Article

Three-Source Partitioning of Methane Emissions from Paddy Soil: Linkage to Methanogenic Community Structure

Jing Yuan 1,2, Xiaomei Yi 1 and Linkui Cao 1,*

1 School of Agriculture and Biology, Shanghai Jiao Tong University, 800 Dongchuan Road, Shanghai 200240, China; yuanjing1203@zju.edu.cn (J.Y.); yixiaomei@sjtu.edu.cn (X.Y.)
2 Institute of Soil and Water Resources and Environmental Science, College of Environmental and Resource Sciences, Zhejiang University, Hangzhou 310058, China
*
Correspondence: clk@sjtu.edu.cn; Tel.: +86-021-34206925

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Abstract: Identification of the carbon (C) sources of methane (CH\textsubscript{4}) and methanogenic community structures after organic fertilization may provide a better understanding of the mechanism that regulate CH\textsubscript{4} emissions from paddy soils. Based on our previous field study, a pot experiment with isotopic \textsuperscript{13}C labelling was designed in this study. The objective was to investigate the main C sources for CH\textsubscript{4} emissions and the key environmental factor with the application of organic fertilizer in paddies. Results indicated that 28.6%, 64.5%, 0.4%, and 6.5% of \textsuperscript{13}C was respectively distributed in CO\textsubscript{2}, the plants, soil, and CH\textsubscript{4} at the rice tillering stage. In total, organically fertilized paddy soil emitted 3.51 kg CH\textsubscript{4} ha\textsuperscript{-1} vs. 2.00 kg CH\textsubscript{4} ha\textsuperscript{-1} for the no fertilizer treatment. Maximum CH\textsubscript{4} fluxes from organically fertilized (0.46 mg m\textsuperscript{-2} h\textsuperscript{-1}) and non-fertilized (0.16 mg m\textsuperscript{-2} h\textsuperscript{-1}) soils occurred on day 30 (tillering stage). The total percentage of CH\textsubscript{4} emissions derived from rice photosynthesis C was 49%, organic fertilizer C < 0.34%, and native soil C > 51%. Therefore, the increased CH\textsubscript{4} emissions from paddy soil after organic fertilization were mainly derived from native soil and photosynthesis. The 16S rRNA sequencing showed Methanosarcina (64%) was the dominant methanogen in paddy soil. Organic fertilization increased the relative abundance of Methanosarcina, especially in rhizosphere. Additionally, Methanosarcina sp. 795 and Methanosarcina sp. 1H1 co-occurred with Methanobrevibacter sp. AbM23, Methanoculleus sp. 25XMc2, Methanosaeta sp. HA, and Methanobacterium sp. MB1. The increased CH\textsubscript{4} fluxes and labile methanogenic community structure in organically fertilized rice soil were primarily due to the increased soil C, nitrogen, potassium, phosphate, and acetate. These results highlight the contributions of native soil- and photosynthesis-derived C in paddy soil CH\textsubscript{4} emissions, and provide basis for more complex investigations of the pathways involved in ecosystem CH\textsubscript{4} processes.

Keywords: organic fertilizer; methane; methanogen; paddy; \textsuperscript{13}C

1. Introduction

Climate change is mainly attributed to rapidly rising atmospheric concentrations of greenhouse gases (GHGs) such as carbon dioxide (CO\textsubscript{2}), H\textsubscript{2}O, methane (CH\textsubscript{4}) and nitrous oxide (N\textsubscript{2}O) [1]. The atmospheric concentrations of CH\textsubscript{4}, the GHGs present at the third-highest concentration (after CO\textsubscript{2} and H\textsubscript{2}O), are influenced by anthropogenic activities. The global warming potential of CH\textsubscript{4} on a 100-year time horizon is 28–34 times higher than CO\textsubscript{2} [2]. Approximately 20% of the global warming that occurred after industrialization has been attributed to atmospheric CH\textsubscript{4} emissions [3]. Paddy soils are the largest anthropogenic source of CH\textsubscript{4}, accounting for approximately 10% of global CH\textsubscript{4}
emissions [4]. Emissions of CH\textsubscript{4} from paddy soils are concentrated in irrigated areas; irrigated paddy soils account for 60% of the total rice harvesting area worldwide, but produce 78% of CH\textsubscript{4} emissions in rice-producing areas [3,5]. Several factors are known to affect paddy CH\textsubscript{4} emissions, including climate (temperature, precipitation, humidity), crops (cultivar, root vitality, aeration), soil characteristics (moisture, O\textsubscript{2}, Eh, pH, texture, organic matter, mineral nutrition), and field management (water management, fertilization, farming operations) [6–10]. Changes to any of these factors can alter the soil microenvironment and in turn affect overall CH\textsubscript{4} emissions [11]. Especially, paddy fields treated with organic fertilizers release more CH\textsubscript{4} than inorganically fertilized and unfertilized soils [12,13].

