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Variations in Methanogenic and Methanotrophic Communities Resulted in Different Methane Emissions from Paddy Soil Applied with Two Types of Manure

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Abstract: Organic manure application is crucial for the maintenance and improvement of soil fertility. However, it inevitably results in increased paddy CH4 emissions, restricting the use of organic manure in the rice fields. In the present study, two kinds of manures, rapidly composted manure (RCM) and non-composted manure (NCM), were investigated through a 19-week greenhouse experiment, during which the dynamics of CH4 emission, soil parameters (DOC, acetate, NH4⁺, NO3⁻, and SO4²⁻), and communities of methanogens and methanotrophs were simultaneously measured. The results showed that NCM significantly enhanced CH4 emission, while RCM decreased CH4 emission by 65.03%; there was no significant difference with the manure-free treatment. In order to well understand the methanogenic process, the seasonal CH4 flux was divided into two periods, namely Stage 1 (before drainage) and Stage 2 (after drainage), on the basis of CH4 emission intensity. The different CH₄ production abilities among the three treatments could contribute to the varied CH4 emissions at Stage 1. The much higher soil DOC concentrations were observed in the manureamended soils (NCM- and RCM-treatments), which could correspondingly lead to the relative higher CH4 emissions compared to the control during Stage 1. Furthermore, the increased methanogenic abundance and the shifted methanogenic archaeal community characterized by the functionally stimulated growth of Methanosarcina genus were observed in the NCM-treated soils, which could consequently result in a higher CH4 emission from the NCM treatment relative to the RCM treatment. As for Stage 2, apart from the significant decrease in soil DOC, the increased contents of soil NO₃⁻ and SO₄²⁻, especially with the RCM-treated soils, were also detected following the drainage, which might retard CH4 production. The lower CH4 emission at Stage 2 could also be attributed to the vigorous aerobic CH4 oxidations, especially in the RCM-treated soils. As a support, the amount of methanotrophs revealed an increasing trend during the late rice growth period, as did the predominance of the methylotrophy of Methylophilaceae species, which showed robust co-occurrence with methanotrophs, inferring interspecies cooperation in methane oxidation.

Keywords: manure; CH4 emission; methanogenic community; methanotrophs; qPCR

1. Introduction

CH₄ is the second-most potent greenhouse gas in the atmosphere, with its concentration increasing steadily from 722 ppb (1750) to 1845 ppb (2016) [1]. Rice paddies are recognized as a major source of CH₄, which accounts for nearly 11% of the total CH₄ emissions [2,3]. China is one of the most important rice-producing countries in the world, accounting for 16% and 28% of the world's rice area and rice production [4]. Whereas, the growing demand for rice to feed the ever-increasing global population challenges the present management strategies in rice cultivation [5]. To guarantee sustainable increases in

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Copyright: © 2023 by the authors. Licensee MDPI, Basel, Switzerland. This article is an open access article distributed under the terms and conditions of the Creative Commons Attribution (CC BY) license (https://creativecommons.org/license s/by/4.0/). rice yield, soil fertility must be maintained. Nowadays, the importance of organic agriculture has been confirmed by more and more research, especially in combination with inorganic fertilization. Incorporation of organic materials into the crop fields can help to enhance plant growth and prevent soil desertification by improving soil structure and fertility by increasing soil carbon (C) content as well as soil microbial diversity and activity [6– 8]. However, there are concerns that organic amendments (OAs) can significantly intensify paddy CH₄ emissions with more C materials introduced [9–11].

The total emission of CH₄ is the balance between its production and oxidation. In paddy fields, during the anaerobic degradation of soil organic matter, sequential reduction of soil NO₃⁻, Mn (IV), Fe (III), and SO₄²⁻ happened, and then methanogenesis became the exclusive process [12]. Methanogenic archaea (methanogens) are the sole CH₄ producers, which produce CH₄ by metabolizing either acetate or H₂/CO₂ that were accumulated from the microbial fermentation of organic matters, mainly polysaccharides, in rice fields [13,14]. At present, all known methanogens fall into the phylum *Euryarchaeota*, where they form seven distinct orders: *Methanobacteriales*, *Methanosarcinales*, *Methanomicrobiales*, *Methanocellales*, and *Methanoplasmatales* [15,16].

The oxidation of CH₄ conducted by methanotrophs plays an important role in controlling paddy CH₄ emissions. It is estimated that almost up to 90% of the CH₄ produced in wetlands can be oxidized before reaching the atmosphere [17,18]. Methanotrophs generally inhabit the rhizosphere and surface soil [19] and utilize CH₄ as their sole carbon and energy sources, including two major groups, namely Type I and Type II methanotrophs. Type I methanotrophs, which belong to Gamma-Proteobacteria, are split into two more groups (Type Ia and Type Ib) and are housed within the family Methylococcaceae, which has fifteen genus: Methylococcus, Methylocaldum, Methylomonas, Methylobacter, Methylomicrobium, Methylosarcina, Methylohalobius, Methylosoma, Methylothermus, Methylomarinum, Methylovulum, Methylogaea, Methylosphaera, Crenothrix, and Clonothrix. The first two genera are also referred to as Type Ib methanotrophs. The Type II methanotrophs belonging to Alpha-Proteobacteria are grouped in two families, Methylocystaceae and Beijerinckiaceae, with Methylocystis, Methylosinus, Methylocella, Methylocapsa, and Methylofer*ula* as the member genus [20]. The CH₄ oxidation process involves an enzyme called methane monooxygenase (MMO), which plays an important role in the initial enzymatic reaction of the aerobic oxidation of CH_4 . Generally, the highly conserved *pmoA* gene that encodes the membrane-bound subunit of MMO is widely used as a phylogenetic marker of methanotrophs in ecological studies [19].

