Chapter 12

Methanogens Harboring in Rice Rhizosphere Reduce Labile Organic Carbon Compounds to Produce Methane Gas

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Additional information is available at the end of the chapter

http://dx.doi.org/10.5772/intechopen.73299

Abstract

Submerged rice paddy soils are one of the major anthropogenic sources of methane (CH_4) emission to the atmosphere. Methane is the second most important greenhouse gas after carbon dioxide. Methanogens are strictly anaerobic microorganisms and CH_4 is the metabolic end product of those methanogens. Methane is produced by methanogens through multi-step enzyme-mediated process. Methanogens convert labile organic carbon compounds in CH_4 and application of organic matter in submerged rice field significantly increased CH_4 emission from soil to the atmosphere. The rate of methanogenesis may be determined by quantifying biomarkers namely methyl coenzyme M reductase A (mcrA) gene and coenzyme M (2-mercaptoethane sulphonate) in soil. Nickel ions are present as cofactor in enzymes involved in methanogenesis. Methane emission can be mitigated by application of EDTA at suitable rate in the soil of submerged rice field.

Keywords: methane emission, methanogens, biomarkers, EDTA application, rice paddy soil

1. Introduction

In the era of development and globalization, emissions of greenhouse gases (GHGs) are unavoidable consequences, and that increases atmospheric temperature causing global warming. A greenhouse gas is a substrate in the atmosphere that absorbs and emits radiation within the thermal range. This process is the fundamental cause of the greenhouse effect and global warming [1]. Without GHGs, the average temperature of earth's surface would be about −18°C (0°F) [2], rather than present average of 15°C (59°F) [3]. The primary GHGs in the earth's atmosphere are water vapor, carbon dioxide (CO_2), methane (CH_4), nitrous oxide (N_2O) and chlorofluorocarbon (CFC).

Human activities since the beginning of the industrial revolution (Taken as year 1750) have resulted 40% increased in the atmospheric carbon dioxide concentration from 280 ppm in 1970 to 400 ppm in 2015 [4]. Carbon dioxide (CO_2) is the most important GHG in atmosphere in terms of its emitted volume. The other GHGs are CH_4, N_2O, CFC compounds etc. Methane is the second most important GHG emitted to the atmosphere on volume basis and it has 25 times higher global warming potential (GWP) as compared to equivalent amount of CO_2 [5]. The half-life of CH_4 in the atmosphere is about 25 years, which is also much higher than that of CO_2. Due to these characteristics, CH_4 is considered as one of the most notorious GHGs having potential of causing global warming to the atmosphere. The CH_4 concentration in the earth's atmosphere has been increased by 150% since 1750. Methane accounts for 20% of the total radiative forcing from the entire long-lived and globally mixed GHGs, excluding water vapor.

2. Chemistry of submerged rice paddy soil

Agriculture is one of the most important anthropogenic sources of CH_4 emission to the atmosphere and about 11% of the total CH_4 is emitted from submerged rice paddy soils. Rice is the staple food for more than half of world population and about 90% of that rice is cultivated under submerged condition [6]. During rice cultivation, rice seedlings are transplanted after flooding the soil, and water is removed (drained out) few days before crop harvesting. Therefore, in case of transplanted rice, soil remains submerged for at least 85–90% of the total cultivation duration. Such submerged rice paddy soil is the most important anthropogenic source of CH_4 emission to the atmosphere.

Submerged condition for such a prolonged duration makes rice paddy soils different from soils of upland crops. Submerged condition cuts off air transportation between soil and atmosphere. Flooding of rice paddy soil disconnects gas exchange between soil and air. Under this situation, molecular diffusion is the main mechanism to enter oxygen and other gases from atmosphere to the interstitial water. However, this process is 10,000 times slower than the diffusion through gas-filled pores in soil [7]. Thus the oxygen diffusion rate suddenly decreases when a soil reaches saturation by water [8]. Evans and Scott [9] noted that the concentration of oxygen in the water used for saturating a soil decreased to one - hundredth of its initial value in 75 minutes. The major characteristics of submerged soils are:

a. Absence of molecular oxygen

Flooding of land disconnected gas exchange between soil and air. Under submerged condition, oxygen along with other atmospheric gases enters into the soil only by molecular diffusion in the interstitial water. It was observed that gas diffusion under submerged soil condition is 10,000 times slower than diffusion through gas-filled pores [7]. Hence, soil of submerged rice paddy soil losses all its molecular oxygen as soil microorganisms use-up the oxygen present in soil within a few hours.

b. Oxidized mud – water interface

A submerged soil; however, is not completely devoid of oxygen. The top-most layer of few-millimeter thick soil, saturated with water (in mud form) remains oxygenated. The chemical properties of this oxidized interface are completely different from underneath top-soil.