The total organic C budget in the paddy ecosystem includes C fixed through rice photosynthesis, as well as native organic C present in soil, rice plant residues and sediments [14]. Decomposition of organic matter by methanogens under anaerobic conditions leads to production of CH\textsubscript{4}. Overall CH\textsubscript{4} emissions from paddy soil are the net result of CH\textsubscript{4} production, oxidation and transportation [15]. There are two major pathways of CH\textsubscript{4} production: acetoclastic and hydrogenotrophic (Figure 1). Acetoclastic methanogens [16,17] use ATP to convert acetate to acetyl phosphate and then remove the phosphate ion via a reaction catalyzed by coenzyme A. CH\textsubscript{4} is formed gradually by processes involving oxidized ferredoxin, tetrahydrosarcinapterin, coenzyme M, and coenzyme B. In contrast, hydrogenotrophic methanogens [18] initially form formylmethane furans (CHOMFR) from H\textsuperscript{+} and methanofuran, then CH\textsubscript{4} is finally generated via a series of complex catalytic reactions. Overall, the metabolic pathways by which methanogens produce CH\textsubscript{4} involve numerous enzymes and require large quantities of electrons, C substrates and energy [19,20]. In most soils, CH\textsubscript{4} is mainly (58–75%) produced from acetate, except in permanent pasture soil (42%) [21]. Hydrogenotrophic methanogenesis produces about 22–37% of CH\textsubscript{4}. Subsequently, the CH\textsubscript{4} produced is partially consumed by methanotrophs via the phosphoribulose or serine pathways [22]; the remainder diffuses into the atmosphere through rice aerenchyma or via ebullition (bubbles of gas in water) [3,23]. When an organic fertilizer is applied to soil (Figure 1), some nutrients are assimilated by the plants, while others would be reserved within the soil and utilized by soil microorganisms. In comparison with chemical fertilization and no fertilization, organic fertilization improves soil quality by enhancing a series of complex microbial metabolic activities, such as sulfate reduction, iron reduction, nitrogen cycle, and carbon cycle [9,24,25]. Ultimately, organic fertilizers provide a large amount of energy and substrates to methanogens and, thus, stimulate production of CH\textsubscript{4} [25]. This effect has been demonstrated in many other studies [26]. In addition, rice plant growth and photosynthesis are also improved by organic fertilization [27]. Consequently, more C-containing compounds are transported from the rice plant tissues into the soil, which eventually increases the availability of C substrates for CH\textsubscript{4} production [9]. Therefore, controlling and limiting the availability of additional C sources to methanogens could potentially mitigate the increased CH\textsubscript{4} emissions observed in organically-managed rice soils.
Studies have emphasized that increased CH\textsubscript{4} emissions from organically fertilized paddy soils are due to the extra C input provided by the organic fertilizer [27–29]. In our previous paddy field study [30–32], we applied four treatments with chemical fertilizer (CT), organic fertilizer (OT), mixed with chemical and organic fertilizer (MT), and no fertilizer (ctrl). Results found that CH\textsubscript{4} emissions were completely higher in OT than that in MT, CT or ctrl. Moreover, soil nitrogen (N), C, phosphate (P), potassium (K), and acetate were the main key environmental factors associated with...
CH₄ emissions and the dominant methanogenic communities. The hydrogenotrophic Methanocella and acetoclastic Methanoseta were the predominant methanogenic communities. Especially, application of organic fertilizer promoted the dominant acetoclastic methanogens, but suppressed the dominant hydrogenotrophic methanogens. While, another study [25] found that the abundance of Methanosarcina and Methanoseta at the rice tillering stage decreased rapidly after application of composted manure compared to non-composted manure treatment. Furthermore, a decrease in Methanosarcina, but increase in Methanobrevibacter, was observed in the composted treatment in contrast to the control treatment [33]. Varied fertilization could have varied effects on CH₄ emissions and methanogens from paddy soil. The transformation in the methanogenic community structure and enhanced availability of C substrates could explain the increased CH₄ production in OT compared to other treatments. However, the specific contribution of the additional C from organic fertilizer on paddy CH₄ emissions are unclear and merit more assessment. We hypothesized there were three C sources responsible for the CH₄ emissions in organically-fertilized paddy soil: rice photosynthesis-derived, native soil C-derived, and organic fertilizer-derived.

The contribution of root-derived CO₂ in paddy ecosystems indicates photosynthesis should be considered as one of the main drivers of soil C flow [14]. Photosynthesis-derived C (including rice residues, root tissues, root CO₂, etc.) is assimilated by methanogenic archaea present on the rice roots [34]. The rates of photosynthesis-derived CH₄ emissions from rice fields are controlled by the abundance of active methanogens on the rice roots [35]. While, the native soil C is also a large C source for CH₄-producing methanogens. Before rice transplantation, paddy soils contain both labile and ‘old’ native C [8]. Different C substrates have stimulating or restraining effects, termed positive or negative priming effects, on the mineralization of native soil organic C [36]. Addition of substrates with relatively high C availability and nutrient contents often exerts a priming effect, and increases microbial activity [37]. The increased soil levels of such easily degraded compounds greatly enhance decomposition of native soil C [38,39]. After addition of organic fertilizer, old soil C becomes more labile and is more easily utilized by microbes. However, the role of old native soil C in the priming of soil organic C as a source of CH₄ production is not well established. Moreover, we also assumed that the C derived from the organic fertilizer can also contribute to the increased CH₄ emissions in paddy soils. However, it is complicated to separate the contributions of soil native C and organic fertilizer C due to the limitation of labelling approaches. Based on our former field research, we applied a pot experiment with isotopic ¹³C labelling in this study. Paddy CH₄ and CO₂ emissions, and some soil variables (N, C, P, K, and acetate) for the whole rice cultivation were firstly investigated. Besides, the relative abundances of methanogenic communities on the roots and in the soil, were measured by microbial 16S rRNA sequencing. Moreover, we calculated the relative contributions of rice photosynthesis-derived, native soil C-derived, and organic fertilizer-derived C to the C sources for CH₄ emissions from paddy soil. The co-occurrence networks between methanogenic species and environmental factors were also analyzed. The objectives of this research were to elucidate: (1) the main C source(s) responsible for the increased CH₄ emissions observed in rice soil after addition of organic fertilizer; (2) the dominant methanogenic community in organically fertilized paddy soils; and (3) the main factors affecting CH₄ effluxes and methanogenic community structures in paddy soil.