Strategies to reduce CH₄ production or enhance CH₄ oxidation can help retard the increased paddy CH₄ emission due to the introduction of OAs, and a large number of relevant experiments have been conducted. The mitigative potential of electron acceptor supplementation has been well confirmed in field and laboratory studies with ferrihy-drite, ammonium sulfate, gypsum, and phosphogympsum amended [21–23]. In those cases, it is predicted that the methanogens are thermodynamically outcompeted for common substrates like H₂ or acetate by ferric iron-reducing bacteria (SRB) or sulfate-reducing bacteria (SRB). Additionally, a few field studies have also demonstrated the importance of the proper pretreatment of the incorporated OAs in effectively mitigating paddy CH₄ emissions. As revealed, the incorporation of composted crop straw and livestock manures can significantly decrease paddy CH₄ emissions compared to untreated manures, with reduction rates ranging between 50% and 90% [9,24,25]. But unfortunately, most of the research did not pay attention to the responses of methanogens and methanotrophs, which is of great significance to better understanding the microbial mechanisms underlying those measures in paddy CH₄ emission control.

In this study, a new type of rapidly composted manure (RCM) was applied to paddy fields. Basically, the manure is prepared in a closed rolling reactor with a catalyst rich in sulfate and nitrogen and an ammonia fixator and treated at 120 °C for just three hours, similar to biochar production. This processing technique realizes the rapid disposal of awkward manures, potentially advancing the sustainable development of the livestock

and poultry industries and the. In our previous study, the decreased CH₄ emission of RCM relative to non-composted manure (NCM) was demonstrated [26]. However, how the methanogens and methanotrophs, both in populations and community structures, as well as the soil parameters, changed along with the cultivation of rice is still unclear. Therefore, a new pot experiment was conducted in a greenhouse, including three treatments: the NCM treatment, the RCM treatment, and the control. The main objectives of this study are: (1) to confirm the mitigation of seasonal CH₄ emission from paddy soils treated with RCM relative to NCM; (2) to determine the shifts of methanogens and methanotrophs along rice cultivation, both including population size and community structure, as well as the variations of corresponding soil parameters; and (3) to eventually explicate the underlying mechanisms that contributed to the significantly lower CH₄ emission of the RCM-treated soils in detail.

2. Materials and Methods

2.1. Greenhouse Experiment

The experiment was set up with three replicates and conducted in the greenhouse of the Institute of Soil Science, Chinese Academy of Sciences, during 19 June 2014 to 27 October 2014. The soil used was collected from the Changshu Agroecological Experiment Station (123°38' E, 31°33' N), Chinese Academy of Sciences, in Jiangsu Province, eastern China. The soil was air dried, ground, sieved through a 5-mm mesh screen, and then thoroughly mixed. Approximately 7 kg of dried soil was put into a quadrate pot (20 × 24 × 20 cm), with a water groove acting as the chamber base in this experiment. The soil was classified as Gletic-Stagnic Anthrosols. The relevant soil properties were as follows: 20.7 g/kg soil organic C (SOC), 2.41 g/kg total N, 1.67 g/kg total P, 27.02 g/kg total K, and pH 7.30.

In this study, two types of cattle manures were used as basal fertilizer, which were collected from WoLvBao Organic Agriculture (Suqian, China). The raw manure (Non-composted manure, NCM) contains total organic C (TOC), 332.75 g/kg; total N, 17.26 g/kg; total P, 12.66 g/kg; total K, 12.78 g/kg; SO_{4²⁻}, 10.43 g/kg; and pH, 7.80. The RCM manure had a pH of 6.67 and contained 335.68 g/kg TOC, 32.65 g/kg total N, 10.19 g/kg total P, 11.5 g/kg total K, and 61.18 g/kg SO_{4²⁻}.

Manures were applied to the pots a week before transplantation, considering the slow release of nutrients in organic fertilizers. Then, twelve 30-day-old seedlings (*Oryza sativa* L.) from four hills were transplanted into each pot 7 days after flooding (7 d). For the whole rice cultivation period, apart from the cattle manures applied at a loading rate of 1% (dry weight) of the total pot soil at the beginning of the experiment, no more chemical fertilizer was amended. During rice growth, all the pots were permanently flooded, with the water level maintained at approximately 3 cm, except for a drainage carried out from 28 July (39 d) to 3 August (45 d). The rice was harvested 126 days after transplantation.

