Effects of Slag and Biochar Application on Methanogen Community Structure in Paddy Soil Postprint

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Date: 2018-05-10T00:00:00+00:00

Abstract

To understand the microbiological mechanisms by which waste amendment treatments affect methane emission fluxes from rice paddies, rice paddies were subjected to single amendments of slag, biochar, and combined amendment treatments. Soil physicochemical properties were analyzed during the jointing stage of early and late rice under these amendment conditions, and PCR-RFLP technology combined with clone sequencing was employed to analyze the composition, diversity, and structure of methanogenic archaeal communities in paddy soil. The results showed that during the early rice jointing stage, the combined amendment treatment significantly increased soil salinity and pH; during the late rice jointing stage, the combined amendment treatment significantly increased soil salinity, while both slag and combined amendment treatments significantly increased pH. The Shannon-Wiener index (H') and Simpson index (D) revealed that slag, biochar, and combined amendment treatments enhanced the diversity of methanogenic archaea in paddy soil. Community composition analysis indicated that paddy soil methanogenic archaea mainly comprised six major groups: Methanomicrobiales, Methanobacteriales, Methanosarcinales, Methanococcales, Methanocellales, and Methanomassiliicoccales, with Methanomicrobiales being the dominant group. At the genus level, community structure analysis revealed that, compared with the control, all three amendment treatments reduced the relative abundance of Methanomassiliicoccus in early rice soil; the biochar treatment also decreased the relative abundance of Methanosarcina. It is preliminarily concluded that these two genera, Methanomassiliicoccus and Methanosarcina, are closely associated with reduced CH4 emissions.

Full Text

Preamble

ACTA ECOLOGICA SINICA (ChinaXiv Partner Journal)
Vol. 38, No. 8, April 2018
DOI: 10.5846/stxb201705210939

Effects of Slag and Biochar Amendments on Methanogenic Community Structures in Paddy Fields

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Abstract

To understand the microbial mechanisms underlying the effects of waste amendments on methane emission fluxes from paddy fields, we investigated the impacts of single and mixed applications of slag and biochar on soil physicochemical properties during the elongation stages of both early and late rice. Using PCR-RFLP and clone sequencing, we analyzed the composition, diversity, and community structure of methanogens in paddy soil. The results showed that during the early rice elongation stage, mixed amendments significantly increased both soil salinity and pH. During the late rice elongation stage, mixed amendments significantly increased soil salinity, while slag and mixed amendments significantly increased soil pH. Shannon and Simpson diversity indices indicated that slag, biochar, and mixed amendments all enhanced methanogen diversity compared to the control. Phylogenetic analysis revealed that methanogenic communities in paddy fields could be subdivided into six groups: *Methanobacteriales*, *Methanosarcinales*, *Methanomicrobiales*, *Methanococcales*, *Methanocellales*, and *Methanomassiliicoccales*, with *Methanomicrobiales* being the dominant group. Compared to the control, all three amendment treatments reduced the relative abundance of *Methanomassiliicoccus* in early rice soil, and biochar amendment reduced the relative abundance of *Methanosarcina*. The reduced relative abundances of *Methanomassiliicoccus* and *Methanosarcina* may have contributed to decreased CH emissions.

Keywords: methanogens; community structures; waste; PCR-RFLP; paddy fields

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Introduction

Methane (CH) is the second most important greenhouse gas after carbon dioxide (CO), contributing approximately 20% to the greenhouse effect. Agricultural activities are the primary source of atmospheric greenhouse gases, with rice paddies representing the most significant agricultural source, accounting for about 10% of global CH emissions. While rice cultivation ensures food production, it also exacerbates greenhouse gas emissions, accelerating global climate warming. Coordinating food production increases with greenhouse gas reduction has important theoretical and practical significance.

Research on applying agricultural and industrial wastes to rice paddies has increased, with biochar and slag being major waste types that can improve soil properties. Biochar, derived from agricultural and woody waste, has shown effectiveness in improving soil physicochemical properties, increasing grain yield, and reducing greenhouse gas emissions. International scholars have found that steel slag application can increase rice yield while reducing CH emissions, with the yield-increasing and emission-reducing effects related to iron oxide and silicon content in slag.

Methanogens are strictly anaerobic microorganisms that serve as specific targets affecting CH production in rice paddies. The functional gene *mcrA* has been widely used to detect methanogen diversity in paddy soils. Major methanogen groups in paddy soils include *Methanobacteriales*, *Methanosarcinales*, *Methanomicrobiales*, and *Methanococcales*. The relative abundance of *Methanosarcina* and *Methanosaeta* in *Methanosarcinales* is closely related to CH production. Water-fertilizer management and rice growth stages can alter methanogen community structure, thereby affecting CH emissions. Substrate type is a key factor influencing methanogen community structure, with acetate and soil organic carbon content playing important roles.

