cycle (Qi et al., 2021), and exploring the mechanism of CH_4 production and CH_4 emission reduction from paddy fields is an important part of the current carbon neutrality in China.

CH_4 emissions from rice fields are the net effects of three processes: CH_4 production, oxidation, and transport from the soil into the atmosphere (Cai et al., 2009), of which CH_4 production is the basic prerequisite for CH_4 emission (Le Mer and Roger, 2001). Methanogens use CH_4 precursors to produce CH_4 under strictly anaerobic conditions (Conrad, 2007). The acetate and CO_2/H_2 were the two major precursors of methanogenesis in paddy ecosystems, and the corresponding methanogenic pathways of paddy soils were acetoclastic and hydrogenotrophic methanogenesis, respectively (Glissmann and Conrad, 2000; Ji et al., 2018b). The acetoclastic and hydrogenotrophic methanogenic pathways would fractionate organic material of a similar signature in ways, resulting in different signatures of product CH_4 (Ji et al., 2018a; Whiticar et al., 1986). This difference in isotopic fractionation could in principle be used to estimate the relative contribution of the two methanogenic pathways to total CH_4 production, which was determined by specific inhibition of acetoclastic methanogenesis with methyl fluoride (CH_3F) (Conrad and Klose, 1999; Glissmann and Conrad, 2000).

China has a large rice planting area with wide distribution and diverse cropping modes, mainly including three cropping modes of Rice-Wheat (RW), Rice-Follow (RF), and Double-Rice (DR). Different cropping modes with various water management and fertilization conditions lead to diverse soil properties (e.g. soil organic matter, soil pH, and soil organic acid, etc.) in paddy soils, changing the supply of methanogenic substances, the community structure, and composition of methanogens, then probably affecting CH_4 production and methanogenic pathways (Fu et al., 2021; Sun et al., 2018;

Wang et al., 1993; Yang et al., 2021). The RF paddy is mainly distributed in the hilly mountainous areas of southwest China, which is flooded all year round resulting in higher CH_4 emissions than other paddy fields (Cai et al., 2000; Mei et al., 1998). The DR paddy was mainly found in the central region south of the Yangtze River, the Pearl River basin, and the Hainan Province. In general, these regions have relatively more precipitations, higher temperatures, and longer duration of rain, which provide favorable conditions for CH_4 production and emission (Mei et al., 1998). However, most of the previous studies on CH_4 production and methanogenic pathway in Chinese paddy soils have focused on the RW cropping mode (Zhang et al., 2011; Zhang et al., 2012), and little is known about RF and DR soils with high CH_4 emissions and large emission reduction potential (Cai et al., 2000; Chen et al., 2013). Furthermore, paddy soils under different cropping modes have various methanogenic communities due to their specific soil conditions, which leads to changes in methanogenic pathways (Jiang et al., 2022; Zhang et al., 2017). However, there is no clear information so far on the targeted comparative study about the microbiological mechanisms between methanogenesis pathways and environmental factors in paddy soils under different cropping modes. Therefore, it is of great guiding significance to carry out the above research in-depth to take appropriate technical measures of microbial regulation for emission reduction.

In this study, we hypothesized that the various soil properties in different cropping modes directly or indirectly affect the abundance, diversity, and community composition of methanogens, thereby regulating the CH_4 production and the methanogenic pathway. Therefore, a microcosm experiment was conducted to determine the CH_4 production potential (MPP), carbon isotope composition ($\delta^{13}C$) of CH_4 production, and the dynamics of communities and composition of methanogens in paddy soils of three cropping modes (RW, RF, and DR) in China. DOC, acetate, soil pH, and other soil physicochemical properties were measured to probe the environmental factors of the methanogenic pathway with different cropping modes. The prime objectives of this research were: 1) to explore MPP and the related key influencing factors in paddy soils with different cropping modes, 2) to reveal the relevant composition and diversity of methanogenic communities in these paddy soils, and 3) to investigate the CH_4 production pathways and the corresponding microbial mechanisms in paddy soils with different cropping modes.

2. Materials and methods

2.1 Soil sample

The soils were sampled at the mature stage of rice from paddy fields under three typical Chinese cropping modes: RW [located in Jurong City, Jiangsu Province, East China (31°57′ N, 119°10′ E)], RF [located in Jianyang City, Sichuan Province, Southwest China (30°39′ N, 104°55′ E)], and DR [located in Yingtan City, Jiangxi Province, Southeast China (28°24′ N, 117°03′ E)]. Soil samples were collected following Soil Agro-Chemical Analyses procedures (Lu, 2000), for each model, cores (0–20 cm) were taken from three representative rice fields according to the distribution method. Ten fresh soil samples were then pooled to form a composite sample. Each soil was sieved (< 2 mm) and split into two subsamples. One subsample was air-dried and stored for incubation studies at room temperature and the other for soil physicochemical properties analysis stored at 4°C.

2.2 Soil incubation

The incubation procedure was the same as described by Ji et al. (2018a). Soil slurries of three cropping modes (RW, RF, and DR) were prepared by mixing 20 g of air-dried soil with 20 mL of deionized, sterile, and anoxic water at a ratio of 1:1. Pre-incubation of the slurries occurred in 120 mL serum bottles to activate soil activity, closed with butyl rubber stoppers at 25 ℃ for 3 days. After pre-incubation, the slurries were flushed with N_2 consecutively six times to purge the air in the mixture of residual O_2 and CH_4 (Cai et al., 2009). Then, they have been incubated anaerobically in the dark at 25 ℃ for 34 days in the absence (no CH_3F addition, treatment control) and the presence of CH_3F [the gas headspace of bottles was supplemented with 1% (treatment 1%) and 2% (treatment 2%)] in triplicates. Notably, acetoclastic methanogens have been described to be inhibited at much lower concentrations of CH_3F than hydrogenotrophic methanogens (Conrad and Klose, 1999). Therefore, when CH_3F is added at a low concentration (0.5–2%), it is generally considered to be an inhibitor of acetoclastic methanogenesis (Conrad and Klose, 1999; Ji et al., 2018a; b).

2.3 Sample collection and analyses

Gas samples were collected every three to four days during the 34-day incubation. The CH_4 and CO_2 concentrations were analyzed with a gas chromatograph (GC) (Agilent 7890B, USA) equipped with a flame ionization detector (FID). After 34 days of incubation, gas samples were collected to

144 analyze $\delta^{13}C$ of CH_4 and CO_2. The stable carbon isotopes of CH_4 and CO_2 were analyzed using the
145 continuous flow technique on a Finnigan MAT 253 Plus Isotope Ratio Mass Spectrometer (Thermo
146 Fisher Scientific, Waltham, USA).
147 Soil properties were determined following Soil Agro-Chemical Analyses procedures (Lu, 2000).
148 Soil pH was measured in a 1:2.5 (v/v) ratio of soil to water (deionized water). Total soil organic carbon
149 (SOC) was analyzed by wet digestion with H_2SO_4-$K_2Cr_2O_7$. Active Fe (Fe(III)) and Mn (Mn(IV)) were
150 extracted with $H_2C_2O_4$-$(NH_4)_2C_2O_4$, and were determined by Inductively Coupled Plasma Mass
151 Spectrometer (Nexion2000, America). NO_3^- and SO_4^{2-} were extracted in a 2 M KCl solution at a
152 soil/water ratio of 1:5, and measured using an ion chromatograph (ICS-5000+, America). Soil cation
153 exchange capacity (CEC) was determined after extraction with 1 mol L^{-1} ammonium acetate. Total
154 nitrogen (TN) was analyzed by an elemental analyzer (Vario MAX).
155 At the beginning and end of all incubation, soil samples were also collected and a portion of them
156 was analyzed DOC and acetic acid content by a total organic carbon/total nitrogen analyzer (Multi NC
157 3100) and by high-pressure liquid chromatography (HPLC) (ELSD/UV, Agilent HPLC 1260),
158 respectively. The other portion was freeze-dried and extracted for total DNA using a FastDNA® SPIN
159 Kit for Soil (MP Biomedicals LLC, USA). DNA concentration and quality were determined using a
160 NanoDrop spectrophotometer (Thermo Fisher Scientific, MA, USA). The abundances of *mcrA* gene
161 were measured by Majorbio (Shanghai, China) via Real-Time qPCR on an Applied Biosystems (ABI)
162 7300 Real-Time PCR system (Thermo Fisher Scientific, MA, USA).
163 The primer pairs mlas-mod/mcrA-rev were used to quantify the copy numbers of *mcrA* gene (Angel
164 et al., 2011). The PCR mixture consisted of 10 μL of ChamQ SYBR Color qPCR Master Mix (2×), 0.8
165 μL of each primer, 0.4 μL of ROX Reference Dye 1 (50×), and 2 μL of DNA template, brought to a
166 final volume of 6 μL with sterile water. The primers MLfF and MLfR were used to amplify the *mcrA*
167 gene fragments targeting the 469–490 bps region. Then a library was constructed by pooling equal
168 amounts of individual barcoded amplicons of *mcrA* and sequenced on an ILLUMINA MISEQ PE250
169 system using a 2 × 300 cycle combination mode by Majorbio (Shanghai, China).

2.4 Bioinformatics analysis

After the sequencing was completed, the raw bacterial sequences were processed using the QIIME pipeline (Caporaso et al., 2010). All reads were subjected to quality control, de novo chimera filtering, singleton filtering, and operational taxonomic units (OTUs) clustering according to the UPARSE pipeline. The effective sequences were clustered into OTUs using a 97% identity threshold, and the chimeras were detected and deleted via USEARCH (Edgar et al., 2011). Sequences that cannot be classified into any known group were considered unclassified, and groups with < 1% average proportion were merged into the "others" taxa. All sequences have been deposited in the NCBI sequence Read Archive (SRA) database under the accession number PRJNA842891.

2.5 Calculations

2.5.1 CH_4 production potential and soil oxidation capacity

CH_4 production potential (MPP) under anaerobic incubation was determined, which was calculated by the following equation (Zhang et al., 2011; Zhang et al., 2010):

$$P = \mathrm{d}c/\mathrm{d}t \times V_H/W_S \times M_r/M_V \times 273/(273+25) \quad (1)$$

where P is MPP ($\mu g\ g^{-1}\ d^{-1}$), $\mathrm{d}c/\mathrm{d}t$ is the rate of CH_4 accumulation ($\mu L\ L^{-1}\ d^{-1}$), V_H is the headspace volume of the serum bottle (L), W_S is dry soil weight (g), M_r is the relative molecular mass of CH_4 (g), M_V is the gas volume of an ideal gas (L).

Soil oxidation capacity (OXC) was calculated using a modification of the equation given by Zhang et al. (2009b):

$$OXC = 5[NO_3^-] + 2[Mn(IV)] + [Fe(III)] + 8[SO_4^{2-}] \quad (2)$$

where brackets denote molar concentrations ($mol\ kg^{-1}$).