2. Results

2.1. Percentage δ¹³C Fractionation

At the regreening stage (Figure 2a,b), CO₂ accounted for 93.42% of δ¹³C fractionation on average in all samples, followed by the rice plants ¹³C (6.50%) and soil ¹³C (0.07%). At the regreening stage the δ¹³CH₄ distribution was no greater than 0.01%. At the tillering stage, the percentages of δ¹³C fractionation in the CO₂, rice plants, soil, and CH₄ were 28.60%, 64.47%, 0.40%, and 6.53%, respectively. The proportion of δ¹³CH₄ in the organic fertilizer treatment was 7.28%, higher than that in the no fertilizer treatment (5.78%). Ultimately, at the end of the booting stage, 86.49–91.38% of ¹³C was
fixed within the rice plants; 0.62–1.08% was stored in the soil, and 2.19–2.98% was transformed and emitted in the form of $^{13}$CH$_4$. Compared to the no fertilizer treatment, the addition of organic fertilizer increased CH$_4$ emissions derived from rice photosynthesis. We calculated organic fertilizer directly accounted for, at most, 0.34% of the C sources of CH$_4$ emissions (Figure 2c). Photosynthesis and native soil C accounted for at least 49.06% and 50.60% of the C sources of CH$_4$ emissions, respectively. Hence, rice photosynthesis-derived and native soil-derived C flow were the two primary C sources of CH$_4$ emissions. To analyze which C sources affected CH$_4$ emissions most, contribution coefficients were generated using the following linear regression equations:

$$
\delta^{13}CH_4 \text{ and rice plant } \delta^{13}C: y_r = 6.00x + 120.47, k_r = 0.17
$$

$$
\delta^{13}CH_4 \text{ and rice soil } \delta^{13}C: y_s = 0.08x + 0.94, k_s = 12.50
$$

Figure 2. Relative abundance of $\delta^{13}$C from cultured rice soil. (a,b) are the distributions of $\delta^{13}$C in the CO$_2$, CH$_4$, rice plant and soil for the average values of organic fertilizer and no fertilizer treatment. (c) shows the total carbon sources for the CH$_4$ emissions in the cultivated rice soil. (d,e) are the linear regressions for the daily $\delta^{13}$CH$_4$ to the daily rice plant $\delta^{13}$C and soil $\delta^{13}$C. Meanwhile, CH$_4$ fluxes, $\delta^{13}$CH$_4$ and rice biomass are shown in Figure S1, CO$_2$, CH$_4$ emissions and $\delta^{13}$C atom percentage (%) in Figure S2, and soil and rice plant $\delta^{13}$C in Figure S3. Results indicated that approximately 49.06% of the carbon in the CH$_4$ was derived from rice photosynthates. Therefore, the rest of the carbon sources would be approximately 50.95%, which should come from organic fertilizer or native soil. The average carbon and nitrogen of the organic manure was 23.05% and 2.07%. We knew that the pure nitrogen was applied at 300 kg·N·ha$^{-1}$. By calculation, the carbon from the organic fertilizer was applied at 2.36 g in the soil. Alternatively, the soil total carbon and bulk density were 1.78% and 1.43 g·cm$^{-3}$, respectively. The total amount of carbon in the topsoil (20 cm) was approximately 359.67 g. Therefore, the carbon ratio of the organic fertilizer and original soil would be 0.66%. Consequently, the carbon source for the CH$_4$ emissions derived from the organic fertilizers would be 0.34% at most, and 50.60% were from the soil at least. This was how we calculated the carbon sources of CH$_4$ in the rice soil after the organic fertilizer was applied.
According to the linear regression analysis, daily $\delta^{13}$CH$_4$ was significantly ($p < 0.01$) and positively dependent on both daily rice plant $\delta^{13}$C and soil $\delta^{13}$C (Figure 2d,e). $K_s$ was higher than $K_r$, revealing that CH$_4$ emissions were more sensitive to soil C fixation than plant photosynthesis C accumulation.