Studies on how waste amendments affect methanogen community composition and structure in paddy soils are limited. Biochar input can improve soil aeration and nutrient content while reducing bulk density and dissolved organic carbon (DOC), thereby inhibiting acetoclastic methanogens like *Methanosarcina* and *Methanosaeta*. Some studies suggest biochar reduces CH emissions by enhancing methanotrophic activity or reducing the *pmoA*/*mcrA* ratio. Most researchers agree that biochar application reduces CH emissions, but the microbial mechanisms require further investigation. For slag, the primary mechanism for CH reduction is thought to be iron oxide acting as an electron acceptor to inhibit methanogen activity, though whether slag also affects methanogen diversity and community structure remains unclear.

Our previous research found that CH emission fluxes during the rice elongation stage are higher than during the milk ripening stage. This study investigated a red soil paddy field in the Fuzhou Plain, analyzing soil physicochemical properties and methanogen community composition and structure during early and late rice elongation stages using the *mcrA* gene as a molecular marker via PCR-

RFLP and clone sequencing. The objective was to correctly assess how waste amendments affect soil properties and their relationship with methanogen diversity, community structure, and CH emission fluxes, providing theoretical support for CH mitigation strategies.

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1. Study Site Overview

The study area was located at the Wufeng Comprehensive Experimental Base of the Rice Research Institute, Fujian Academy of Agricultural Sciences, in the southern branch of the Fuzhou Plain of the Min River estuary (25°59 44.12 N, 119°38 35.50 E). The site has an elevation of 3–5 m and a subtropical monsoon climate. See Figure 1 [Figure 1: see original paper] for the sampling location map.

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2. Amendments

Slag composition: SiO (27.7%), CaO (35.7%), SO (1.3%), Fe O (6.2%), P O (0.1%), MgO (4.3%), and other components (2.7%).

Biochar composition: N (1.4%), P (1.0%), K (1.8%), C (56.6%), S (0.6%), Mg (1.0%), Ca (0.5%), and other components (0.2%). Biochar was sieved through a 2 mm mesh before use.

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3. Rice Varieties

Early rice: Hesheng 10 (developed by Jiangxi Academy of Agricultural Sciences)
Late rice: Qinxiangyou 17 (developed by Fujian Academy of Agricultural Sciences)

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4. Amendment Application Methods

The experiment was conducted during early and late rice growing seasons in 2015. Late rice was planted in the same experimental area after early rice harvest, with no amendments applied during the previous early rice season. Four treatments were established: control (no amendment), slag application, biochar application, and mixed slag+biochar application. Amendment rates were 8 t/ha for single applications and 8 t/ha each for slag and biochar in the mixed treatment (total 16 t/ha). Amendments were applied once to the plow layer (0–15 cm) before rice transplanting. Each plot was 30 m² with three replications in a randomized block design. Plots were separated by 0.5 cm thick, 30 cm deep PVC sheets to prevent water and material exchange.

5. Soil Sample Collection

During the elongation stages of early and late rice, soil samples were collected from each plot using a soil corer (0–15 cm). Samples were placed in sterile bags and transported to the laboratory. Each sample was divided into two portions: one for physicochemical analysis and another stored at -80°C for DNA extraction and methanogen analysis.

6. Soil Physicochemical Analysis

Soil pH was measured using an IQ150 pH meter (IQ Scientific Instruments, USA). Electrical conductivity was measured to characterize soil salinity. Bulk density was determined by the ring knife method, and water content by aluminum box drying. Soil organic carbon (SOC) and total nitrogen (TN) were measured using a Vario MAX CN elemental analyzer (Elementar, Germany).

7. DNA Extraction and Methanogen Analysis

7.1 Soil Total DNA Extraction

Total DNA was extracted from 0.5 g fresh soil using an E.Z.N.A. Soil DNA Kit (OMEGA). DNA purity and concentration were assessed using a NanoDrop spectrophotometer (USA). The A /A ratio of 1.8–2.0 indicated high-quality DNA suitable for PCR amplification.