2.2. Methane Emission

CH₄ flux was measured after flooding. A bottom-open PVC chamber ($20 \times 24 \times 80$ cm) equipped with a battery-driven fan, a thermometer, and a sampling septum was used for gas sampling. Water was added to the groove to seal the chamber. 20 mL gas samples were collected at 0, 5, 10, 20, and 30 min with a syringe and injected into pre-evacuated 18 mL vials for CH₄ analysis (Agilent 7890, Palo Alto, CA, USA). The gas sampling interval was 3–7 days, and the sampling time was fixed between 8:00 and 10:00 am. The CH₄ emission rate was calculated by a linear regression of CH₄ concentration over time.

The CH₄ emission rate was calculated as follows: $F = Q \times V/A \times (dc/dt) \times 273/T$ [9]. Where F is the emission rate; Q is the CH₄ density (0.714 kg/m³); V is the chamber volume; A is the surface area enclosed by the chamber; dc/dt is the CH₄ increase rate; and T is the air temperature of the chamber. Cumulative CH₄ emissions were calculated by linear interpolation of daily CH₄ emissions during the monitoring period.

2.3. Soil Sampling and DNA Extraction

Soil was sampled with a core sampler on days 7 d (26 June), 21 d (1 July), 39 d (28 July), 90 d (16 September), and 133 d (29 October), five times in total. The third and fourth samplings corresponded to the maximum rice tillering stage and the grain filling stage, respectively. Genomic DNA was extracted from 0.5 g of moist soil using the FastDNA® SPIN Kit for soil (MP Biomedicals, Santa Ana, CA, USA) according to the manufacturer's protocol. The extracted DNA was dissolved in 100 μ L of TE buffer, quantified by a NanoDrop ND-1000 spectrophotometer (Thermo Scientific, Waltham, MA, USA), and stored at -20 °C until use.

2.4. Chemical Analysis of Soil Samples

The collected soil samples were slightly dried and sieved through a 2-mm mesh screen. Soil dissolved organic carbon (DOC) and inorganic nitrogen (NH₄⁺ and NO₃⁻) were determined according to the methods described by Li et al. [27]. Briefly, fresh moist soil (10 g) was shaken using a reciprocating shaker for 30 min with double-distilled water (20 mL), and the extracts were then centrifuged at 4000 rpm for 20 min and filtered through the 0.45 μ m membrane. The DOC in the extract was analyzed by Multi N/C 3000 total organic carbon (TOC) analyzer (Analytik Jena AG, Jena, Germany). The extracts were also analyzed by ion chromatography Dionex ICS-1100 (Thermo Scientific, Waltham, MA , USA) for the determination of SO₄²⁻ [28]. In addition, acetate in the filtrate was analyzed using an Agilent 7890A GC with a Headspace Sampler Agilent 7697A (Agilent Technologies, Palo Alto, CA, USA). Ten grams of soil were suspended in 40 mL of KCI (2M) and shaken for 1 h. After filtration, the NH₄⁺ and NO₃⁻ from the supernatant were analyzed using an autoanalyzer (Skalar Scan++, Skalar, Breda, The Netherlands).

2.5. Real-Time Quantitative PCR

For each soil, qPCR was used to determine the abundance of the methanogenic archaeal 16S rRNA (primer set 1106F (TTWAGTCAGGCAACGAGC)/1378R (TGTG-CAAGGAGCAGGGAC)), methanotrophic pmoA gene (primer set A189 (GGNGACTGG-GACTTCTGG)/mb661 (CCGGMGCAACGTCYTTACC)) [29], and sulfate-reducing bacte-DSR1-F+ rial dsrAB (primer set (ACSCACTGGAAGCACGGCGG)/DSR-R (GTGGMRCCGTGCAKRTTGG) [30] genes. The target gene copy numbers were quantified by a C1000[™] Thermal Cycler equipped with a CFX96[™] Real-Time system (Bio-Rad, Hercules, CA, USA). A 20 µL reaction mixture containing 10 µL of SYBR Premix Ex Taq™ (TaKaRa, Kyoto, Japan), primer sets (0.5 μ M each), and a 1.0 μ L template containing approximately 10 ng of DNA was set up. The negative control was run with water as the template instead of soil DNA extract. Plasmids carrying the target gene were extracted and purified. Then, the plasmid DNA content was quantified by NanoDrop and diluted 10-fold to generate a 7-point standard curve. The specificity of the target gene amplification was confirmed by melting curve analysis, and the expected sizes of the PCR amplicons were checked by agarose gel electrophoresis. Real-time PCR was performed in triplicate, and amplification efficiencies of 97–102% were obtained with R² values of 0.990– 0.998. The final number of target genes was obtained by calibrating the extracted total DNA concentrations and soil water content.