2.5.2 The relative contribution of acetoclastic methanogenesis

The relative contribution of CO_2/H_2 and CH_3COOH to CH_4 production in paddy soils was determined by applying the stable carbon isotope method (Sugimoto and Wada, 1993), which assumed that total CH_4 production was equal to the sum of acetate fermentation ($CH_{4(ac)}$) and CO_2/H_2 reduction ($CH_{4(CO_2)}$):

$$CH_4 = CH_{4(ac)} + CH_{4(CO_2)} \quad (3)$$

The contribution of acetate to total CH_4 production (f_{ac}) was calculated by the following (Sugimoto

198 and Wada, 1993):

199 $$f_{ac} = CH_{4(ac)} / [CH_{4(ac)} + CH_{4(CO_2)}] \times 100\% \quad (4)$$

200 The $\delta^{13}CH_4$ of the evolved CH_4 primarily depended on the relative contribution of the two main
201 methanogenic pathways, according to the isotopic mass balance (Tyler et al., 1997):

202 $$\delta^{13}CH_4 = \delta^{13}CH_{4(ac)} \times f_{ac} + \delta^{13}CH_{4(CO_2)} \times (1 - f_{ac}) \quad (5)$$

203 where $\delta^{13}CH_{4(ac)}$ refers to $\delta^{13}C$ values of CH_4 produced by acetate; $\delta^{13}CH_{4(ac)}$ is assumed a fixed value
204 (Bilek et al., 1999; Nakagawa et al., 2002; Sugimoto and Wada, 1993; Tyler et al., 1997) or varies
205 within a certain range (Sugimoto and Wada, 1993), which assumed to be –37‰ and –43‰ (Zhang et
206 al., 2012; Zhang et al., 2016).

207 $\delta^{13}CH_{4(CO_2)}$ referred to $\delta^{13}C$ values of CH_4 produced by CO_2/H_2 reduction, which was calculated by
208 the following equation (Sugimoto and Wada, 1993):

209 $$\delta^{13}CH_{4(CO_2)} = (\delta^{13}CO_2 + 1000)/\alpha_{(CO_2/CH_4)} - 1000 \quad (6)$$

210 where $\delta^{13}CH_{4(CO_2)}$ is calculated from $\delta^{13}C$ (‰) and $\alpha_{(CO_2/CH_4)}$ for CO_2 obtained from anaerobic
211 incubation of soils without the addition of CH_3F (Fig. S1). The carbon isotope fractionation factor
212 during H_2/CO_2 reduction ($\alpha_{(CO_2/CH_4)}$) for methanogenesis was defined by Nakagawa et al. (2002):

213 $$\alpha_{(CO_2/CH_4)} = (\delta^{13}CO_2 + 1000)/[\delta^{13}CH_{4(CO_2)} + 1000] \quad (7)$$

214 where $\delta^{13}CH_{4(CO_2)}$ is the $\delta^{13}C$ of CH_4 obtained by anaerobic incubation of soil with the addition of
215 CH_3F (Fig. S1).

216 **2.6 Statistical analysis**

217 The correlations between different parameters were assessed using Spearman's correlation analysis.
218 Differences in MPP, $\delta^{13}C$ of CH_4 production, *mcrA* gene abundance, and the relative abundance of
219 dominant methanogens among treatments were analyzed using the one-way analysis of variance
220 (ANOVA). The alpha diversity of methanogens was evaluated by Shannon index and Chao 1
221 estimator. Redundancy analysis (RDA) was used to study the relationship between methanogenic
222 community composition and environmental factors. Analysis of similarities (ANOSIM) was
223 performed to determine the differences between and within groups, and the Bray-Curtis dissimilarity
224 matrix was used to perform nonmetric multidimensional scaling analyses (NMDS). Network analysis

about methanogens associations based on the Spearman correlation was performed using Microeco bioinformatics cloud (https://bioincloud.tech/).

3. Results

3.1 Soil properties

The physicochemical properties of three cropping mode paddy soils were shown in Table 1. RF soil was alkaline clay soil (pH = 7.70), and RW and DR soils were acidic silt and sandy soils, respectively. The lowest soil pH (4.89) was detected in DR soil. RF soil possessed higher SOC, TN, clay, Mn(IV), NO_3^-, SO_4^{2-}, and CEC content, while it showed much lower sand and Fe(III) content than that of the other two soils. DR soil possessed little clay, Mn(IV), SO_4^{2-},OXC, and CEC content, and RW soil possessed little NO_3^- content. Spearman correlation coefficients indicated that soil pH was positively correlated with CEC, and soil clay content ($p < 0.01$, Fig. 1).

3.2 The cumulative concentration of CH_4 production and MPP

The cumulative concentration of CH_4 production increased rapidly in the early stage of incubation, then gradually stabilized in the later stage of incubation (Fig. 2A–C). However, it varied in paddy soils with different cropping modes, with the maximum and minimum peak values of CH_4 production cumulative concentrations in DR (150 460 μmol mol^{-1}) and RF (80 493 μmol mol^{-1}), respectively. Compared to the control treatment, the addition of 1% and 2% CH_3F resulted in a pronounced partial inhibition of CH_4 production, significantly reducing CH_4 production cumulative concentration (Fig. 2A–C). DR soil had the highest MPP at 30.7 μg g^{-1} d^{-1}. Compared with DR soils, the MPP of RW and RF soils was lower by 57% and 66%, respectively ($p < 0.05$, Fig. 2D). The MPP of different cropping modes with 1% and 2% CH_3F treatment ranged from 0.98 to 7.51 μg g^{-1} d^{-1}. Moreover, MPP negatively correlated with soil pH and soil clay content ($p < 0.01$, Fig. 1).

3.3 Soil DOC and acetate concentration

The contents of DOC and acetate before incubation in the three cropping modes were 181.1 to 443.4 mg kg^{-1} and 28.24 to 157.3 mg kg^{-1}, respectively (Fig. 3). In addition, the DOC and acetate contents decreased significantly after incubation ($p < 0.05$). The highest DOC content was observed in DR soil (255 mg kg^{-1}), which was 51% and 17% higher than that in RW and RF soils, respectively ($p < 0.05$, Fig. 3A). The DOC contents of all cropping modes increased with the CH_3F addition ($p < 0.05$). The

acetate content ranged from 5.68 to 7.19 mg kg^{-1}, with the highest content in DR and the order by DR > RW > RF ($p > 0.05$, Fig. 3B). Similar to DOC contents, the acetate contents of all cropping mode soils increased with the CH_3F addition ($p < 0.05$).

3.4 The $\delta^{13}CH_4$, $\delta^{13}CO_2$, $\alpha_{(CO_2/CH_4)}$, and f_{ac} values

After anaerobic incubation, the values of $\delta^{13}CH_4$ in paddy soils under different cropping modes ranged from –54.4‰ to –43.9‰, and the values of $\delta^{13}CO_2$ ranged from –26.3‰ to –18.7‰ (Table 2). RF soil had the highest $\delta^{13}CH_4$ value, which was more positive than RW and DR soils by 10.5‰ and 7.8‰, respectively ($p < 0.05$). However, it exhibited a much lower $\delta^{13}CO_2$ value in RF soil, being more negative than RW and DR by 7.6‰ and 5.2‰, respectively ($p < 0.05$). Both $\delta^{13}CH_4$ and $\delta^{13}CO_2$ values in the addition of CH_3F were far lower than that of the control treatment ($p < 0.05$), with values of –82.3‰ to –68.0‰ and –28.2‰ to –21.1‰, respectively.

The values of $\alpha_{(CO_2/CH_4)}$ in RW, RF, and DR soils ranged from 1.049 to 1.050 and from 1.057 to 1.062 in the addition of 1% and 2% CH_3F, respectively (Table 2). Compared to DR soil, the $\alpha_{(CO_2/CH_4)}$ values of RW and RF soils were significantly lower ($p < 0.05$). The f_{ac} values for RW, RF, and DR soils ranged from 39% to 60%, from 80% to 98%, and from 52% to 75% when $\delta^{13}CH_{4(ac)}$ values were –37‰ to –43‰, respectively. There were significant differences in f_{ac} depending on the cropping mode, and f_{ac} values for RF soil were much larger than those for RW and DR soils ($p < 0.05$).

3.5 The abundance and community composition of methanogens

RF soil had the lowest *mcrA* gene abundance among the three cropping modes (Fig. 4). Compared to the RF soil, the abundance of *mcrA* gene in RW and DR soils increased by 29% and 40% ($p < 0.05$), respectively. The addition of CH_3F significantly reduced *mcrA* gene abundance by 51–90% ($p < 0.05$). The abundance of *mcrA* gene had a strong positive correlation with the MPP, while significantly negatively correlated with soil pH, CEC, and clay content ($p < 0.001$, Fig. 1).

The number of OTUs in a single sample ranged from 168 to 275 (Table 3). Further, the Shannon indices and Chao 1 estimators were calculated to evaluate the richness and diversity of methanogens in soils under different cropping modes. A significant difference in Shannon indices was observed in RF soil compared with RW and DR soils ($p < 0.05$). The number of OTUs and Chao 1 estimator in RW

soil was significantly higher than that in RF and DR soils ($p < 0.05$). Correlation analysis showed that the number of OTUs and Shannon index negatively correlated with the MPP ($p < 0.01$), whereas the Chao 1 estimator positively correlated with f_{ac} ($p < 0.05$, Fig. 1).

The NMDS based on Bray-Curtis distances indicated that there was a significant variation in the community structure of methanogens in paddy soils under the three different cropping modes (Fig. S2, $p < 0.001$). To more completely interpret the composition of the methanogenic community under three cropping modes, the relative abundance of methanogens at which was given as the percentage of different methanogens in total methanogens provided (Figs. 5 and S3). No matter at order, family, and genus levels, the methanogens in different cropping mode soil samples varied in community composition, but each cropping mode with different CH_3F addition possessed similar community composition. At the order level (Fig. S3A), the dominant methanogens in paddy soils under different cropping modes mainly included *Methanosarcinales* (15–91%), *Methanobacteriales* (3–38%), *unclassified_p_Euryarchaeota* (4–21%), *norank_p_Euryarchaeota* (1–22%), *Methanocallales* (1–4%), *Methanomassiliicocales* (1–3%), as well as *Methanmicrobiales* (1–2%). At the family level (Fig. S3B), five known methanogen communities dominated in paddy soils under different cropping modes: *Methanosarcinaceae*, *Methanobacteriaceae*, *Methanocellaceae*, *Methanomassiliicoccaceae*, and *Methanotrichaceae*. The composition of methanogens differed significantly at the genus level under different cropping modes. For instance, *Methanosarcina* was the dominant methanogen in RW and RF soils, while *Methanobacterium* was in DR soils (Fig. 5). Furthermore, the relative abundance of *Methanosarcina* was negatively correlated with MPP and *mcrA* gene abundance, while positively correlated with soil pH (Fig. S4). The relative abundance of *Methanobacterium* positively correlated with MPP and *mcrA* gene abundance ($p < 0.05$), but negatively correlated with soil pH ($p < 0.05$).