2.2. Methanogenic Community Structures

After 16S rRNA sequencing, the differential abundance analysis of each sample was compared using volcano plots and a Venn plot (Figure 3a–d). More differences in the OTUs were observed between the non-root soil than root soil samples overall. The NMDS and RDA plots (Figure 3e,f) also revealed that microbial community structures in root soil samples differed from non-root soil samples, especially after organic fertilizer was applied. At the taxonomic class level (Figure 4a), Euryarchaeota (48.56%), Crenarchaeota (3.45%), Thaumarchaeota (1.54%), and other non-ranked archaeal communities (38.86%) were identified in all treatments. At the genus level (Figure 4b), eight genera of methanogens were identified: 63.89% Methanosarcina, 8.64% Methanoculleus, 6.42% Methanobacterium, 1.87% Methanomethylovorans, 0.06% Methanoseta, 0.04% Methanosphaera, 0.38% Methanobrevibacter, 0.65% Methanolobus, and 40.50% unknown methanogens. Methanosarcina was the dominant methanogenic community. Methanosarcina had a higher relative abundance in O_root soil (87.75%) than the other samples. In addition, the relative abundance of Methanoculleus, the second largest methanogenic community, was higher in the no fertilizer treatment than organic fertilizer treatment. RDA (Figure 3f) and networks (Figure 5) were generated to analyze the correlations between the methanogenic species and environmental variables, and indicated the methanogenic community structures were susceptible to soil available phosphate (AP), available potassium (AK), available nitrogen (AN), soil C, CO$_2$ efflux, rice C, and biomass. Moreover, Methanobacterium palustre co-existed with Methanoculleus sp. 25XMc2, Methanoseta sp. HA, and Methanosphaera sp. ISO3-F5. Methanosarcina sp. 795 primarily co-occurred with Methanobrevibacter sp. AbM23, Methanoculleus sp. 25XMc2, Methanoseta sp. HA, Methanosarcina sp. 1H1 and Methanobacterium sp. MB1. Additionally, Methanosarcina sp. 1H1 also frequently co-occurred with Methanobrevibacter sp. AbM23, Methanoculleus sp. 25XMc2, and Methanoseta sp. HA. Most environmental factors were positively related to the methanogenic communities, except for Methanobrevibacter ruminantium, which was negatively correlated with acetate, $\delta^{13}$CO$_2$, TC, AK, TN, and soil $\delta^{13}$C. Overall, soil C—which was strongly, positively related to Methanosarcina sp. 795, Methanobacterium sp. MB1, Methanosarcina sp. 1H1, Methanoseta sp. HA, uncultured Methanomethylovorans sp., uncultured Methanosarcina sp. and an uncultured methanogen—was the environmental factor that most strongly influenced paddy methanogenic community structures.
Figure 3. Profiles of soil samples from 16S rRNA sequencing. O_non-root, OTUs from the rice non-root soil of organic fertilizer treatment. C_non-root, OTUs from the rice non-root soil of no fertilizer treatment. O_root, OTUs from the rice root soil of organic fertilizer treatment. C_root, OTUs from the rice root soil of no fertilizer treatment. After sequencing, we obtained 645 series of OTUs. The volcano plots (a–e) illustrated that the OTUs were significantly enriched (red) and depleted (blue) for each sample. Each point represented an individual OTU, and the X-axis indicated the abundance fold change between two soil samples. Using the OTU abundance from the C_non-root soil or the C_root soil as the control and an adjusted p-value cutoff of 0.1, “down OTUs (Down)”, “up OTUs (up)” and “none down or up OTUs (None)” specifically represent OTUs that increase and decrease significantly in relative abundance from different soil samples, respectively. The Venn plot (d) involved four sets of ellipses: O_non-root, C_non-root, O_root, and C_root. Numbers within the diagrams indicated whether the OTUs were shared among treatments or unique to one given treatment. In addition, the Nonmetric Multidimensional Scaling (NMDS e) analysis was performed to reveal the distances within the samples. The NMDS analysis is not affected by numerical sample distance and only considers the size of the relationship between each other. The RDA plot (f) was to analyze the correlations between the methanogenic community structure and variables. CO₂ emission of the chamber in the whole system, the CO₂ flux of the chamber in the whole system, CO₂ flux, the CO₂ flux of the chamber in the whole system, calculated by the sum of CO₂ flux. TN, soil total nitrogen. Biomass, the dry weight of rice plant. AK, soil available potassium. CH₄ flux, the CH₄ flux of the chamber in the whole system. Acetate, soil acetate. TC, soil total carbon. δ¹³CH₄, the δ¹³CH₄ abundance in the whole system. Soil δ¹³C, soil δ¹³C abundance. G1, Methanosarcina; G2, Methanolobus; G3, Methanomethylovorans; G4, Methanoculleus; G5, Methanobacterium; G6, Methanobrevibacter; G7, Methanospirillum; G8, Methanosaeta.
However, the extent to which organic fertilizer increases CH$_4$ emissions remains unknown. By calculation, it was 10.24 g organic fertilizer C:soil C ratio was 0.66%. If mineralization of the organic fertilizer transformed all of the added C, 6.10% of the C is involved in CH$_4$ production. If mineralization of the organic fertilizer transformed 50% of the added C, 3.05% of the C is involved in CH$_4$ production. The remainder of the CH$_4$ emissions may originate from the native soil or rice roots. In addition, the applied dose of organic fertilizer was same to the local N fertilization of 300 kg·N·ha$^{-1}$. By calculation, it was 10.24 g organic fertilizer N:soil N. The red lines, negative correlations. The blue lines, positive correlations. The red circles, methanogenic species. The green square, factors.

**Figure 5.** Co-occurrence networks. Acetate, soil acetate. $\delta^{13}$CO$_2$, the $\delta^{13}$CO$_2$ abundance in the whole system. TC, soil total carbon. AK, soil available potassium. TN, soil total nitrogen. AP, soil available phosphate. $\delta^{13}$CH$_4$, the $\delta^{13}$CH$_4$ abundance in the whole system. Soil $\delta^{13}$C, soil $\delta^{13}$C abundance. AN, soil alkali-hydrolyzable nitrogen. The red lines, negative correlations. The blue lines, positive correlations. The black lines, correlations among methanogens. The thickness of lines, the value of correlations. The red circles, methanogenic species. The green square, factors. Methanobrevibacter palustre, Taxonomy ID 2171 in NCBI. Methanosphaera sp. ISO3-F5, taxonomy ID 1,452,353 in NCBI. Methanosarcina sp. 795, taxonomy ID 1,653,396 in NCBI. Methanobacterium sp. MB1, taxonomy ID 1,379,702 in NCBI. Methanosarcina sp. 1H1, taxonomy ID 1,882,224 in NCBI. Methanosaeta sp. HA, taxonomy ID 1,562,961 in NCBI. Methanobrevibacter ruminantium, no taxonomy ID in NCBI. Methanoculleus sp. 25XMc2, taxonomy ID 1,898,331 in NCBI. Methanobrevibacter sp. AbM23, taxonomy ID 1,452,345 in NCBI. Methanolobus psychrophilus, taxonomy ID 420,950 in NCBI.
3. Discussion

3.1. Rice Photosynthesis-Derived and Native Soil-Derived Methane Emissions

Addition of organic manure is commonly implemented in rice agriculture to improve soil fertility and recycle agricultural waste, but inevitably enhances CH$_4$ production in flooded paddy soil [40,41]. However, the extent to which organic fertilizer increases CH$_4$ emissions remains unknown. This study indicates that approximately 49% of CH$_4$ emissions from rice paddy soil are derived from rice photosynthesis, and the application of organic fertilizer increases photosynthesis-derived CH$_4$ emissions in rice soil. The remainder of the CH$_4$ emissions may originate from the native soil or organic fertilizer, and this needs further calculation. In this study, the applied dose of organic fertilizer was same to the local N fertilization of 300 kg N ha$^{-1}$. By calculation, it was 10.24 g organic fertilizer, which included 2.36 g C. While, it was 359.67 g C in the topsoil (20 cm). Therefore, the fertilizer C:soil C ratio was 0.66%. If mineralization of the organic fertilizer transformed all of the fertilizer-derived C to CH$_4$ (which is unlikely) [38], CH$_4$ emissions derived from the organic fertilizer would account for only 0.34% of total CH$_4$ emissions at most. Consequently, at least 50.60% of the C sources for CH$_4$ emissions must be derived from the native soil. Therefore, the increased CH$_4$ emissions observed in organically-fertilized paddy soil can ascribed to enhanced rice photosynthesis and improved soil fertility.