2.6. PCR and Illumina Miseq Pyrosequencing

For the soil samples, the above-mentioned primer sets 1106F/1378R and 519F (CAGCMGCCGCGGTAATWC)/907R (CCGTCAATTCMTTTRAGTTT) were used to amplify the methanogenic archaeal and bacterial 16S rRNA gene fragments of approximately 280 bp and 400 bp, respectively, for sequencing on the Illumina Miseq pyrosequencing platform. We chose soils sampled on 7 d, 39 d and 133 d for bacterial 16S rRNA gene analysis. The oligonucleotide sequences included a 5-bp barcode fused to the forward primer as follows: barcode + forward primer. PCR was carried out in 50-µL reaction mixtures

with the following components: 4 μ L (initial 2.5 mM each) of deoxynucleoside triphosphates, 2 μ L (initial 10 μ M each) of forward and reverse primers, 2 U of Taq DNA polymerase with 0.4 μ L (TaKaRa, Kyoto, Japan), and 1 μ L of template containing approximately 50 ng of genomic community DNA. For the methanogenic archaeal 16S rRNA gene, the PCR conditions were as follows: 35 cycles of denaturation at 95 °C for 45 s, annealing at 55 °C for 45 s, and extension at 72 °C for 60 s, with a final extension at 72 °C for 10 min. As for the bacterial 16S rRNA gene, 35 cycles (95 °C for 45 s, 56 °C for 45 s, and 72 °C for 60 s) were performed with a final extension at 72 °C for 7 min. The bar-coded PCR products from all samples were normalized to equimolar amounts and purified before pyrosequencing.

2.7. Processing of the Pyrosequencing Data

The methanogenic archaeal and bacterial 16S rRNA gene data were processed using the Quantitative Insights Into Microbial Ecology (QIIME) 1.4.0-dev pipeline [31]. Briefly, raw data were quality filtered (>25 quality score and 200 bp in length), denoised, and then binned into OTUs at a 97% similarity level. The sequence with the highest abundance was selected from each OTU as the representative sequence of that OTU. Taxonomic analysis was conducted by comparing it with the Silva 104 database. PyNAST and QIIME were used to align representative sequences and remove chimeric sequences, respectively. A total of 721,250 methanogenic sequences and 398,775 bacterial sequences were obtained, respectively. The raw sequences were deposited in the NCBI SRA with an accession number of SRP388055.

2.8. Statistical Analysis

The statistical analysis was performed using SPSS v 13.0 (SPSS Inc., Chicago, IL, USA). The data were expressed as the means \pm standard deviation (SD). The letters above the error bars in the figure indicate significant differences between treatments. The mean separation was assessed by Tukey's test. Differences at *p* < 0.05 were considered statistically significant. The data were visualized by ImageGP[32].

A partial least squares path model (PLS-PM) was performed to infer the potential direct and indirect effects of NCM and RCM amendment, soil properties, microbial abundance, methanogenic community, and methanotrophic community on total CH₄ emissions at five sampling time points. Soil properties are latent variables measured by concentrations of NO_{3⁻}, NH_{4⁺}, DOC, acetate, and SO_{4²⁻}; microbial abundances are latent variables reflected by abundances of three functional microbial groups: methanogenic and methanotrophic communities. The quality of the PLS-PM is evaluated by the goodness of fit (GOF) index, which >0.7 means a good overall prediction performance of the model, and R^2 values represent the variance of dependent variables explained by the inner model [33].

3. Results

3.1. CH₄ Emission

Similar CH₄ flux patterns were found from three treatments, with the amplitudes varying (Figure 1a). Generally, no obvious CH₄ emission was observed at the beginning (before 17 d) of the experiment, followed by steadily increased emissions, with the emission peaks appearing on 33 d Thereafter, the drainage carried out between 39 d and 45 d resulted in significantly decreased CH₄ emission rates for all three treatments. In addition, pulse CH₄ emissions were monitored during the drainage. The average CH₄ fluxes before drainage were approximately 9.73, 6.30, and 3.55 mg m⁻² h⁻¹ for NCM-, RCM-, and control treatments, respectively. After drainage, slight variations of the CH₄ emission rates were measured, and the values ranged between 0.09 and 3.57 mg m⁻² h⁻¹.

In order to have a clear vision of the CH₄ fluxes with three different treatments, the whole rice growing season was divided into two parts, namely Stage 1 (before drainage) and Stage 2 (after drainage). In general, vigorous CH₄ emissions were observed in Stage 1, accounting for 52.6%, 96.8%, and 32.9% of the total CH₄ emissions of the NCM-, RCM-, and control treatments, respectively. In comparison with the control, NCM and RCM applications significantly increased the total CH₄ emission of Stage 1, with an increase of 279.4% and 120.8%, respectively (Table S1). However, for Stage 1, approximately 34.3–55.4% of CH₄ emissions were mitigated with the RCM treatment compared to the NCM treatment. On the contrary, the cumulative CH₄ emission of three treatments during Stage 2 decreased in the following order: NCM < Ctrl < RCM. The CH₄ emission with the RCM-treated soils was weak, and the total CH₄ emissions from the RCM-treated soils compared to the NCM treatment at Stage 1 and Stage 2, respectively, accounted for 21.9% and 46.7% of the total emitted CH₄ of the NCM treatment, indicating the main mitigation occurred at Stage 2 for RCM.



Figure 1. Seasonal variations of CH₄ flux and the cumulative CH₄ emissions of different treatments. (a) Seasonal variations of CH₄ flux. The zoom-in view for the late rice cultivation period was displayed on the right. (b) Cumulative CH₄ emissions. Different letters above the error bars indicate significant differences (p < 0.05).

3.2. Soil Chemical Characteristics

Regardless of treatments, soil DOC represented an accumulation during the first three weeks when soils were subjected to flooding (Figure 2a). After the maximum recorded at the time point of 21 d, the DOC concentrations began to decrease gradually. Applications of NCM and RCM significantly increased soil DOC concentration during rice cultivation relative to the control (p < 0.05), except for a minimum detected from the RCM-treated soils on 90 d. When harvested (133 d), relatively similar DOC concentrations were observed among the three treatments, but at a low level.