7.2 Methanogen *mcrA* Gene Amplification

The methanogen-specific *mcrA* gene was amplified using primers ME1 (GCMATGCARATHGGWATGTC) and ME2 (TCATKGCRTAGTTDGGRTAGT). The 25 L reaction mixture contained: 2.5 L 10× buffer, 2 L dNTPs, 0.5 L each primer, 0.5 L DNA template, 0.25 L Ex Taq polymerase, and ddH O. PCR conditions: 5 min at 95°C; 30 cycles of 45 s at 95°C, 48°C for 90 s, 72°C for 1 min; final extension at 72°C for 10 min. Amplified products (~760 bp) were verified by agarose gel electrophoresis.

7.3 Clone Library Construction

PCR products were purified using a SanPrep gel extraction kit (Sangon, China) and ligated into pMD18-T vector (TaKaRa), then transformed into *E. coli* competent cells. Transformed cells were plated on LB medium with ampicillin. White colonies were selected to construct clone libraries. Colony PCR was performed using M13-47/RV-M primers to identify recombinants.

7.4 Restriction Fragment Length Polymorphism (RFLP) Analysis

Positive clones were cultured in liquid medium, and plasmids were extracted. The 10 L enzyme reaction contained: 1 L 10× buffer, 1 L 0.1% BSA, 0.5 L *Msp*I restriction enzyme (TaKaRa, Japan), 2.5 L plasmid DNA, and 6 L ddH O. Digested fragments were separated by 2% agarose gel electrophoresis. Clones with identical restriction patterns were grouped as one operational taxonomic unit (OTU).

7.5 Sequencing and Phylogenetic Analysis

Representative clones from each OTU were sequenced (Sangon Biotech). Sequences were compared against GenBank using BLAST. Similarity analysis was performed using Clustal X, and phylogenetic trees were constructed using MEGA 4.0 with the neighbor-joining method (bootstrap = 1000).

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8. Data Analysis

Diversity indices (Shannon-Weiner index H and Simpson index D) were calculated using Bio-dap software. Coverage C was calculated as: $C = (1 - n/N) \times 100\%$, where n is the number of single-occurrence OTUs and N is the total clone number. SPSS 17.0 was used for one-way ANOVA to test treatment differences, and Excel 2003 for data organization.

Results

1. Soil Physicochemical Properties

Soil properties during early and late rice elongation stages are shown in Table 1 . During early rice:

- Mixed amendment significantly increased soil salinity (0.57 mS/cm) by 185.00% and pH (7.41) by 17.06% compared to control (0.20 mS/cm, pH 6.33).
- Slag and biochar amendments increased pH by 2.89% and 5.60%, respectively.
- Biochar and mixed amendments increased SOC by 5.82% and 33.96%, and C/N ratio by 1.07% and 27.80%, respectively.

During late rice:

- Mixed amendment significantly increased salinity (0.47 mS/cm) by 104.35%.
- Slag and mixed amendments significantly increased pH (7.21) by 18.59%.
- Biochar and mixed amendments increased SOC by 2.80% and 33.38%, and C/N ratio by 0.62% and 24.48%, respectively.

Overall, amendments consistently increased soil salinity, pH, SOC content, and C/N ratio, with mixed amendment showing the most pronounced effects.

2. Soil DNA Extraction and Methanogen *mcrA* Gene Amplification

Total DNA was successfully extracted from all 8 soil samples (4 treatments × 2 seasons). DNA concentrations ranged from 102.4–136.1 ng/ L with A /A ratios of 1.8–2.0, meeting PCR requirements (Table 2). The *mcrA* gene was successfully amplified, producing ~760 bp fragments matching the expected size (Figure 2 [Figure 2: see original paper]).

3. Clone Library Construction and Diversity Analysis

RFLP analysis of *mcrA* clones yielded 18–22 OTUs per treatment. Diversity analysis showed high library coverage (87.50–95.92%) (Table 3). Shannon indices were higher in all amended soils compared to control, with mixed amendment showing the greatest increase (early rice: 2.98 vs 2.31; late rice: 2.79 vs 2.55). Simpson indices were lower in amended soils, indicating reduced species evenness. These results demonstrate that amendments increased methanogen diversity while decreasing evenness.

4. Phylogenetic Analysis

Sequencing of representative clones revealed methanogen sequences with 87–99% similarity to GenBank references. Phylogenetic analysis (Figures 4–5 [FIGURE:4, FIGURE:5]) identified 11 genera across 6 orders:

- *Methanomicrobiales* (dominant): *Methanoregula* (30.47–72.30%), *Methanoculleus* (10.32–33.63%)
- *Methanobacteriales*: *Methanobacterium*, *Methanobrevibacter*
- *Methanosarcinales*: *Methanosarcina* (0.85–25.00%)
- *Methanocellales*: *Methanocella*
- *Methanococcales*: *Methanococcus*
- *Methanomassiliicoccales*: *Methanomassiliicoccus*

Community composition varied by season and treatment (Figures 6–7 [FIGURE:6, FIGURE:7]). *Methanoregula* was dominant in all samples. *Methanoculleus* was unique to late rice soils, likely due to seasonal climate differences affecting hydrogenotrophic methanogen activity.