Previous studies showed that up to 60% of CH$_4$ originated from rice photosynthesis [34]. Atmospheric CO$_2$ is brought into the rice during photosynthesis and partially utilized by methanogens, especially Methanocellales (Rice Cluster I) and Methanosetaeae [35,42]. However, not all soil methanogens can survive in rice roots and, thus, use organic C substrates to produce CH$_4$ [34]. The contribution of rice photosynthesis to CH$_4$ emissions in paddy fields was found to be influenced by the community abundances and activities of methanogens in the rice roots [14]. Previous $^{13}$CO$_2$ labelling studies showed more than 85% of the $^{13}$C accumulated in the aerial parts of the plant, approximately 10% was transferred to the roots, less than 2% was transferred to the soil, and only 0.3% was transferred to CH$_4$ [43]. At the end of cultivation, we found 86.49–91.38% of the $^{13}$C was present in the plants, 0.62–1.08% in the soil, and 2.19–2.98% in CH$_4$. Thus, rice photosynthesis-derived C is an important source of metabolic substrates for methanogens. In addition, $\delta^{13}$CH$_4$ and rice biomass were higher in the organic fertilizer treatment than no fertilizer treatment, especially at the tillering stage (Supplemental Figure S1). This indicates CH$_4$ emissions increased with the rice photosynthetic activity [44]. However, C originating from rice photosynthates is just one component of CH$_4$ emissions. The C substrate utilization efficiency of methanogens may be a more important factor that determines the increase in CH$_4$ emissions from organically fertilized paddy soil.

Apart from the organic fertilizer- and rice photosynthesis-derived CH$_4$ emissions, 50.60% of C sources for total CH$_4$ emissions were derived from the native soil. This suggests that native or pre-existing soil C became more labile to microbial metabolism after organic fertilizer was applied [38]. The utilization efficiency of soil C substrates or native C sources was previously found to be of pivotal importance to CH$_4$ emissions from rice soil [34]. However, the mechanisms by which native C sources transform to active C and finally convert into CH$_4$ are unknown and merit further research. Essentially, CH$_4$ emissions from paddy fields are the result of a variety of complex soil microbial processes, including CH$_4$ production in flooded anaerobic microsites and consumption (oxidation) in aerobic microsites [22,23]. In our previous research, we found soil C, N, potassium, phosphate, and acetate were strongly related to CH$_4$ emissions during all stages of rice growth, especially after organic fertilizer was applied [31]. This suggests that the ability of organic fertilizer to increase CH$_4$ emissions is not due to the introduction of extra C sources, but instead was due to the alterations to soil properties. Therefore, the increase in CH$_4$ emissions induced by the addition of organic fertilizer are primarily due to changes in labile soil characteristics (N, P, K, and acetate) that affect the availability of soil C substrates to methanogens and other microbial communities in paddy soil.
3.2. Acetoclastic Methanogens Dominate Rice Soil after Application of Organic Fertilizer

Methanogens occur widely in anaerobic environments because of their unique ability to harvest energy using CO$_2$ (electron acceptor) and/or methylated compounds as an energy source [45,46]. Despite the widespread presence of methanogens, global CH$_4$ production primarily stems from subsurface sediments that contain a small proportion of the methanogenic population, corresponding to <1% of the total microbiome and a small fraction of archaea [47]. The dynamics of methanogenesis and methanogen community structure in flooded rice fields depend on various parameters, such as soil properties, rice cultivar, fertilization, seasonal stage, crop rotation, and climate change [33]. Analysis of methanogenic community structure and function in paddy fields will provide a better understanding of the ecology of methanogens and their CH$_4$ production potential [3,6,23]. This is essential to formulate a strategy to mitigate CH$_4$ emissions from rice agriculture. Methanogens are classified into three categories: hydrogenotrophic, acetoclastic, and methylotrophic [11,16]. Acetoclastic and hydrogenotrophic methanogens were the primary physiological groups found in rice fields [48,49]. Acetoclastic methanogens convert acetic acid to CH$_4$ and CO$_2$ and contain two families, Methanosarcinaceae and Methanosaetaceae [48]. However, Methanosarcinaceae exhibit hydrogenotrophic and acetotrophic nutritional modes [50]. We found Methanosarcina dominated the organically fertilized rice soil, with a higher relative abundance in rice root soil than non-root soil. In rice soil, CH$_4$ is produced by methanogens inhabiting the rhizosphere and non-rhizosphere bulk soil, as well as the surfaces of the roots [6,34,51]. In contrast to the non-rhizospheric or bulk region, transport of atmospheric O$_2$ via the rice aerenchyma to the roots and the release of dissolved organic compounds in the form of root exudates occur in the rhizosphere region [34]. Thus, the microorganisms responsible for the degradation and utilization of substrates in these regions show a heterogeneous community composition. Another acetoclastic methanogen, Methanosaeta, was the predominant methanogenic community identified across all rice soil treatments in our former field research [31]. The environmental factors affecting methanogenic community dynamics—such as rice plant biomass, soil available nutrients, and CO$_2$ and CH$_4$ emissions in rice fields—have been previously discussed. Several researchers have proven that soil characteristics control methanogenic community structures [9,10,52]. We found organic fertilization led to expansion of dominant acetoclastic methanogens due to the increased availability of soil nutrients, including soil-available N, P, and K. We assume that production of CH$_4$ from decomposition of acetate could be dramatically increased by organic fertilization, and this hypothesis merits further research.