Variations in the concentrations of soil acetate were also monitored over time. In contrast to the dynamics of DOC in Figure 2a, generally, the acetate concentration gradually increased with rice growth for these three treatments (Figure 2b). For the control, apart from a slight decline from 7 to 21 d, the soil acetate contents gradually increased, which has been more apparent lately. Consistent with the case of DOC, NCM and RCM applications also led to significantly increased soil acetate concentrations, and the acetate concentrations from the NCM treatment seemed higher than those from the RCM treatment (p > 0.05).

Concentrations of soil nitrate (NO₃⁻) and ammonium nitrogen (NH₄⁺) during rice growth changed in opposite ways (Figure 2c). Generally, the soil NH₄⁺ concentrations showed a decreasing trend and remained at low levels during the late rice growth period. NCM and RCM amendments significantly increased soil NH₄⁺ concentrations (p < 0.05) compared to those of the control for the first three sampling dates. Moreover, the NH₄⁺ concentration with the RCM treatment was approximately twice that with the NCM treatment. There was no significant difference observed in the soil NO₃⁻ concentration among the three treatments before drainage (p > 0.05), and the values were always low. After drainage, the soil NO₃⁻ concentrations began to increase, which was remarkably apparent with the RCM-treated soils and much higher than those from the other two treatments (p < 0.05).

The SO₄²⁻ concentrations from these three treatments varied in similar patterns to those of NO₃⁻ (Figure 2d). Before drainage, the SO₄²⁻ concentration gradually decreased and then apparently started to increase, following the reflooding of soils. Both the NCM treatment and the control contained a similar amount of SO₄²⁻, which was much lower than that of the RCM treatment (p < 0.05). Relatively large amplitudes of the soil SO₄²⁻ content variations were observed with the RCM-treated soils.



Figure 2. The dynamics of soil dissolved organic carbon (DOC) (**a**), acetate (**b**), NH_{4^+} , and NO_{3^-} . The full line and the dotted line refer to NH_{4^+} and NO_{3^-} , respectively. (**c**) and $SO_{4^{2^-}}$ (**d**) concentrations.

3.3. Abundance of Functional Microbial Groups

The abundance of the methanogens from soils treated with NCM ranged between 0.95 and 1.54×10^9 /g and was consistently higher than that from RCM treated soils (0.65– 1.20×10^9 /g) and the control (0.51– 1.27×10^9 /g), during the cultivation of rice (Figure 3a). However, no significant difference was found between the RCM treatment and the control in the abundance of soil methanogens (p > 0.05), except for an apparent increase in the control at the beginning of the rice tillering stage (21 d) (p < 0.05).

Variations of the number of methanotrophs from all treatments were similar, with the abundance gradually decreasing before drainage while obviously increasing from 90

to 133 d (Figure 3b). It is noteworthy that, at the initial stage (7 d), relatively similar abundances of the methanotrophs were found with all three treatments, and then the methanotrophic numbers decreased, with the RCM treatment containing the lowest methanotrophic abundance on 21 and 39 d. However, the number of methanotrophs in the soils treated with RCM emerged to be the largest on 90 d. At maturation, the methanotrophic abundances of the three treatments reached peaks, with the largest number observed in the NCM-treated soils.

The population size of sulfate-reducing bacteria (SRB) varied in similar ways as the changes in the methanotrophs. As flooding continued, the SRB abundance showed a decreasing trend until the drainage was carried out at 39 d (Figure 3c). Then, after drainage, the SRB abundance began to increase again and reached its maximum at the harvest. Similarly, an apparent increase in the number of SRB was observed over 90 d in soils treated with the RCM.



Figure 3. Copy numbers of the methanogenic archaeal 16S rRNA genes (**a**), the methanotrophic *pmoA* genes (**b**), and the *dsr* gene (**c**) of paddy soils under different treatments along rice cultivation. Significant differences are indicated by different letters above the error bars (p < 0.05).

3.4. Community Compositions of Methanogens and Methanotrophs

Pyrosequencing revealed that the soil methanogenic archaeal community of all treatments during the whole rice growing period was dominated by genera of *Methanosarcina* (12.2–45.4%), *Methanocella* (14.4–29.4%), *Methanosaeta* (12.2–26.9%), and *Methanobacterium* (11.7–21.0%), whose relative abundances could represent serious shifts with the cultivation of rice, as illustrated in Figure 4a. *Methanosarcina* was the most predominant methanogenic group for soils amended with NCM, while the proportions largely varied, ranging between 23.0 and 45.4%. In contrast, the RCM-treated soils were dominated by *Methanocella* (24.3–29.4%), with the percentage varying slightly. The methanogenic species in the control seemed evenly distributed, with almost no dominant methanogenic species observed. In addition, the relative abundance of *Methanosarcina* from the NCM treatment and the control showed a similar decreasing trend with the growth of rice, which was more apparent in the NCM treatment and not found in the RCM treatment. Besides, the *Methanosaeta* seemed to increase at the late rice growing stage, while the *Methanobacterium* decreased within all three treatments.