The bio-plot of RDA analyses illustrated that the eight environmental factors explained 84.11% of the cumulative variance for the first principal component, and more importantly, the soil pH had significant effects on the variation of methanogenic community structure (Fig. 6). Network analysis showed, compared with RW and DR soils, the number of lines of methanogens strongly increased in RF soil (Fig. 7, Table S2), which meant that the interaction between methanogens in RF soil was closer than the other two soils. In addition, *Methanosarinales* had the highest relative abundance in all

paddy soils and were negatively associated with most methanogens, especially in RW and RF soils.

4. Discussion

4.1 Effect on MPP

MPP in double-season rice fields (RW and DR) was significantly higher than in single-season rice (RF) fields (Fig. 2D). The possible reason was that the abundance of methanogens in RW and DR soils was higher than that in RF soil (Fig. 4). Soil organic matter and soil organic acids are the important precursors of methanogenesis for consumption by methanogens (Ding and Cai, 2002; Glissmann and Conrad, 2002). They are mainly derived from carbon input, e.g., root stubble and the content of root exudate that is affected by cropping modes (Jiang et al., 2022). In theory, both DR and RW were planted with crops harvested twice a year (Zhang et al., 2017), resulting in more readily available root stubble and root exudate than RF soil. As such, the large carbon sources provided an abundant substrate for CH_4 production, resulting in higher MPP in DR and RW soils (Fig. 2D). More importantly, DR was characterized by flooding in both early and late rice seasons, while RW was in a dry-wet alternation of winter wheat and summer rice (Zhang et al., 2017). It was reported that flooded fields in the previous rice season would increase CH_4 production in the second season (Zhang et al., 2011), which supported the MPP in DR soil was higher than that in RW soil (Fig. 2D). Therefore, CH_4 emissions in RW soil (He et al., 2022; Zhang et al., 2017) was often observed to be much lower than those in DR soil (Wang et al., 2018; Zhong et al., 2021).

In addition to the different straw incorporation and water management, such cropping modes inevitably lead to significant differences in soil physicochemical properties, like as soil pH, soil texture, and so on (Table 1), which could affect CH_4 production by regulating methanogens (Wang, 2015). We found that soil pH was negatively correlated with *mcrA* gene abundance and the relative abundance of most methanogens (Figs. 1 and S4), suggesting that soil pH probably affects MPP by regulating the abundance and community composition of methanogens (Dubey et al., 2013). Generally, different methanogens have certain differences in soil pH preference, and the optimal pH for *Methanosarcinales* and *Methanobacteriales* is from 4.16 to 4.38 and from 3.64 to 4.04, respectively (Galand et al., 2003; Yavitt et al., 2006). Thus, in alkaline RF soil, *Methanosarcinales* and *Methanobacteriales* were also the dominant methanogens (Fig. S3A), and the abundance of *mcrA* gene

was far lower than that in acid RW and DR soils (Fig. 4), which might be one of the reasons for the lower MPP of alkaline RF soil.

Soil texture might be another influencing factor for the MPP. First, clay soil has a strong retention effect on the organic matter resulting in a low supply of organic substrate for methanogens, reducing CH_4 production and emission (Kim et al., 2018; Mitra et al., 2002). Indeed, the MPP was negatively correlated with soil clay content, and the highest soil clay content in RF had the lowest MPP (Figs. 1 and 2). Second, Mitra et al. (2002) showed that soil CEC affected different redox indices by stepwise multiple regression analyses, and then had a significant effect on MPP. Soil CEC also affects CH_4 oxidation by mediating the NH_4^+ behavior. Low soil CEC leads to a weaker soil buffering capacity and a lower NH_4^+ concentration, which has a strong inhibitory effect on the microbial oxidation of CH_4 (De Visscher et al., 1998). Therefore, these reasons might explain the negative correlation between soil CEC and MPP in this study (Fig. 1). Third, the higher OXC values usually maintain higher levels of redox potential (Eh) in paddy soil (Zhang et al., 2009b), which does not conducive to CH_4 production (Zhang et al., 2015). The OXC of RW and RF soils was significantly higher than that of DR soil (Table 1) further verifying the lower MPP of RW and RF soils than DR soil in this study (Fig. 2D).

4.2 Effect on methanogenic pathway

In consistency with our hypothesis, f_{ac} values were different under various cropping modes (Table 2), which might due to the various substrate content among different cropping modes. Indeed, in paddy fields, acetate was the most important substrate for the acetoclastic methanogens pathway (Ji et al., 2018b). In our study, both DOC and acetate contents in RW soil were lowest, and the availability of substrates for acetoclastic methanogens resulted in the lowest f_{ac} value (Fig. 3, Table 2). Furthermore, the content of acetate in RF soil was the highest among the three soils, and RF soils had the highest SOC (Table 1) with large number carbon, which also could provide rich methanogenic substrates for acetoclastic methanogens (Luo et al., 2022).

In this study, the acetoclastic methanogen *Methanosaeta* was the dominant methanogen in RF soil, and the f_{ac} value of RF alkaline soil was highest relative to those of RW and DR acidic soils (Table 2). Lee et al. (2014) and Yang et al. (2021) reported that *Methanosaeta* was the dominant methanogen in soils with soil pH of 6.0 to 8.0. Additionally, Liu and Ding (2011) also found that hydrogenotrophic

methanogens were dominant under acidic conditions. Thus, the f_{ac} decreased in acidic DR soil dominated by hydrogenotrophic methanogens, while increased in alkaline RF soil dominated by acetoclastic methanogens (Table 2). Our results were consistent with previous studies and further demonstrated that the f_{ac} was affected by soil pH by influencing the compositions of methanogens (Lee et al., 2014; Liu and Ding, 2011).

Based on the results of previous studies in Thailand, the USA, Japan, Italy, and China, f_{ac} values of paddy soils varied with different cropping modes, ranging from 10% to 108% (Table S1). Our results showed that f_{ac} values of the three cropping modes were all within the range of these studies, but there were some differences between the f_{ac} values of our study and previous studies under the corresponding cropping modes (Table 2). For instance, compared with the corresponding DR mode (Yingtan) in China, the f_{ac} values in Thailand paddy were higher (Table S1), which may be due to the enhanced activity of methanogens and the promoted methanogenic pathway as affected by higher temperature in Thailand (Fey and Conrad, 2000). In addition, soil texture could change the microbial community structure and function (Zhang et al., 2007), which was probably related to the differences in soil organic carbon and soil carbon-nitrogen ratio in different soil grain size components (Zhou et al., 2002). Xiao et al. (2019) reported that *Methanosarcina* had high activity in clay soil, which might be the reason that f_{ac} values were highest in clay RF soil (Table 2). Thus, differences in soil texture under the same cropping mode might also contribute to differences in methanogenic pathways (Allison and Prosser, 1991; Qian and Cai, 2010).

Generally, $\delta^{13}CH_{4(ac)}$ and $\alpha_{(CO_2/CH_4)}$ values have a strong influence on f_{ac} values (Zhang et al., 2009a). Numerous studies have shown that $\delta^{13}CH_{4(ac)}$ values measured in paddy soils are usually in the range of –43‰ to –37‰ (Conrad et al., 2002; Fey et al., 2004; Krüger et al., 2002; Nakagawa et al., 2002), and the $\alpha_{(CO_2/CH_4)}$ value was usually assumed as a fixed value (Bilek et al., 1999; Conrad et al., 2002; Nakagawa et al., 2002). However, the $\alpha_{(CO_2/CH_4)}$ value in paddy soils varied greatly with the differences in soil properties and soil carbon isotope in different cropping modes (Zhang et al., 2012; Zhang et al., 2009a). In this study, the $\alpha_{(CO_2/CH_4)}$ value in paddy soil under different cropping modes was calculated using Eq. (7) by the CH_3F inhibitor method (Fig. S1), ranging from 1.049 to 1.062 (Table 2), which

was consistent with previous studies (Zhang et al., 2016). Therefore, our study provided a more detailed reference for soil $\alpha_{(CO_2/CH_4)}$ value in three typical cropping modes (RW, RF, and DR) in China. The f_{ac} values of RW, RF and DR were from 27% to 36%, from 78% to 96%, and from 44% to 57%, respectively, when the commonly $\alpha_{(CO_2/CH_4)}$ (1.045) selected to quantify the methanogenic pathways of different cropping modes (Fey et al., 2004). The results indicate that the relative contribution of acetate to total methanogenesis was mainly dependent on cropping modes rather than on the values of $\alpha_{(CO_2/CH_4)}$ cited.

4.3 Effect on methanogenic communities

A higher abundance of *mcrA* gene was observed in DR mode than that in RW and RF modes (Fig. 4). Previous studies have shown that DR mode had less soil carbon loss and higher SOC stock than RF and RF modes (Cha-un et al., 2017; Sun et al., 2019). Therefore, the higher abundance of *mcrA* gene in DR soil than in the other two soils, which possibly driven by nutrient availability due to the different soil properties among various cropping modes (Jiang et al., 2022). In theory, DR mode, a biannual rice cultivation mode, had higher residual carbon (stubble and root), organic acid remaining, and more favorable anaerobic conditions for methanogens in the soil. It will inevitably lead to the release of more liable carbon and acetate by residual decomposition (Jiang et al., 2022), thus potentially supplying sufficient available substrates for methanogen and increasing the abundance of *mcrA* gene. Furthermore, RF mode had less fertilizer application and less stubble compared with RW and DR modes, which may lead to fewer types and quantities of methanogenic substrates, and then affect the low diversity and abundance of methanogens (You et al., 2022), which is also confirmed by the low Shannon index and Chao 1 estimator observed in our study (Table 3).

The methanogenic community compositions varied among the three cropping modes, the dominant methanogen of RW was *Methanosarcina*, while the dominant methanogen of RF and DR soil were *Methanosarcina* and *Methanobacterium* (Fig. 5). The detected dominantly methanogens in the soils coincided with previous research in rice fields (Jiang et al., 2022; Wang et al., 2021). Among the different cropping modes, unique geographical features determined that the soil pH of the RF soil was significantly higher than that of RW and RF soils (Table 1). This indicated that different types of

methanogens might have different preferences for soil pH, with acetoclastic methanogens being more active under weak acidic and alkaline conditions and hydrogenotrophic methanogens being more active under acidic conditions (Kotsyurbenko et al., 2007). In this study, the relative abundance of acetoclastic methanogen (*Methaosarcina*) in RF alkaline soil was higher than that in RW and DR acidic soils, while the relative abundance of hydrogenotrophic methanogen (*Methanobacterium*) in RW and DR soils was higher than that in RF soil (Fig. 5). The findings indicated that differences in soil pH driven by cropping modes might be the underlying cause of methanogenic communities.