Discussion

1. Methanogen Groups in Paddy Soil

Methanogens are classified into 6 orders and 10 genera. Our study detected 11 genera across 6 orders, consistent with previous reports. *Methanomicrobiales* was the dominant order (49.57–79.06%), followed by *Methanocellales* (10.32–

33.63%). *Methanobacteriales* are thermophilic methanogens found in high-latitude permafrost and deep-sea hydrothermal vents; we did not detect them, likely due to unsuitable temperature conditions.

At the genus level, *Methanoregula*, *Methanoculleus*, and *Methanosarcina* were dominant, matching previous studies. Variations in community composition across studies likely reflect differences in soil type, climate, and management practices. Seasonal changes also affect community structure, with *Methanocella* and *Methanosarcina* being dominant during flowering and heading stages in some studies, while *Methanomicrobiales* dominated during elongation stages in our study.

2. Effects of Amendments on Methanogen Diversity

Soil pH (6.9–7.2) and salinity directly influence methanogen activity. Biochar and mixed amendments increased pH due to alkaline functional groups neutralizing soil protons. Optimal pH for methanogens is 6.0–7.5; values outside this range inhibit growth. Salinity increases can inhibit methanogen activity and alter community structure by affecting electron acceptor availability.

Amendments increased SOC content and C/N ratio. Biochar carbon is highly stable and recalcitrant, making it less available to microbes. Our previous study found biochar and mixed amendments decreased bacterial abundance but increased fungi:bacteria ratios, suggesting amendments alter microbial activity and stimulate changes in methanogen diversity.

Comparing diversity indices, all amendments increased Shannon diversity, with mixed amendment showing the strongest effect. This may be due to increased substrate complexity and altered competitive interactions among methanogens.

3. Effects of Amendments on Community Composition

Amendments affected community composition differently: - **Slag** increased *Methanococcus* abundance - **Biochar** increased *Methanoregula* but decreased *Methanosarcina* - **Mixed amendment** decreased *Methanomassiliicoccus* abundance

These effects likely operate through multiple mechanisms: 1. **Chemical properties**: Biochar' s stability and slag' s iron oxide content alter substrate availability 2. **pH and salinity**: Increased pH and salinity create selective pressures 3. **Electron acceptors**: Iron oxide in slag may directly inhibit methanogenesis

Methanomassiliicoccus is a methylotrophic methanogen that reduces methanol to methyl-coenzyme M. Iron oxide can directly inhibit this pathway, explaining its reduced abundance in amended soils. This reduction may contribute to decreased CH emissions.

Methanosarcina is an acetoclastic methanogen with high CH production potential. Biochar' s recalcitrant carbon is not easily degraded to acetate, limiting

substrate for *Methanosarcina* and reducing its abundance. This mechanism may be key to biochar' s CH mitigation effect.

4. Comparison of Single vs. Mixed Amendments

Mixed slag+biochar application showed greater CH reduction than single amendments, demonstrating synergistic effects. Mixed amendment more strongly increased soil salinity, SOC, TN, and C/N ratio, and supported higher methanogen diversity. The combination likely integrates the benefits of both materials: biochar' s pH buffering and carbon stabilization, and slag' s iron oxide electron acceptor capacity. The higher application rate (16 t/ha vs. 8 t/ha) may also contribute to enhanced effects, though further research is needed to separate rate effects from material interactions.

Conclusion

1. Mixed slag+biochar amendment significantly increased soil salinity during both rice seasons and increased pH during late rice season. Biochar and mixed amendments increased SOC content and C/N ratio.
2. All amendments increased methanogen diversity while decreasing species evenness, with mixed amendment showing the strongest effects.
3. Methanogen communities were dominated by *Methanomicrobiales*, with *Methanoregula* as the predominant genus. Amendments reduced the relative abundance of *Methanomassiliicoccus* (early rice) and *Methanosarcina* (biochar treatment).
4. Amendments likely reduce CH emissions by altering soil physicochemical properties (especially salinity and pH), changing substrate availability, and shifting methanogen community composition away from high-production genera.

These findings provide mechanistic insights into how waste amendments mitigate CH emissions from paddy soils, supporting their use as climate-smart agricultural practices.

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Note: Figure translations are in progress. See original paper for figures.