The total bacterial sequences assigned to each family were classified at genus level, and sequences belonging to methanotrophs were selected. The relative abundance of each methanotrophic genera is illustrated in Figure 4b. It was observed that the composition of methanotrophs varied over time, especially at the rice maturation stage. At the beginning of the experiment, the methanotrophic community structures of all treatments seemed relatively similar; only the genus less dominant differed. Type II methanotrophs, especially Methylocystis and other undefined genus from the family of Methylocystaceae, overwhelmingly dominated the paddy methanotrophic community before drainage, accounting for approximately 60.3–73.7% of the total methanotrophs. However, the structure of the methanotrophic community significantly changed at the late growing stage of rice (*p* < 0.05), as shown in Figure 4b on 133 d. The dominance of Type II methanotrophs decreased, ranging from 19.9% to 48.8% among the three treatments. Interestingly, data from 133 d revealed that methylotrophs of the family Methylophilaceae emerged to be the most abundant group, with the proportions increasing from the initial 7.4%, 6.9%, and 8.4%, respectively, to 34.1%, 73.5%, and 37.0% for the treatments of NCM, RCM, and control. It deserved to be mentioned that, in the mature stage, the methylotrophs of the soils treated with RCM seemed to greatly grow and ultimately dominate the methanotrophs.



Figure 4. A 100% stacked column chart of the relative abundances of the dominant methanogenic (**a**) and methanotrophic (**b**) genera derived from 16S rRNA genes of paddy soils under different treatments along rice cultivation. The relative abundances of the methylotrophs of Methylophilaceae were also displayed, as they cooperated with methanotrophs in methane oxidation.

3.5. Shifts in the Communities of Methanogens and Methanotrophs

The variations in the paddy methanogenic archaeal and methanotrophic communities of the different samples along rice cultivation were statistically evaluated using a principal component analysis (PCA) of the weighted pairwise UniFrac community distances, which are displayed in Figure 5. PCA revealed that the methanogenic communities from the NCM-treated soils apparently shifted with the rice cultivation, which seemed much different from those of the other treatments (Figure 5a). The samples from the RCM treatment and the control were grouped together and dissimilated from samples of the NCM treatment, which could indicate the much similar methanogenic communities between the RCM treatment and the control. Moreover, the methanogenic communities of the RCM treatment and the control slightly shifted over time, especially for those of the RCM treatment.

As shown in Figure 5b, the soil samples from the first two sampling dates were gathered for all three treatments, suggesting similar and less varied methanotrophic communities in the three treatments. However, all soil samples on 133 d separated from the rest of the samples, indicating the methanotrophic communities largely changed, which was more apparent for the RCM-treated soils.



Figure 5. The community compositional structures of the methanogens (**a**) and methanotrophs (**b**) in the paddy soils as indicated by principal component analysis (PCA) of the weighted pairwise UniFrac community distances between different treatments along rice cultivation.

3.6. Potential Direct and Indirect Effects of NCM and RCM Amendments on Total CH₄ Emission

PLS-PM indicated that, for five sampling time points, all predictor variables explained 91.3%, 90.5%, 89.4%, 95.7%, and 93.5% of variations in total CH₄ emission, respectively. Both NCM and RCM amendments showed significant effects on soil properties, except for 7 d, when just RCM application significantly affected soil SO₄²⁻ concentration (Figure 6a). Amendment of NCM exerted significant impacts on soil methanogenic communities for 7 d, 21 d, 39 d and 90 d, while the effects of RCM amendment were non-significant for all time.



Figure 6. The partial least squares path models (PLS-PM) illustrate the direct and indirect effects of NCM and RCM, soil properties, microbial abundance, methanogenic community, and methanotrophic community (except 21 d and 90 d) on total CH₄ emissions at 7 d (**a**), 21 d (**b**), 39 d (**c**), 90 d (**d**), and 133 d (**e**), respectively. Soil properties are latent variables measured by SO₄^{2–} concentrations for 7 d (**a**); NO₃[–], NH₄⁺, DOC, acetate, and SO₄^{2–} concentrations for 21 d (**b**) and 133 d (**e**); NO₃[–], DOC and acetate concentrations for 39 d (**c**); and NO₃[–], NH₄⁺, DOC, and SO₄^{2–} concentrations for 90 d (**d**). Microbial abundances are latent variables reflected by the abundances of three functional microbial groups: methanogens, SRB, and methanotrophs, with the abundances of methanotrophs and SRB excluded for 7 d and 39 d, respectively. The main soil properties and microbial abundances are selected through PLS-PM analysis, and microbial communities are the first PCA axis of methanogenic and methanotrophic communities. The numbers on the arrowed lines indicate the normalized direct (above) and indirect (below) path coefficients. *R*² below the latent variables represents the variations of dependent variables explained by the inner model. The GOF index represents the goodness of fit. Asterisks represent significant effects. *, *p* < 0.05; ***, *p* < 0.001.