Different cropping modes have different water and fertilizer management and climatic conditions, which change the composition of methanogens and also affected the interaction of methanogens (Gu et al., 2022). Network analysis showed the RF soil had the most lines, indicating that the interaction of methanogens in both RW and DR soils was weaker than that in RF soil (Fig. 7, Table S2). The reason might be due to the anaerobic environment conducive to the retention of methanogens activities and the growth of methanogens in flooded RF soil (Xu et al., 2020; Zhang et al., 2017). The methanogens population could increase in flooded RF soil (Bhullar et al., 2013; Pavlostathis and Giraldo, 1991), thus forming a more complex microbial network. In addition, since only one crop is harvested in RF soil, N fertilizer application is much less than that in DR and RW soils in which two crops are harvested. This must theoretically lead to less root exudate. In other words, RF soil has less methanogenic substrate than both DR and RW soils. Interestingly, we found that RF soil had a higher SOC content (Table 1), but had relatively lower available organic carbon dominated by the unique clay characteristics (Ding and Cai, 2003). These results probably lead to an insufficient supply of methanogenic substrates for RF soils, which also explained the lowest MPP of RF soils among the three cropping modes (Fig. 2). Meanwhile, highly active *Methaosarcina* consumed large amounts of methanogenic substrates, resulting in a complex antagonistic interaction with other methanogens (Fig. 7).

The effect of CH_3F as an inhibitor on CH_4 oxidation and production in rice fields was first reported by Frenzel and Bosse (1996). Subsequently, Conrad and Klose (1999) systematically investigated the effects of different concentrations on CH_4 production and proposed that it inhibits only the acetoclastic methanogenesis in a certain concentration range, which could be well used for the study of the

methanogenesis pathway. However, how CH_3F inhibited acetoclastic methanogenesis by affecting methanogens had not been observed so far. We observed that the addition of CH_3F resulted in a strong decrease in the abundance of *mcrA* gene by one order of magnitude (Fig. 4) and in the relative abundance of acetoclastic methanogen (Fig. 5). The finding confirmed that CH_3F addition inhibited methanogenesis mainly by reducing the abundance of acetoclastic methanogen (Conrad and Klose, 1999; Ji et al., 2018a). However, 1% CH_3F addition significantly changed the relative abundances of *Methanosarcinales* and *Methanobacterials* in RF and DR soils, but not in RW soils (Fig. S3). Additionally, a 2% CH_3F addition significantly changed the relative abundances of *Methanosarcinales* and *Methanobacterials* in RW soil. The response of soil methanogens composition to CH_3F varied among cropping modes and the sensitivity of soil to CH_3F differed among cropping modes. These phenomena demonstrated that the difference in the inhibitory effect of CH_3F addition probably attributes to the difference in acetoclastic methanogens under different cropping modes.

5. Conclusion

Our findings suggest that the potential of CH_4 production, the pathway of methanogenesis, and the composition of methanogenic community were different under the three typical rice cropping modes in China. The carbon isotope fractionation during hydrogenotrophic methanogenesis was 1.049–1.062, making the acetoclastic methanogenesis in paddy soils range from 39% to 98%. The CH_4 production potential was largely controlled by soil pH and *mcrA* gene abundance while the relative contribution of acetoclastic methanogenesis was probably affected by soil pH and methanogenic community. Moreover, it was found that CH_4 production potential was positively correlated with the abundance of *mcrA* gene and *Methanobacterium* (hydrogenotrophic methanogens), respectively. More importantly, soil pH was negatively correlated with the abundance of *mcrA* gene while positively related to *Methanosarcina* (acetoclastic methanogens). The results indicated that both CH_4 production potential and acetoclastic methanogenesis would mainly be regulated by the abundance and community of methanogens driven by soil pH under the different rice cropping modes.

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Conflict of Interest

The authors declare no conflict of interest relevant to this study.

Authors' contributions

Wanyu Shen wrote the main manuscript text, Yang Ji and Guangbin Zhang modified the manuscript, Qiong Huang and Xiaoli Zhu assisted with data analysis, Jing Ma and Hua Xu supervised the data. All authors read and approved the manuscript.

Data Availability Statement

Research data are not publicly shared, please contact corresponding author for enquiries.

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Table 1 The physicochemical properties of paddy soils in the three typical rice cropping modes in

683 China. Data are presented as the mean ± SD (n = 3).

684

685 **Table 2** Values of $\delta^{13}CH_4$, $\delta^{13}CO_2$, $\alpha_{(CO_2/CH_4)}$, $\delta^{13}CH_{4(CO_2)}$, and f_{ac} in three cropping mode paddy soils.
686 Data are presented as the mean ± SD (n = 3). The 1% and 2% indicate the proportions of CH_3F
687 addition. Different uppercase and lowercase letters denote significant differences among different
688 cropping modes and different proportions of CH_3F added treatment at $p < 0.05$, respectively. [a, b, c]
689 These values were calculated under different proportions of CH_3F addition with Equations 7, 6, and 5,
690 respectively.

691

692 **Table 3** Alpha diversity of *mcrA* gene in paddy soils under different cropping modes. Data are
693 presented as the mean ± SD (n = 3). Different uppercase and lowercase letters denote significant
694 differences among different cropping modes and different proportions of CH_3F added treatment at $p <$
695 0.05, respectively. The 1% and 2% indicate the proportions of CH_3F addition.

696

697 **Fig. 1** The heatmap of correlations among soil physicochemical properties, soil oxidation capacity
698 (OXC), CH_4 production potential (MPP), and the diversity and abundance of *mcrA* gene in paddy soils
699 under different cropping modes. Red circles represent the positive correlation, blue circles represent
700 the negative correlation, and the size of the solid circle represents the size of the correlation
701 coefficient. *, **, and *** represent significant differences between each two variables at $p < 0.05$, $p <$
702 0.01, and $p < 0.001$, respectively.

703

704 **Fig. 2** The cumulative concentration of CH_4 production (A, RW; B, RF; and C, DR) and MPP (D) in
705 paddy soils under different cropping modes. Data are presented as the mean ± SD (n = 3). Different
706 uppercase and lowercase letters denote significant differences among different cropping modes and
707 different treatments at $p < 0.05$, respectively. The 1% and 2% indicate the proportions of CH_3F
708 addition.

709

710 **Fig. 3** The contents of DOC (A) and acetate (B) in paddy soils under different cropping modes. Data

711 are presented as the mean ± SD (n = 3). Different uppercase and lowercase letters denote significant
712 differences among different cropping modes and different proportions of CH_3F added treatment at $p <$
713 0.05, respectively. The 1% and 2% indicate the proportions of CH_3F addition, BI represents the before
714 the inducation experiment.
715
716 **Fig. 4** The abundance of *mcrA* gene in paddy soils under different cropping modes. Data are presented
717 as the mean ± SD (n = 3). Different uppercase and lowercase letters denote significant differences
718 among different cropping modes and different proportions of CH_3F added treatment at $p < 0.05$,
719 respectively. The 1% and 2% indicate the proportions of CH_3F addition.
720
721 **Fig. 5** Relative abundance of methanogens under different cropping modes at the genus level. Data are
722 presented as the mean ± SD (n = 3). The 1% and 2% indicate the proportions of CH_3F addition.
723
724 **Fig. 6** Redundancy analysis (RDA) ordination plots showing the relationship between the community
725 structure of methanogenic and environmental factors in paddy soils under different cropping modes at
726 genus level. The 1% and 2% indicate the proportions of CH_3F addition.
727
728 **Fig. 7** Network analysis of the correlation of methanogens in paddy soils under different cropping
729 modes (A, RW; B, RF; and C, DR). Each circle indicates a different individual. The node size
730 represents the node degree (a larger size indicates a higher degree). The color of the lines represents
731 positive (red) or negative (dark cyan) correlation. Only significant correlations ($p < 0.05$) are shown.
732

Figure 1.