Under the current taxonomic system in the Silva or NCBI databases, Methanosarcina sp. 795 and Methanosarcina sp. 1H1 had a symbiotic relationship with Methanobrevibacter sp. AbM23, Methanoculleus sp. 25XMc2, Methanosaeta sp. HA, and Methanobacterium sp. MB1, as well as some uncultured methanogenic species. Kouzuma et al. [53] found that the mcrA phylogenetic tree constructed for Methanosarcina showed Methanosarcina sp. 795 was affiliated with Methanosarcina thermophila in an enrichment culture from an acetate-fed thermophilic digester in Canada. Methanosarcina sp. 795 was also very closely related to Methanosarcina thermophila CHT1-55 isolated in France [54]. Close relatives of Methanosarcina thermophila have been widely detected in thermophilic anaerobic conditions worldwide. These observations suggest this taxon plays an important ubiquitous role in anaerobic digesters, regardless of geographical location. In this study, Methanosarcina sp. 795, as well as Methanosarcina sp. 1H1 were found in rice soil. Iijiri et al. [55] cultivated the methanogen 1H1 strain for approximately nine months to study a pure isolation of spherical cells. Phylogenetic analysis of the 16S rRNA gene showed strain 1H1 was closely related to Methanosarcina mazer. The Methanosarcina mazer isolate could grow on H$_2$/CO$_2$, acetate, methanol, dimethylamine, and trimethylamine, but not on formate, dimethylsulfide, ethanol, 1-propanol, 2-propanol, cyclopentanol, 1-butanol, or 2-butanol. As shown in Figure 5, Methanosarcina sp. 795 and Methanosarcina sp. 1H1 had a strong symbiotic relationship. In their role as hydrogenotrophic methanogens, CO$_2$ is first reduced and activated to formyl-methanofuran with reduced ferredoxin (Fd$_{red}$) as the electron donor [56]. However, during acetoclastic methanogenesis, acetate is first activated [42], then converted with ATP and coenzyme A (CoA) to acetyl-CoA, which is
then split by the CODH/acetyl-CoA synthase complex. The methyl group is transferred to H₄MTP, which is tetrahydrosarcinapterin (H₄SPT) in *Methanosarcina*. H₄SPT is then converted to CH₄ via a process similar to the CO₂ reduction pathway (Figure 1). The carbonyl group is oxidized to CO₂, thus providing the electrons for methyl group reduction. However, cytochrome-containing *Methanosarcina* (including *Methanosarcina acetivorans*, *Methanolobus tindarius*, and *Methanothrix soehngenii*) produce less CH₄ than hydrogenotrophic methanogens under many environmental conditions [46]. This indicates many *Methanosarcina* would switch to the acetoclastic pathway in the presence of sufficient acetate to produce CH₄ [48]. However, the functional role of *Methanosarcina* and main active species involved in CH₄ production are still unknown. Other minor groups could be more active. In the future, the contributions of different methanogenic groups need to be verified.

4. Materials and Methods

4.1. Experimental Site

The experimental soil was from a typical paddy field located at the Irrigation Technology Research Station (121.12° E, 31.15° N, altitude 5 m) in Qingpu, Shanghai, China. The site experiences a subtropical humid monsoon climate with an annual average daily temperature of 15.8 °C, annual average rainfall of 1300 mm, and 1400 h of sunshine per year. Rotation between winter wheat cv. Yangmai-5 and summer rice cv. Huayou-14 was the major cropping system since 2009 [30,32]. The local nitrogen fertilization is 300 kg·ha⁻¹ for the total paddy season. After rice harvesting in October 2016, we dug about 300 kg soil (0–20 cm) with hoes and shovels from the field. Before pot cultivation, we took 500 g soil for air-drying and passed through a 2 mm sieve to measure the physicochemical characteristics [30]. The soil had a pH of 7.71, EC of 0.11 mS·cm⁻¹, bulk density of 1.43 g·cm⁻³, total nitrogen (TN) content of 0.43%, total carbon content (TC) of 1.78%, available phosphorus content of 19.53 mg·kg⁻¹, and available potassium content of 84.34 mg·kg⁻¹. The fresh soil was directly taken to the greenhouse. Every 20 kg soil was packed into a column-shaped bucket.

4.2. Experimental Design

The experiment was conducted with four treatments and three replicates: 1) no fertilizer as a control (CN), 2) no fertilizer +¹³CO₂ (CY), 3) organic fertilizer (ON), and 4) organic fertilizer +¹³CO₂ (OY). The organic fertilizer was chicken manure with moisture of 23%, TN content of 19 g·kg⁻¹, TC content of 202 g·kg⁻¹, total phosphorus content of 13 g·kg⁻¹, and total potassium content of 29 g·kg⁻¹. It was supplied by Sennong Environmental Protection Technology Co. Ltd. (Shanghai, China). Two days before rice transplantation, 10.24 g organic fertilizer were applied to each pot (calculated according to the same dose as the local N fertilization of 300 kg·N·ha⁻¹). Afterwards, thirty-day-old rice seedlings (cv. Huayou-14) with uniform growth tendency (approximately 15 cm tall) were transplanted to the fresh soil. Every pot had three clusters of rice seedlings (a cluster = 3 rice seedlings). The paddies were flooded with 5 cm water layer during the whole pot experiment. The greenhouse was maintained at 20–35 °C and a daytime light intensity of 35 kLux. After rice transplanting, the pots were placed into the 490 L incubation chamber (Figure S1). Generally, the rice growth is classified into seven stages: regreening, tillering, jointing, booting, heading, filling, and maturing. Paddy CH₄ fluxes have been shown to be significantly higher from the regreening to booting stages than others [30,31,57]. In this experiment, rice was cultivated from the regreening to booting stage, lasting for 70 days.

Isotopic labelling was generated inside the chamber via the reaction between the hydrochloric acid (HCl) and Na₂¹³CO₃ (99.80% purity; Cambridge Isotope Laboratories Inc., Tewksbury, MA, USA). The Na₂¹²CO₃ was applied as the control. Beforehand, 10 g of Na₂¹³CO₃ or Na₂¹²CO₃ were put inside the chamber. The 200 mL 1 mM HCl solution was then gently added to the chamber through a tube (Figure S1). The chamber was kept sealed during the labelling experiment. The specific experimental stages were listed below:
(1) Rice regreening stage (first stage, 9 d). Twelve pots containing rice plants with homogeneous growth were selected for the first stage of $^{13}\text{C}$ labelling. The first day of labelling was set as day 1 and labelling ended on day 10.