4. Discussion

4.1. RCM Application Significantly Decreases Paddy CH₄ Emission

Livestock manures contain high amounts of labile organic C and nutrients, which are regarded as valuable organic fertilizers in agriculture, favoring crop growth as well as improving soil structure and fertility [34,35]. As anything has two sides, the high C content of the livestock manure could otherwise inevitably lead to vigorous paddy CH₄ emissions when applied to the rice fields [11,36], triggering concerns about global warming. However, the substantial CH₄ emission resulting from the incorporation of the raw manure could be mitigated when that is properly pretreated. In the present study, a new rapid

composting method was utilized to handle the raw cattle manures, which can promise the rapid disposal of vast amounts of ever-growing waste generated from intensive livestock and poultry operations. CH₄ emission fluxes with all three treatments were simultaneously measured during the whole rice cultivation, revealing that the RCM-treated rice soils emitted almost the same amount of CH₄ in comparison with that from the control, whereas approximately double CH₄ emission was detected with the NCM-treated rice soils (Figure 1a). Therefore, referring to the above-mentioned results, we can conclude that RCM seems to be more superior relative to raw manure as an organic amendment, which can effectively enhance rice growth, increase grain production (Table S2), and emit less CH₄. Numerous studies have also shown that composting manure, irrespective of whether it is straw or livestock manure, can effectively reduce CH₄ emissions compared to raw manures when used in paddies [9,24,26]. Field experiments conducted by Kim et al. [24] reveal that the application of composted manures reduces CH₄ emissions by up to 50% compared to air-dried manures without pretreatment.

4.2. Insights into the CH₄ Emission Patterns of Soils Treated with NCM and RCM

In the present study, organic manures from NCM and RCM were amended as basal fertilizers without other fertilizers being applied during the whole rice growth period. Before transplantation, all the pots were subjected to flooding for one week, considering the slow release of nutrients contained in the organic manures. As shown in Figure 1a, the CH4 emissions from all three treatments during the early 20 days after flooding were relatively lower, which could be partially attributed to the weak CH₄ transport of rice seedlings as the rice aerenchyma tissues were not well developed then [37]. It is estimated that approximately 90% of the emitted paddy CH4 is released to the atmosphere through plantmediated transport, with only 2-3% percolating through flooded water [38]. In addition, the relatively low temperature during the early experiment could also limit CH4 production. Then, CH₄ emission rates significantly increased, with the appearance of three CH₄ flux peaks, despite those being much less conspicuous in the control (Figure 1a). It is reported that the occurrences of such CH4 emission peaks correspond to maxima in air temperature [39]. Whereas, the relatively higher CH4 emissions observed in Stage 1 with the NCM- and RCM-treated soils could be ascribed to the extra application of organic manures. It is noteworthy that relatively lower CH₄ emissions were detected with the RCMtreated soils compared to those from the NCM-treated soils, and the possible underlying reasons will be interpreted below.

After drainage, the intensity of paddy CH₄ emissions significantly decreased (Figure 1a), with emission rates consistently maintaining low levels, which was extremely apparent in the RCM treatments. Moreover, the CH₄ fluxes between the NCM and Ctrl treatments were very similar, as shown in the zoomed-in view shown on the right. The almost comparative CH₄ fluxes between the NCM and Ctrl treatments could indicate the consumption of the labile C contained in the NCM and the CH₄ production during late rice cultivation, which mostly originated from degradations of exudates and autolysis products of roots. Studies have shown that the presence of rice plants promoted the emission of CH₄ at the rice mature stage [40,41], indicating the important influence of rice roots on CH₄ production in the late growth stage of rice. Whereas, the situation was strange with the RCM-treated soils, whose rice biomasses were 68.7% and 35.4% higher than those of the Ctrl and NCM treatments (data not shown), respectively, which means relatively higher root exudates, but there was almost no CH₄ emission detected with the RCM treatment. Thus, we speculated that either reduced CH₄ production or increased CH₄ oxidation conducted by methanotrophs could take place in the RCM-treated soils.

4.3. The Lower CH₄ Production with the RCM Treatment at Rice Growth Stage 1 Led to The Lower CH₄ Emissions Relative to the NCM Treatment

DOC is the most labile part of soil organic C, which is an important C source and easily accessible for soil microorganisms. A large amount of research has demonstrated that CH₄ emission rates positively correlate with DOC concentrations in soil [24,42]. Soil DOC is the major C source for methanogens and is considered an important factor affecting paddy CH₄ emissions. The dynamics of soil DOC concentrations during rice cultivation are depicted in Figure 2a. Amendment of NCM and RCM significantly increased soil DOC concentration, except in the case of the RCM treatment at 90 d, ultimately resulting in higher emissions of CH₄ by increasing CH₄ production relative to Ctrl.