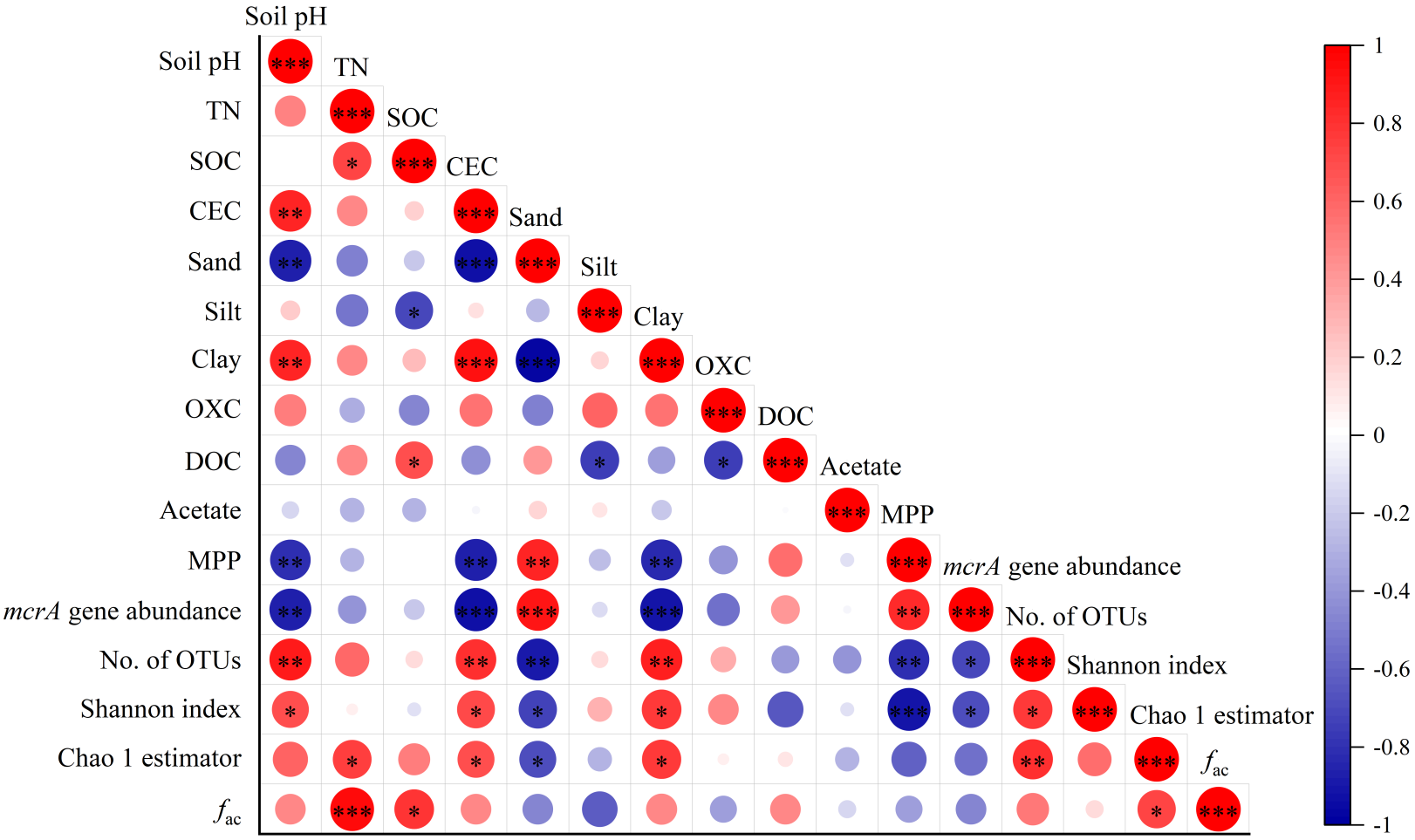
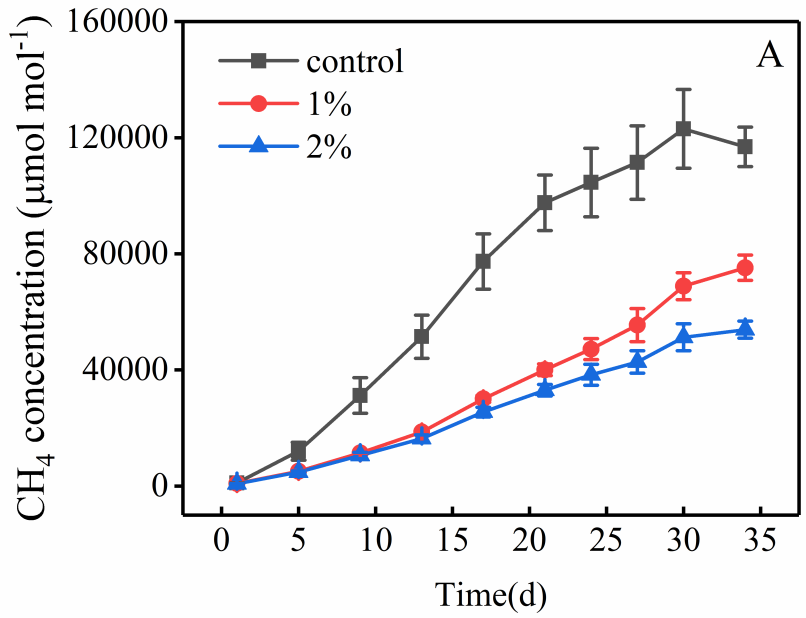
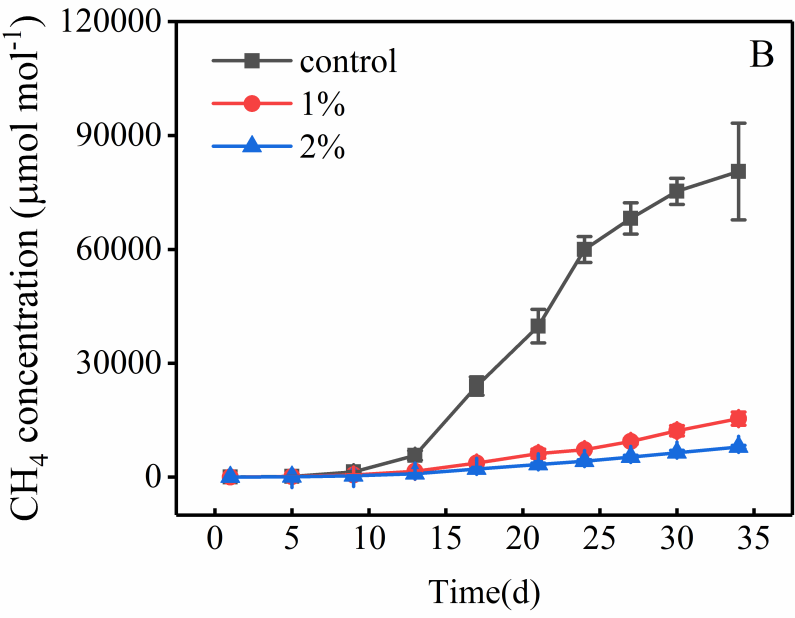
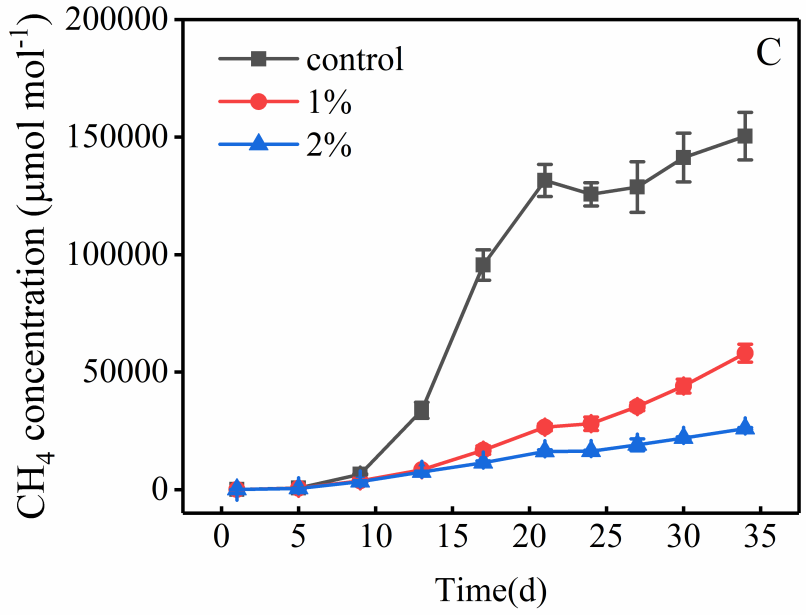

Soil pH
TN
SOC
CEC
Sand
Silt
Clay
OXC
DOC
Acetate
MPP
mcrA gene abundance
No. of OTUs
Shannon index
Chao 1 estimator
f_ac
1
0.8
0.6
0.4
0.2
0
-0.2
-0.4
-0.6
-0.8
-1

Figure 2.


A
control
1%
2%
CH4 concentration (μmol mol-1)
Time(d)
0
40000
80000
120000
160000
0
5
10
15
20
25
30
35


B
control
1%
2%
CH4 concentration (μmol mol-1)
Time(d)
0
30000
60000
90000
120000
0
5
10
15
20
25
30
35


C
control
1%
2%
CH4 concentration (μmol mol-1)
Time(d)
0
50000
100000
150000
200000
0
5
10
15
20
25
30
35

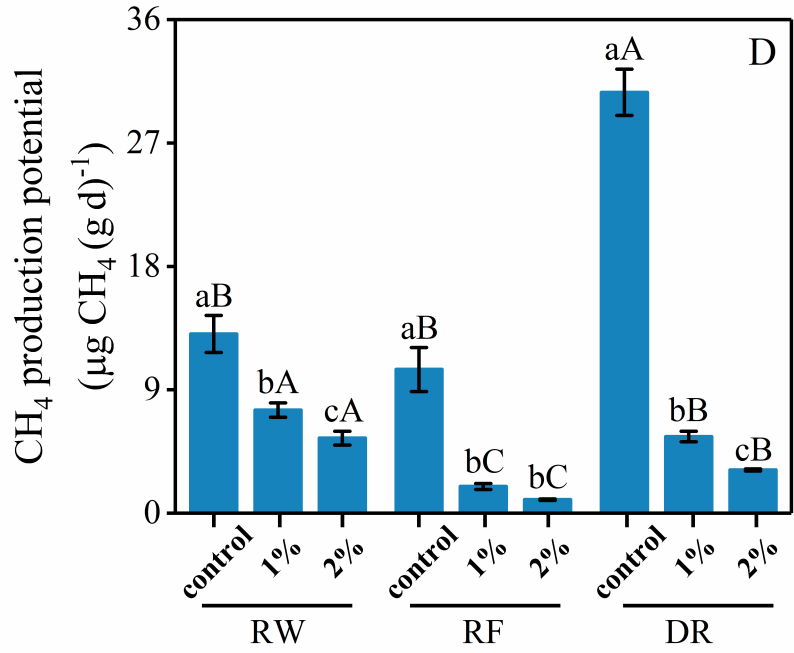
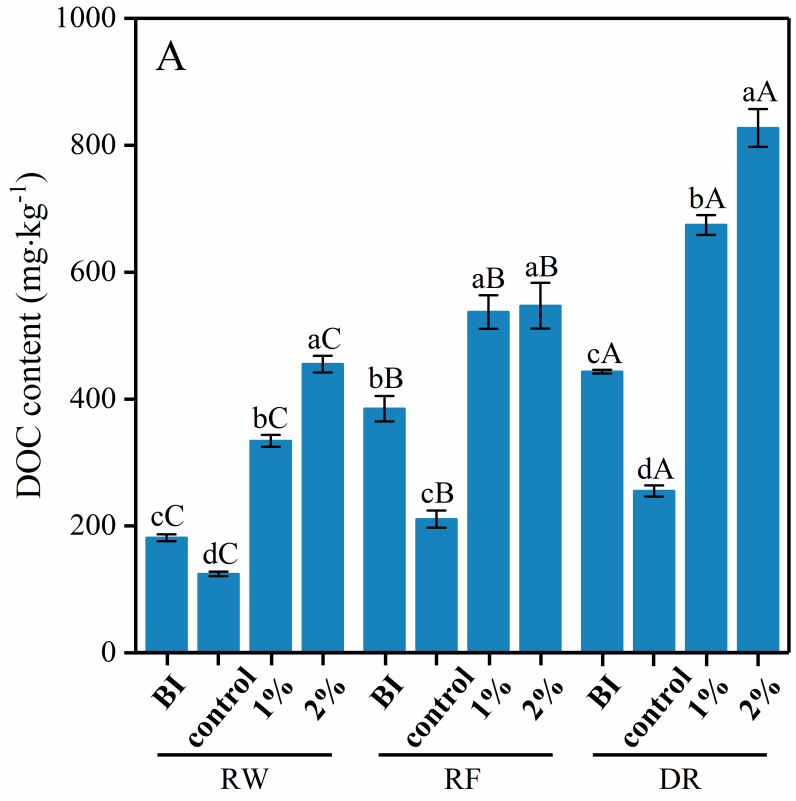

D
CH4 production potential
(μg CH4 (g d)-1)
0
9
18
27
36
aB
bA
cA
aB
bC
bC
aA
bB
cB
control
1%
2%
control
1%
2%
control
1%
2%
RW
RF
DR

Figure 3.