(2) Rice tillering stage (second stage, 27 d). On day 11, new 12 pots containing rice plants with homogeneous growth congruent with the growth trends of the previous stage were selected for the second stage of labelling. Labelling lasted from day 11 until day 37.

(3) Rice booting stage (third stage, 33 d). On day 33, 12 pots containing rice plants with homogeneous growth were selected for the third stage of labelling, which lasted from day 38 to day 70.

4.3. $\text{CH}_4$ and $\text{CO}_2$ Sampling

Gas samples were collected from the incubation chamber at 0, 6, 12, 24, 36, 48, and 72 h after the initiation of labelling, then once every five days (at 9:00–9:30) until the end of labelling. Measurements included $\delta^{13}\text{CH}_4$, $\delta^{13}\text{CO}_2$, total $\text{CH}_4$, and total $\text{CO}_2$. The concentrations of $\text{CH}_4$ and $\text{CO}_2$ were analyzed at the Analytical Platform of Shanghai Jiao Tong University using an Agilent 7820A gas chromatograph (Agilent, Santa Clare, CA, USA). Gas fluxes and emissions were calculated as described by Yuan et al. [30]. Additionally, $\delta^{13}\text{CH}_4$ (accuracy $\delta^{13}\text{C} \pm <0.5\%$) and $\delta^{13}\text{CO}_2$ (accuracy $\delta^{13}\text{C} \pm <0.3\%$) were analyzed by Huakejingxin Technology Co., Ltd. (Shenzhen, China) using a Delta V™ Isotope Ratio Mass Spectrometer (Thermo Fisher Scientific, Grand Island, NY, USA).

(1) $\delta^{13}\text{C}$ was calculated using Equation (3): 
\[
\delta^{13}\text{C} (\%) = 1000 \times (R_{\text{sample}} - R_{\text{standard}}) / R_{\text{standard}}
\]

where $\delta^{13}\text{C} (\%)$ is the abundance of the isotope composition, $R_{\text{sample}}$ is the $^{13}\text{C}_{\text{sample}} / ^{12}\text{C}_{\text{sample}}$ for each sample, and $R_{\text{standard}}$ is the $^{13}\text{C}_{\text{standard}} / ^{12}\text{C}_{\text{standard}}$ of the international common C isotope analysis standard (usually the $^{13}\text{C} / ^{12}\text{C}$ ratio of Pee Dee Belemnite [PDB]).

(2) Daily accumulation of $\delta^{13}\text{C}$ was calculated using Equation (4):
\[
V (\% \cdot \text{d}^{-1}) = (\delta^{13}\text{C}_2 - \delta^{13}\text{C}_1) / t
\]

where $\delta^{13}\text{C}_2$ and $\delta^{13}\text{C}_1$ are the $\delta^{13}\text{C}$ values of the first and last day of each stage, respectively, and $t$ is number of experimental days for each stage.

(3) $\delta^{13}\text{CH}_4$ per rice biomass was calculated using Equation (5):
\[
\text{Rice } \delta^{13}\text{CH}_4 (\% \cdot \text{g}^{-1}) = \text{Final } \delta^{13}\text{C} / S_{\text{chamber}} / W_{\text{rice}}
\]

where rice $\delta^{13}\text{CH}_4 (\% \cdot \text{g}^{-1})$ is $\delta^{13}\text{CH}_4$ per unit rice biomass; final $\delta^{13}\text{C}$ is the ultimate $\delta^{13}\text{C}$ for the organic fertilizer and no fertilizer treatments in the rice soil; $S_{\text{chamber}}$ is the area of the chamber, (0.49 m$^2$); $W_{\text{rice}}$ (g·m$^{-2}$) is the weight of the rice biomass.

(4) Contribution coefficients for $\delta^{13}\text{CH}_4$ responding to rice plant or soil were calculated using Equations (6)–(9):
\[
y_r = a_r x + b_r
\]
\[
y_s = a_s x + b_s
\]
\[
K_r = 1 / a_r
\]
\[
K_s = 1 / a_s
\]

where $y_r$ is the daily rice plant $\delta^{13}\text{C} (\% \cdot \text{d}^{-1})$; $y_s$ is daily rice soil $\delta^{13}\text{C} (\% \cdot \text{d}^{-1})$; $x$ is daily $\delta^{13}\text{CH}_4 (\% \cdot \text{d}^{-1})$; $K_r$ is taken as the contribution coefficient of rice C accumulation to CH$_4$ emission; $K_s$ is taken as the contribution coefficient of rice soil C fixation to CH$_4$ emission.
4.4. Plant and Soil Collection

On the last day of each experimental labelling stage, the rice plants and soil were collected. Plant samples (grains, leaves, shoots, and roots) were oven-dried at 60 °C. Soil samples were homogenized, air-dried, crushed and passed through a sieve (<2 mm). Soil texture was determined using the Bouyoucos hydrometer method. To assess pH and electrical conductivity (EC), soil samples were vigorously mixed with deionized water at a 1:2.5 solid:water ratio for 20 min and assessed using digital meters (Elico Model LI-120, Elico Pvt. Ltd., Hyderabad, India). Organic C was determined using the Walkley–Black method. The δ13C and total C content of the plant and soil samples were determined at the Instrumental Analysis Center of Shanghai Jiao Tong University using an elemental isotope mass spectrometry analyzer (Vario EL III/Isoprime, Agilent, Santa Clara, CA, USA).