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c. Exchanges between mud and water

The presence of molecular oxygen in the soil-water interface makes it a sink of several redox reactions in soil and controls availability of phosphate and other nutrients in submerged soil. The presence of oxygen in the soil-water interface profoundly affects the N economy of submerged rice paddy soils. Ammonium-N released from broadcasted chemical fertilizer or from applied organic matter is converted to nitrate in the oxygenated interface on soil.

d. Soil reduction

An acute reduced state makes the major difference between chemical reactions of a submerged soil and aerated soil. Excluding the thin oxygenated layer in the soil-water interface, submerged soils have a negative oxidation-reduction potential (Eh value) due to anaerobic condition. Under such condition, dominant form of elements are NH_4^+, H_2S, Mn^{2+}, Fe^{2+} and CH_4 instead of NO_3^-, SO_4^{2-}, Mn^{4+}, Fe^{3+} and CO_2.

2.1. Oxidation – reduction (redox) potential

Under submerged condition, aerobic soil microorganisms consume oxygen during their metabolism and that in turn gradually depletes oxygen pool making the soil anaerobic in reaction [10]. The redox potential (E_h) value in submerged soil starts decreasing after 3–4 days of flooding and sharply decreases with time. The Eh values in submerged anaerobic soils vary around −200 eV values throughout the rice cultivation duration [11]. Such anaerobic reducing environment is one of the prime factors for determining the rate and quantity of CH_4 production in rice paddy soil.

3. Methanogens and CH_4 production

The average global CH_4 emission from rice fields is approximately 20–40 Tg CH_4 $year^{-1}$, which accounts for 11% of the total anthropogenic CH_4 emissions [12]. It had already been reported that rice production will be increased from 473 million tons of 1990 to approximately 781 million tons by 2020 to fulfill the food demand of the world population and that proportionately increase CH_4 emission from rice paddy soils by 40–50% [13].

Methane is mainly produced during decomposition of organic matter by strictly anaerobic methanogens under intense reduced condition [14]. At the initial state of rice cultivation, the rate of CH_4 emission is generally low; however, the flux gradually increases with plant development and with enhanced anaerobic condition [15, 16]. Anaerobic conditions of submerged rice paddy soil favors CH_4 production and the highest CH_4 emission is generally observed after the soil E_h value dropped below −200 eV [17].

Both cold- and hot-water extractable organic carbon (C) compounds are labile fraction of soil organic C. Low molecular weight organic compounds namely low molecular weight organic acids, carbohydrates are considered as labile organic C compounds in soil [18]. Labile organic C compounds rather than total organic C pool acts as the energy source for heterotrophic microorganisms like methanogens in soil [19]. Methane is the metabolic end product

of methanogens [20] and methanogens reduce simple carbonaceous compounds namely CO_2, carbohydrates and/ or simple carboxylic acids like formate, acetate through multi-step enzyme-mediated methanogenesis to generate ATP and to produce CH_4 as the end product.

Methanogens are generally specific to their substrate requirement. Based on the ability to utilize carbonaceous compounds as energy source, methanogens may be classified as acetophilic methanogens and hydrophilic methanogens [21]. The acetophilic methanogens transform acetate ions into CH_4, while the hydrophilic methanogens utilize hydrogen and CO_2 as their energy source.