A
DOC content (mg·kg-1)
1000
800
600
400
200
0
cC
dC
bC
aC
bB
cB
aB
aB
cA
dA
bA
aA
BI
control
1%
2%
RW
RF
DR

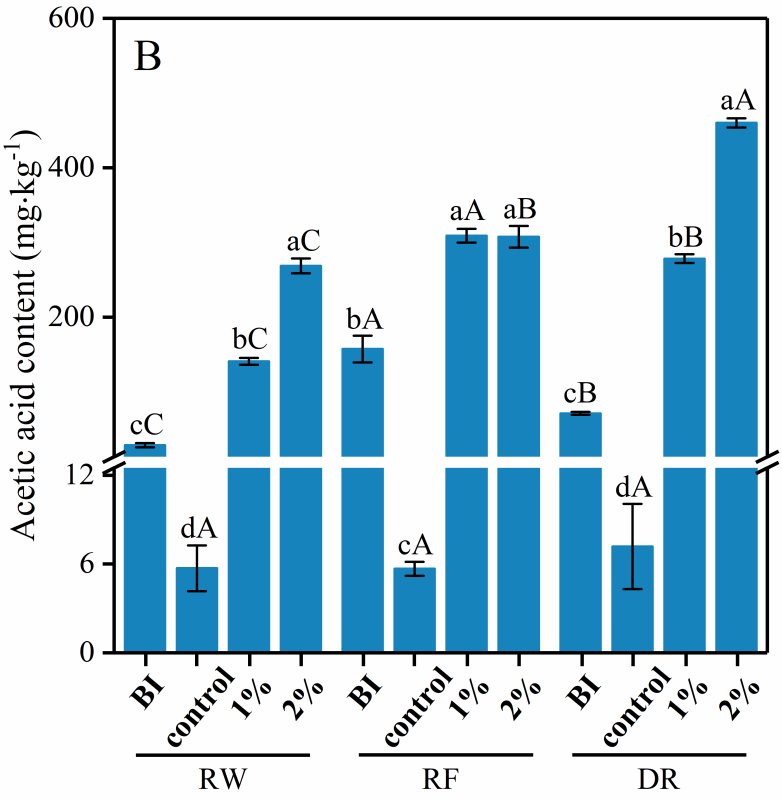

B
Acetic acid content (mg·kg-1)
600
400
200
12
6
0
cC
dA
bC
aC
bA
cA
aA
aB
cB
dA
bB
aA
BI
control
1%
2%
RW
RF
DR

Figure 4.

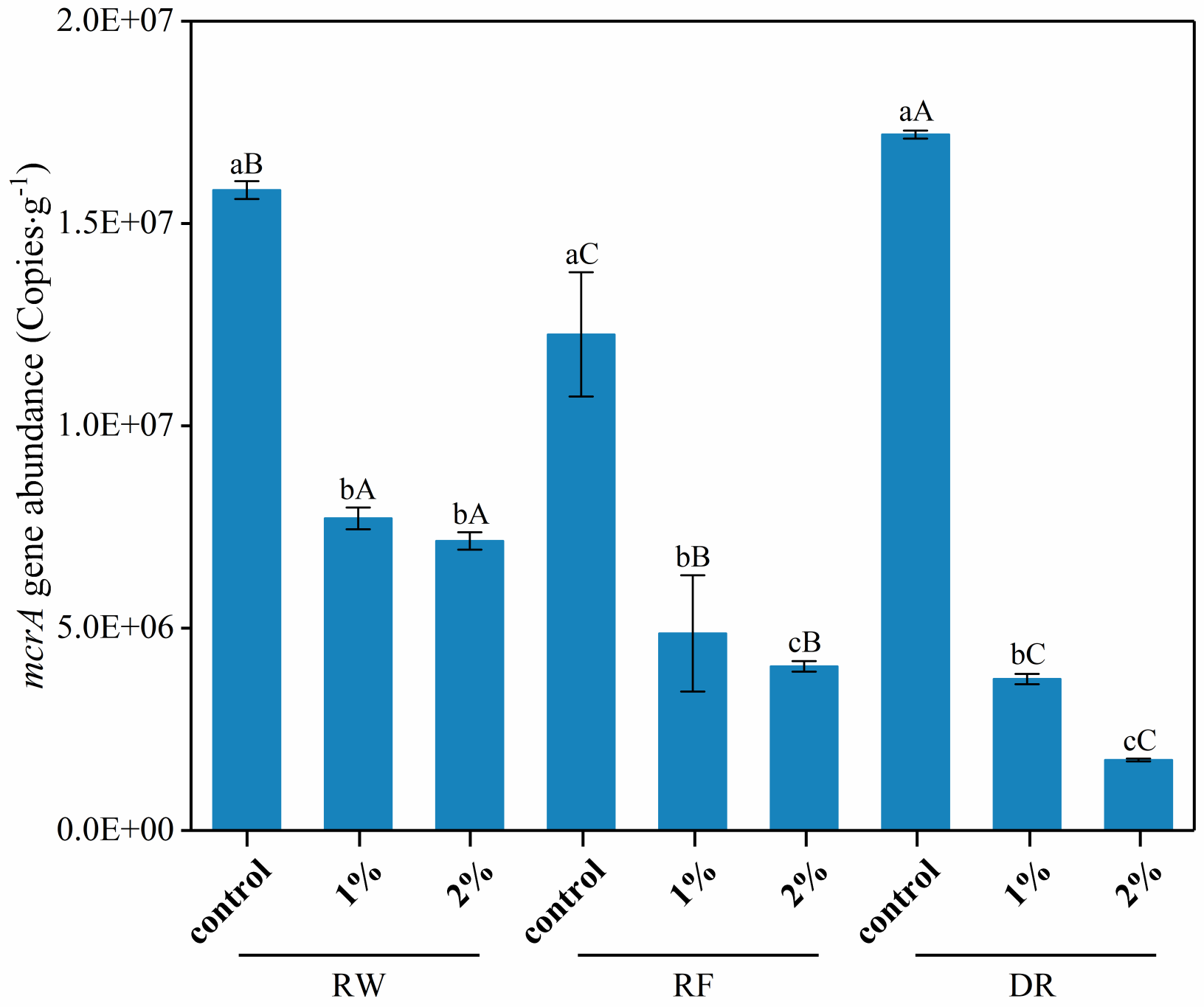

2.0E+07
1.5E+07
1.0E+07
5.0E+06
0.0E+00
mcrA gene abundance (Copies·g-1)
aB
bA
bA
aC
bB
cB
aA
bC
cC
control
1%
2%
control
1%
2%
control
1%
2%
RW
RF
DR

Figure 5.

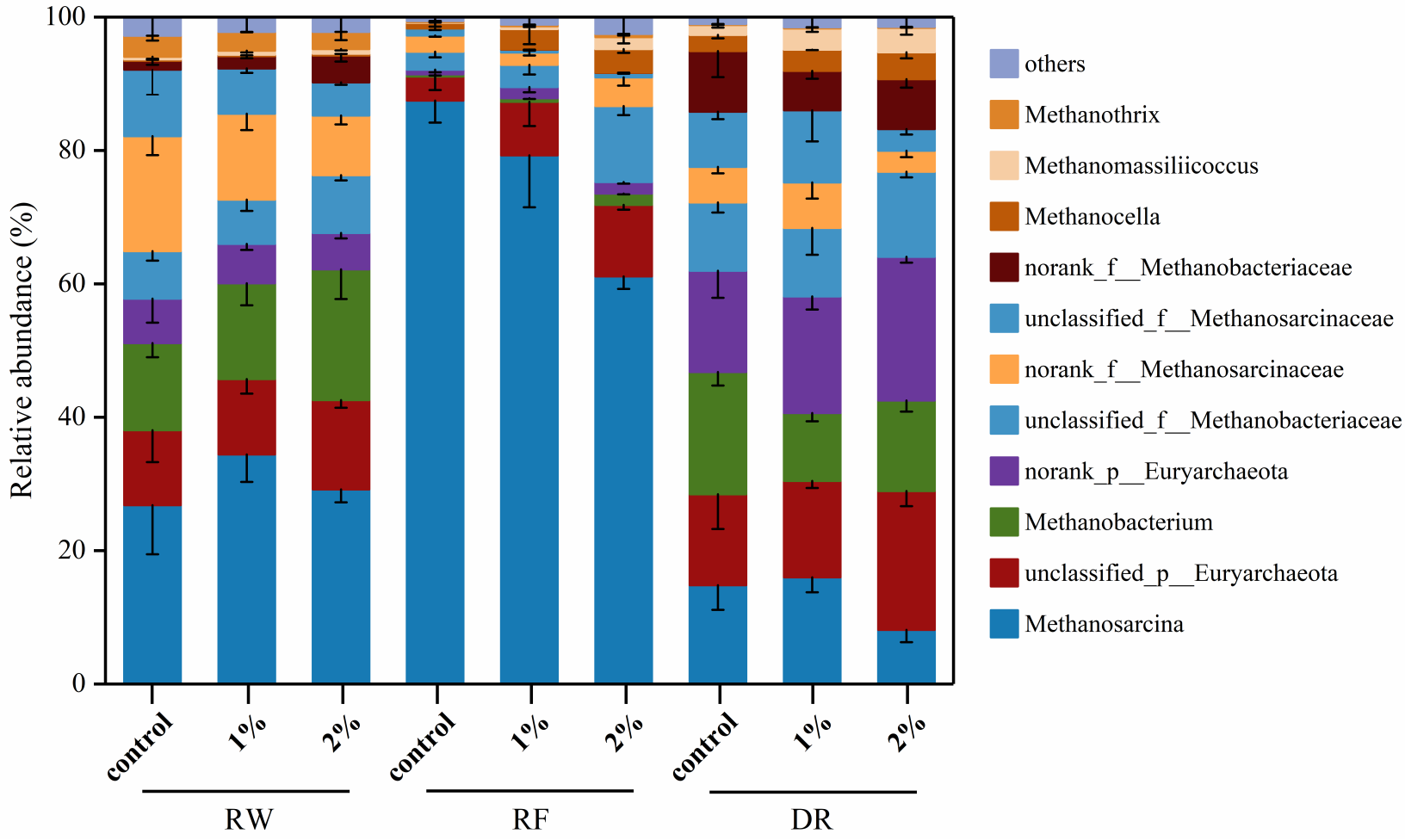

Relative abundance (%)
100
80
60
40
20
0
control
1%
2%
control
1%
2%
control
1%
2%
RW
RF
DR
others
Methanothrix
Methanomassiliicoccus
Methanocella
norank_f__Methanobacteriaceae
unclassified_f__Methanosarcinaceae
norank_f__Methanosarcinaceae
unclassified_f__Methanobacteriaceae
norank_p__Euryarchaeota
Methanobacterium
unclassified_p__Euryarchaeota
Methanosarcina

Figure 6.