Additionally, the lower CH₄ emission from the RCM-treated soils relative to that from the NCM-treated soils seemed strange, considering the similar DOC concentrations between these two treatments. However, this paradoxical phenomenon points toward the significance of the functionally diverse microorganisms that are involved in the production and oxidation of paddy CH4. First, methanogens are the sole producers of paddy CH4, broadly classified into two groups: hydrogenotrophic and acetoclastic methanogens, which can obtain energy from the reduction of CO₂ with H₂ and the cleavage of acetate, respectively [14]. Organic manure amendments introduce more C into soils, which can provide sufficient C substrates for methanogens, potentially influencing both the abundance and composition of the methanogens and finally having an impact on paddy CH4 emissions. A field study conducted by Feng et al. [29] reveals that decreased soil DOC content, resulting from elevated ground-level O₃, reduces both the abundance and diversity of the dominant methanogen of Methanosaeta, consequently contributing to the reduced CH4 emission in the plots under elevated ground-level O3. In the present study, the amendment of NCM seemed to significantly stimulate the growth of methanogens, whereas no obvious enhancement was observed in the methanogenic archaeal population size of the RCM-treated soils over the whole rice growth stage (Figure 3a). Moreover, pyrosequencing results further revealed that NCM application also resulted in apparent shifts in the paddy methanogenic composition, selectively enhancing the growth of the methanogens of Methanosarcina; whereas, the same variation was not found in both the RCM and Ctrl treatments (Figure 4a). It is well known that Methanosarcina methanogens are metabolically versatile, using both CO₂ and acetate as C sources, and can rapidly grow to become the dominant genera [43]. It is estimated that acetoclastic methanogenesis contributes more than 67% of the paddy CH₄ emissions. Thus, as corresponding results show, the lower relative abundance of Methanosarcina in RCM-treated soils may consistently lead to lower CH4 emissions. Second, the methanotrophic numbers of the RCM-treated soils appeared lower than those of the NCM-treated soils for Stage 1, indicating the lower CH₄ oxidation ability of the RCM-treated soils. Additionally, PCA results also displayed a similar community structure of methanotrophs between all three treatments (Figure 5b). Therefore, we can conclude that, for Stage 1, the relatively lower CH4 production ability led to the lower CH4 emission of the RCM treatment compared to that of the NCM treatment.

4.4. The Enhanced CH₄ Oxidation with the RCM Treatment at Rice Growth Stage 2 Contributed to the Lower CH₄ Emission

After drainage, dramatically decreased CH₄ fluxes were observed in all three treatments, and almost no CH₄ emission was detected in the RCM-treated soils. Usually, soil drainage always results in changed soil conditions, primarily increased soil Eh, re-oxidation of soil reductants [19]. In the present study, at the late rice growth stage, the concentrations of NO₃⁻ and SO₄²⁻ showed apparent increasing trends, which were especially intensive in the RCM-treated soils (Figure 2c,d). The increased NO₃⁻ and SO₄²⁻ contents could partially be responsible for the extremely low CH₄ emissions of RCM treatment. On one hand, the high content of NO₃⁻ and SO₄²⁻ could retard the decrease of soil Eh, which could inhibit the CH₄ production of RCM-treated soils as the reduced conditions needed have not been formed [21]. On the other hand, the abundant inorganic acceptors such as NO_{3^-} and $SO_{4^{2^-}}$ may activate the corresponding nitrate- and sulfate-reducing bacteria, effectively competing C substrates with methanogens [44]. Coincidentally, as support, both an apparent drop in DOC concentration (Figure 2a) and a significant increase in *dsr* gene copies (Figure 3c) were observed at 90 d with the RCM-treated soils.

Furthermore, the apparent CH₄ emission reduction of RCM treatment at rice growth Stage 2 might be related to the predominance of the methylotrophs belonging to the family *Methylophilaceae* (Figure 4b). More recently, a CH₄ consumption metabolic mode linked to alternative electron donors, such as nitrate/nitrite-dependent anaerobic/microaerobic bacterial methane oxidation in freshwater environments, was proposed [45]. A number of studies have already revealed the cooperation between the *Methylopcoccaceae* and the *Methylophilaceae* in response to both CH₄ and NO₃⁻ stimuli, implying the importance of such syntrophism in CH₄ consumption [46,47]. Krause et al. [48] deduced the cross-feed-ing mechanism and verified methanol as the dominant carbon and energy source the present study, after drainage, the soil NO₃⁻ from all three treatments showed obvious accumulations for the symbiotic growth of *Methylophilaceae*. This, to some extent, could explain the relatively lower CH₄ emission from the RCM treatment at Stage 2.

5. Conclusions

NCM is a valuable organic fertilizer that contains rich, labile organic C and nutrients. However, the application of NCM to rice soils significantly enhanced CH₄ emission by both promoting the growth of methanogens and shifting the methanogenic archaeal composition, primarily stimulating the growth of *Methanosarcina*, during whole rice cultivation. On the contrary, the incorporation of RCM did not increase CH₄ emissions, which were almost the same as those of the control. Unlike the NCM-treated soils, neither the stimulation of the total methanogenic population size nor the specific methanogenic species, such as *Methanosarcina*, was detected with the RCM-treated soils during the rice growth, which primarily resulted in the low CH₄ production at Stage 1. In addition, the extremely low CH₄ emission from the RCM-treated soils during the late rice growth period (Stage 2) could partially be attributed to aerobic/anaerobic oxidation of CH₄, as the methylotrophy of *Methylophilaceae* species appeared dominant. As a summary, the statistical results of contrasting NCM and RCM, respectively, with control at five sampling time points based on a *t*-test are presented in Table S1.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/agronomy13051268/s1, Table S1: Statistic results of contrasting NCM and RCM, respectively, with control on five sampling days based on t-test.; Table S2: Effects of different treatments on rice biomass.

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