4.5. DNA Extraction and MiSeq Sequencing of 16S rRNA

At the tillering stage, the rice rhizosphere (root) and non-rhizosphere (non-root) soils were collected separately and labelled as C_non-root, C_root, O_non-root, and O_root. All soil samples were placed into sterile sealable plastic bags and stored at −70 °C. Total soil DNA was extracted from 0.5 g rhizosphere soil using BioFast Soil Genomic DNA Extraction kits (Tiangen Co., Ltd., Beijing, China). The extracted DNA was eluted in 100 µL Elution Buffer and stored at −20 °C until the MiSeq Illumina analyses. Amplicon library preparation and Illumina MiSeq sequencing were performed by Majorbio Bio-Pharm Technology Co., Ltd. (Shanghai, China).

Amplification of archaeal 16S rRNA gene fragments was conducted using the primers 1106F/1378R (F: TTWAGTCAGGCAACGAGC and R: TGTGCAAGGAGCAGGGAC) [58] targeting the V4 region (280 bp product). In addition to the 16S target-specific sequence, these primers also contain adaptor sequences to allow uniform amplification of a library with high complexity to enable downstream NGS sequencing on an Illumina MiSEQequation (PCR was conducted using a TransGen AP221-02 reaction kit (TransStart Fastpfu DNA Polymerase, Majorbio Inc., Shanghai, China) on an ABI GeneAMP® 9700 (Thermo Fisher Scientific, Grand Island, NY, USA) with initial denaturation at 95 °C for 90 s, followed by 35 cycles of 95 °C for 30 s, 55 °C for 30 s and 72 °C for 90 s, and a final extension at 72 °C for 6 min. Three replicates were assessed for each sample and the triplicate PCR products for each sample were mixed and separated on 2% agarose gels to recover the PCR products. The amplicons were purified using an AxyPrepDNA gel recovery kit (AXYGEN Co., Ltd., Tewksbury MA, USA) and quantified using the QuantiFluor™-ST Blue system (Promega Co., Ltd. Madison, WI, USA). Finally, the samples were pooled at equimolar concentrations (>2 nM) and sequenced on separate MiSeq runs using a 2 × 300 bp paired end protocol. Finally, the TruSeq™ DNA Sample Prep Kit (Majorbio Inc., Shanghai, China) was used for MiSeq library construction.

The paired end reads obtained from the MiSeq sequencing were first spliced according to the overlap relationship, and quality control and filtration of sequence quality were conducted to distinguish the sample reads, followed by cluster and taxonomy analyses. The double-ended FASTQ sequences were filtered using the sliding window method, and Trimomatic and FLASH software programs were used to connect the sequences that passed the quality filter. The FASTA and qual files from Mothur were converted to FASTQ format using USEARCH (Version 7.1) [59]. Downstream processing and operational taxonomy unit (OTU) picking were performed using UPARSE [60]. Barcodes and primers from the merged sequences were removed. Sequence reads < 200 bp were discarded. After dereplication and abundance sorting keeping singletons, sequences with a minimum of 97% similarity were clustered into OTUs using the average neighbor algorithm. Taxonomy was assigned using the Silva database with PyNAST at an 80% confidence threshold [52,61]. The sequence data are available from the NCBI Nucleotide Archive database under project no. PRJNA450890 and accession number SRP142386.
4.6. Statistical Analysis

The experiment was conducted with three replicates for each treatment. SPSS 22.0 software (IBM, NY, USA) was used to analyze the differences between treatments using one-way analysis of variance (ANOVA) and the Pearson correlation test. Figures were generated using Origin 8.0 (OriginLab Co., Northampton, MA, USA). The Venn diagram was generated using the ‘Vennerable’ package in R (MathSoft Co., Needham, MA, USA). QIIME was used to calculate the weighted pairwise Uni-Frac distances for community comparisons, which were visualized using non-metric multidimensional scaling (NMDS) plots in PRIMER v6 [61]. Clustering was performed using the ‘complete linkage’ method, and the heatmap based on clustering was constructed with the package hclust in R [62]. Redundancy analysis (RDA) was performed to summarize the variations in methanogenic communities that could potentially be explained by the variables (treatments, \( \text{CH}_4 \) efflux and all physicochemical parameters) using CANOCO 4.5 software (Microcomputer Power, Ithaca, NY, USA) [31]. Network analysis was conducted on the methanogenic OTUs and soil properties using the maximal information coefficient (MIC) implanted in python. The MIC is a highly informative score that reveals the strength of linear and non-linear associations among variables. Only 24 OTUs belonging to the methanogenic community were identified at the species level. Therefore, all the relationships were used for network construction in Cytoscape v.3.2.1 [63]. Network topological characteristics were calculated using the NetworkAnalyzer tool in Cytoscape.

5. Conclusions

C sources derived from rice photosynthesis and native soil are the two-primary pathways for \( \text{CH}_4 \) emissions from paddy soil. Daily \( \delta^{13} \text{CH}_4 \) accumulation was positively dependent on daily rice plant \( \delta^{13} \text{C} \) and soil \( \delta^{13} \text{C} \). Moreover, the increase in \( \text{CH}_4 \) emissions observed in organically fertilized soil was sensitive to soil C fixation. After 16S rRNA sequencing, we found organic fertilization led to significantly different microbial community structures in both the root and non-root areas of rice soil. Acetoclastic \textit{Methanosarcina} was the dominant methanogenic community in rice soil overall. \textit{Methanosarcina} sp. 795 and \textit{Methanosarcina} sp. 1H1 co-occurred with \textit{Methanobrevibacter} sp. AbM23, \textit{Methanoculleus} sp. 25XMc2, \textit{Methanosaeta} sp. HA, and \textit{Methanobacterium} sp. MB1, as well as other unknown and uncultured methanogens. The increase of \( \text{CH}_4 \) emissions and variance of methanogenic community structure observed after addition of organic fertilizer to rice soil were primarily due to the increases in labile soil C, N, P, K, and acetate.

Supplementary Materials: Supplementary materials can be found at http://www.mdpi.com/1422-0067/20/7/1586/s1.

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