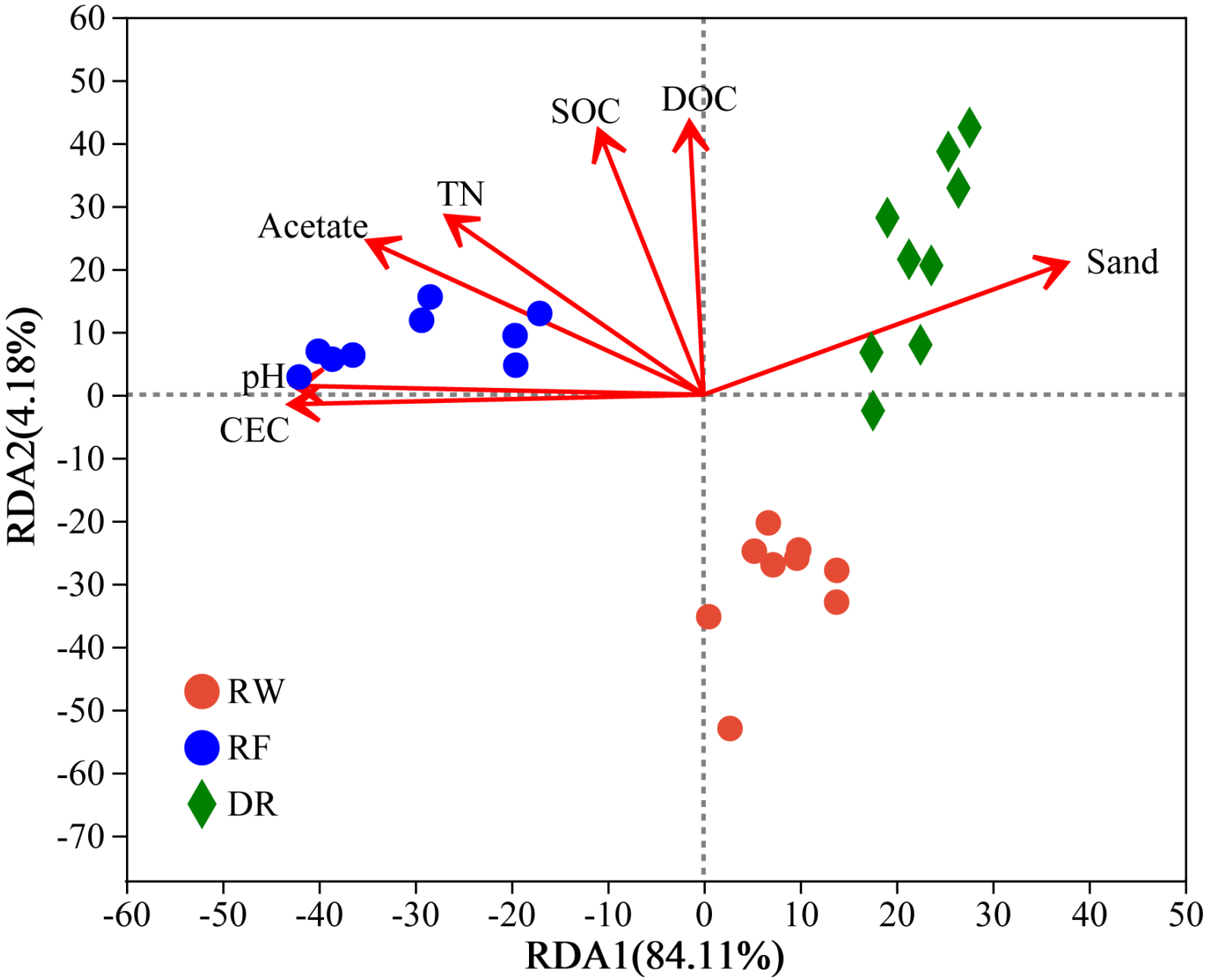
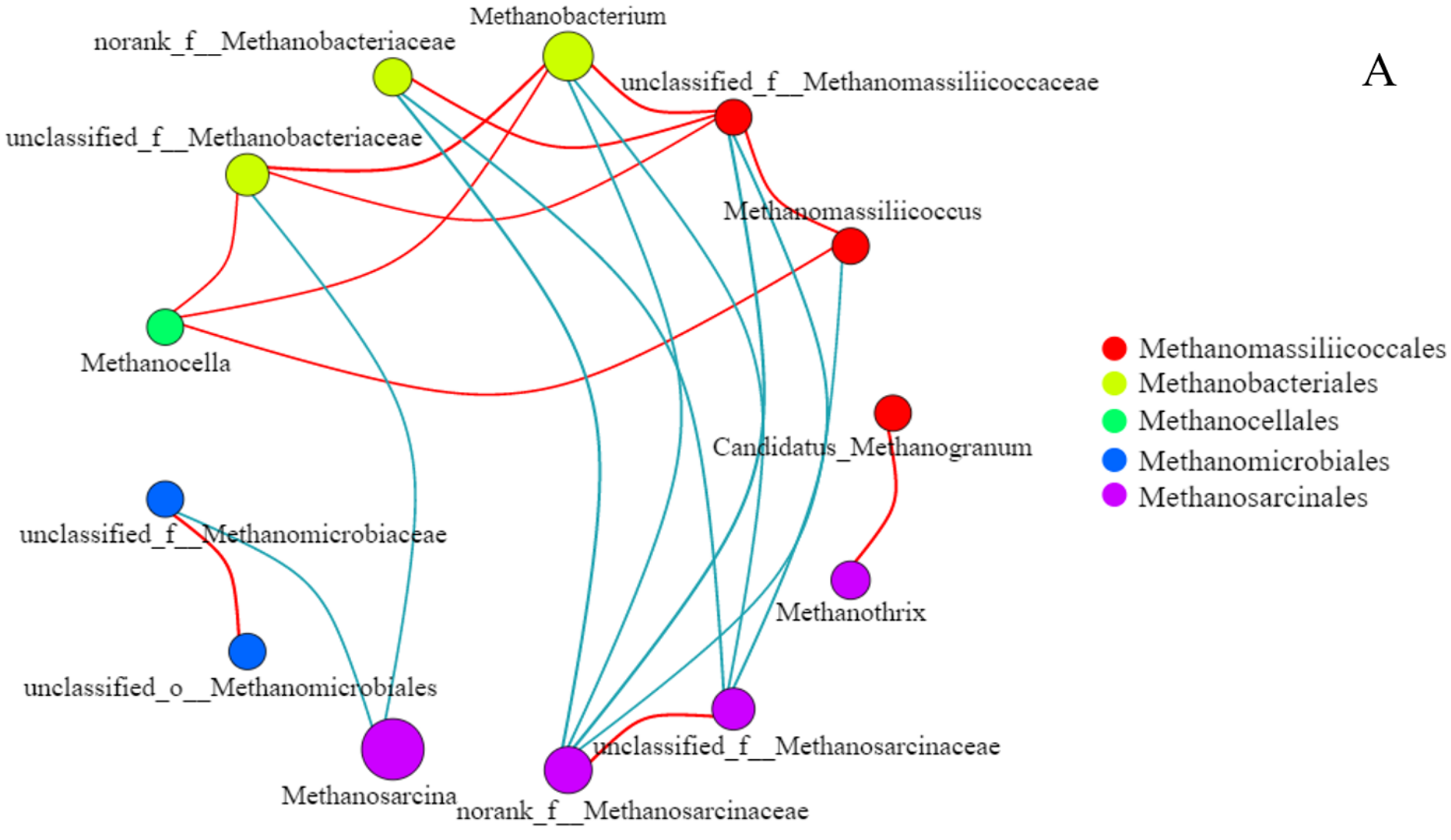
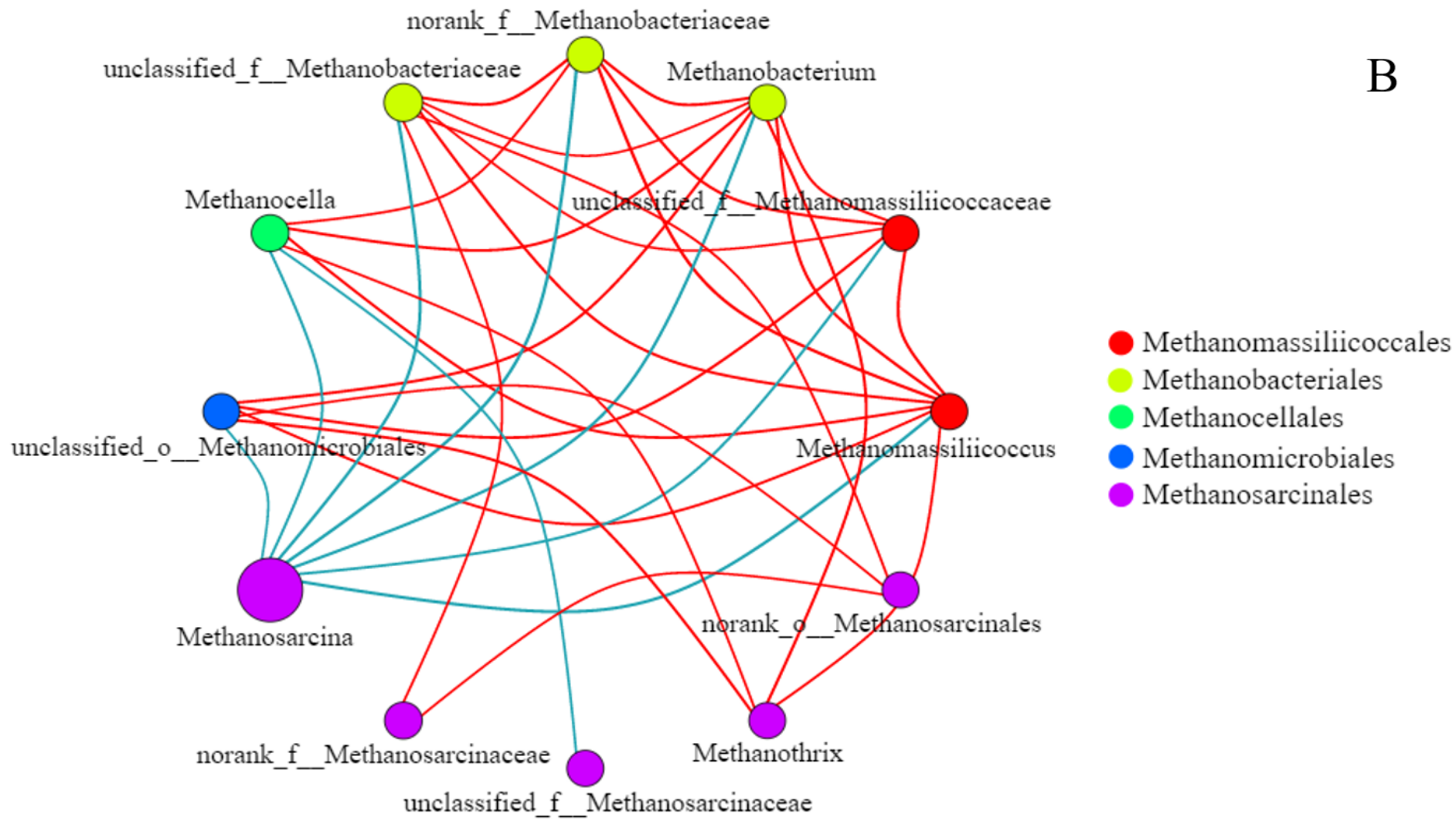

SOC
DOC
TN
Acetate
Sand
pH
CEC
RW
RF
DR
RDA2(4.18%)
RDA1(84.11%)
60
50
40
30
20
10
0
-10
-20
-30
-40
-50
-60
-70
-60
-50
-40
-30
-20
-10
0
10
20
30
40
50

Figure 7.

A


Methanobacterium
norank_f__Methanobacteriaceae
unclassified_f__Methanomassiliicoccaceae
unclassified_f__Methanobacteriaceae
Methanomassiliicoccus
Methanocella
Candidatus_Methanogranum
unclassified_f__Methanomicrobiaceae
Methanothrix
unclassified_o__Methanomicrobiales
unclassified_f__Methanosarcinaceae
Methanosarcina
norank_f__Methanosarcinaceae
Methanomassiliicoccales
Methanobacteriales
Methanocellales
Methanomicrobiales
Methanosarcinales

B


norank_f__Methanobacteriaceae
unclassified_f__Methanobacteriaceae
Methanobacterium
Methanocella
unclassified_f__Methanomassiliicoccaceae
unclassified_o__Methanomicrobiales
Methanomassiliicoccus
Methanosarcina
norank_o__Methanosarcinales
norank_f__Methanosarcinaceae
Methanothrix
unclassified_f__Methanosarcinaceae
Methanomassiliicoccales
Methanobacteriales
Methanocellales
Methanomicrobiales
Methanosarcinales

C

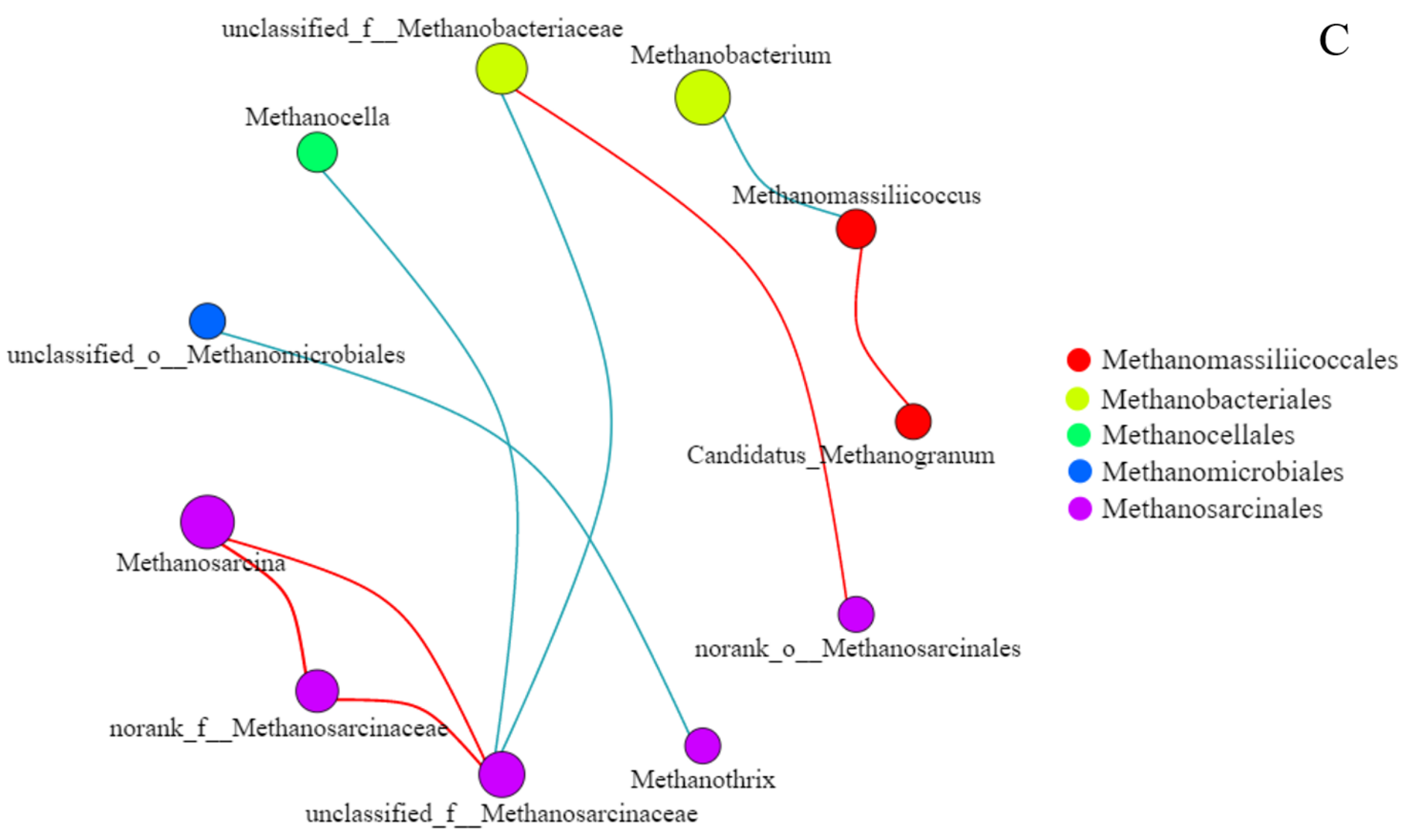

unclassified_f__Methanobacteriaceae
Methanobacterium
Methanocella
Methanomassiliicoccus
unclassified_o__Methanomicrobiales
Candidatus_Methanogranum
Methanosarcina
norank_o__Methanosarcinales
norank_f__Methanosarcinaceae
Methanothrix
unclassified_f__Methanosarcinaceae
Methanomassiliicoccales
Methanobacteriales
Methanocellales
Methanomicrobiales
Methanosarcinales

Table 1 The physicochemical properties of paddy soils in the three typical rice cropping modes in China. Data are presented as the mean ± SD (n = 3)

Soils	SOC ($g\ kg^{-1}$)	TN ($g\ kg^{-1}$)	pH	$\delta^{13}C$ (‰)	Percentage of a particle of different size (%)			Fe (III) ($mg\ kg^{-1}$)	Mn (IV) ($mg\ kg^{-1}$)	NO_3^- ($mg\ kg^{-1}$)	SO_4^{2-} ($mg\ kg^{-1}$)	OXC ($mol\ kg^{-1}$)	CEC ($cmol\ kg^{-1}$)
					Sand	Silt	Clay						
RW	12.8±0.1	1.25±0.1	5.59±0.1	–26.2±0.2	36.9±0.4	39.8±0.3	23.3±0.6	4560±380	180±20	0.03±0.01	79.6±5.3	0.095±0.007	12.2±0.1
RF	19.8±0.3	2.31±0.4	7.70±0.3	–21.2±0.1	31.8±0.6	31.4±0.1	36.8±0.6	2940±60	250±20	1.01±0.02	292±9.2	0.086±0.002	22.7±0.4
DR	19.7±0.1	1.80±0.1	4.89±0.1	–28.8±0.2	50.5±1.1	31.3±1.0	18.2±0.6	3350±280	40±10	0.33±0.07	32.2±5.3	0.064±0.005	7.48±0.2

Table 2 Values of $\delta^{13}CH_4$, $\delta^{13}CO_2$, $\alpha_{(CO_2/CH_4)}$, $\delta^{13}CH_{4(CO_2)}$, and f_{ac} in three cropping mode paddy soils

Cropping	$\delta^{13}CH_4$ (‰)			$\delta^{13}CO_2$ (‰)			$\alpha_{(CO_2/CH_4)}$ [a]		$\delta^{13}CH_{4(CO_2)}$ [b]		$\delta^{13}CH_{4(ac)}$	f_{ac} (%) [c]	
mode	control	1%	2%	control	1%	2%	1%	2%	1%	2%		1%	2%
RW	–54.4±0.2aC	–68.0±0.7bA	–74.5±1.2cA	–18.7±0.5aA	–21.1±0.2bA	–21.7±0.2bA	1.050±0.001bA	1.057±0.001aB	–65.7±0.4aA	–71.7±0.4bA	–37‰	39±1.2eB	50±0.9eA
											–43‰	50±1.4dB	60±1.0dA
RF	–43.9±1.2aA	–73.7±1.2bC	–81.3±0.3cB	–26.3±0.6aC	–28.2±0.2bC	–28.0±0.3bC	1.049±0.002bA	1.058±0.001aB	–71.8±0.6aC	–79.8±0.6bC	–37‰	80±3.8bA	84±3.1bA
											–43‰	97±4.3aA	98±3.4aA
DR	–51.7±0.5aB	–71.2±1.2bB	–82.3±0.4cB	–21.1±0.6aB	–25.1±0.1bB	–25.5±0.2bB	1.050±0.001bA	1.062±0.001aA	–67.5±0.6aB	–78.2±0.6bB	–37‰	52±2.6dB	64±1.8dA
											–43‰	64±3.0cB	75±1.9cA

Data are presented as the mean ± SD (n = 3). The 1% and 2% indicate the proportions of CH_3F addition. Different uppercase and lowercase letters denote significant differences among different cropping modes and different proportions of CH_3F added treatment at $p < 0.05$, respectively. [a, b, c] These values were calculated under different proportions of CH_3F addition with Equations 7, 6, and 5, respectively

Table 3 Alpha diversity of *mcrA* gene in paddy soils under different cropping modes

Cropping modes	Treatment	Effective sequence	No. of OTUs	Shannon index	Chao 1 estimator
RW	control	8145	275±26.2aA	4.01±0.21aA	350±16.7aA
	1%	8145	260±15.1aA	3.89±0.07aA	319±2.95bA
	2%	8145	266±5.69aA	3.82±0.20aAB	330±13.5abA
RF	control	8145	172±9.29bB	3.04±0.06bB	235±30.5aB
	1%	8145	179±1.00bB	2.99±0.28bB	219±15.1aB
	2%	8145	220±8.62aB	3.65±0.05aB	260±19.5aB
DR	control	8145	193±16.2aB	3.98±0.04bA	233±31.2aB
	1%	8145	185±6.66abB	4.10±0.05aA	200±14.0abB
	2%	8145	168±3.06bC	3.93±0.05bA	187±6.57bC

Data are presented as the mean ± SD (n = 3). Different uppercase and lowercase letters denote significant differences among different cropping modes and different proportions of CH_3F added treatment at $p < 0.05$, respectively. The 1% and 2% indicate the proportions of CH_3F addition