4. Factors affecting CH_4 production in soil

Methanogenesis or the process of CH_4 production is an enzyme-mediated multi-step biochemical process and kinetics of this process depends on several factors. The most important and prominent factor of CH_4 production is the availability of initial carbonaceous compounds in soil. Addition of organic materials in flooded rice field promotes CH_4 emission (**Figure 1**) by providing readily available C source to methanogens [22, 23]. Improvement in crop production by organic amendment conflicts with mitigation strategies of CH_4 emission [24]. Hence, it may be believed that methanogens degrade applied organic matter to produce CH_4 under strictly anaerobic conditions [14]. However, this is the exaggeration of the truth.

Application of organic matter increases total population and activity of microorganisms including methanogens in rice paddy soil [25]. Therefore application of organic substrates significantly increases CH_4 emission from rice field [26]. However, methanogens does not have potential to degrade carbonaceous polymers like cellulose due their inability to produce cellulase enzyme. In fact, there is a synergistic effect of cellulolytic microorganisms on methanogens and CH_4 formation in submerged rice paddy soil. Cellulose, a 1,4-β-linked glucan, contributes 20–30% of the organic biomass [27] and application of organic matter provides a significant C source in the form of cellulose to soil microbial community in soil.

The hydrolysis of carbonaceous polymers (mainly cellulose) is an important pathway to convert added organic C into CH_4 and anaerobic cellulolytic microorganisms play a significant role in that process [28]. Incubation of rice paddy soil with different amounts of carboxymethyl cellulose (CMC) under anaerobic condition in a close-vessel produced variable amount of CH_4 after 3 days (**Figure 2**). The amount of generated CH_4 within that period was proportional to the quantity of added CMC in soil. Therefore, cellulolytic materials of applied organic substrates were initially degraded by cellulolytic microorganisms into low molecular weight organic acids and/ or carbohydrates, which are then utilized by methanogens to produce CH_4 under anaerobic condition of submerged rice paddy soils.

In submerged rice paddy soil, the flux of CH_4 emission depends on the amount as well as nature of applied organic matter [29]. The rate of CH_4 emission is dependent on the nature i.e. degree of stabilization of applied organic matter. During decomposition, carbonaceous compounds like cellulose and hemicelluloses are readily stabilized through mineralization and converted into humified substrates. Therefore, composts contain lesser amount of easily

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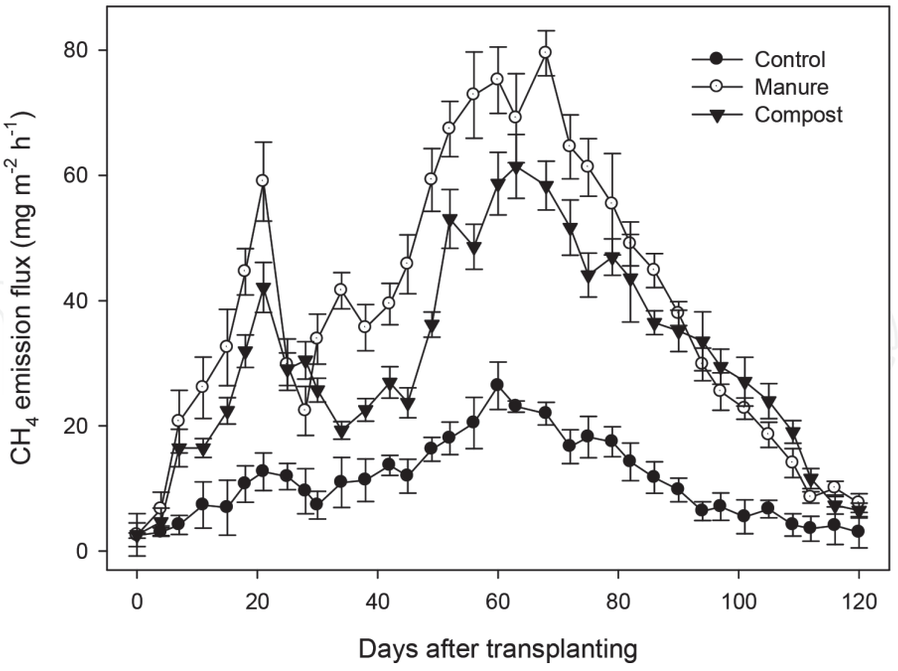

Figure 1. Changes in methane emission flux from rice paddy soils as affected by air-dried and composted dairy cow manures.

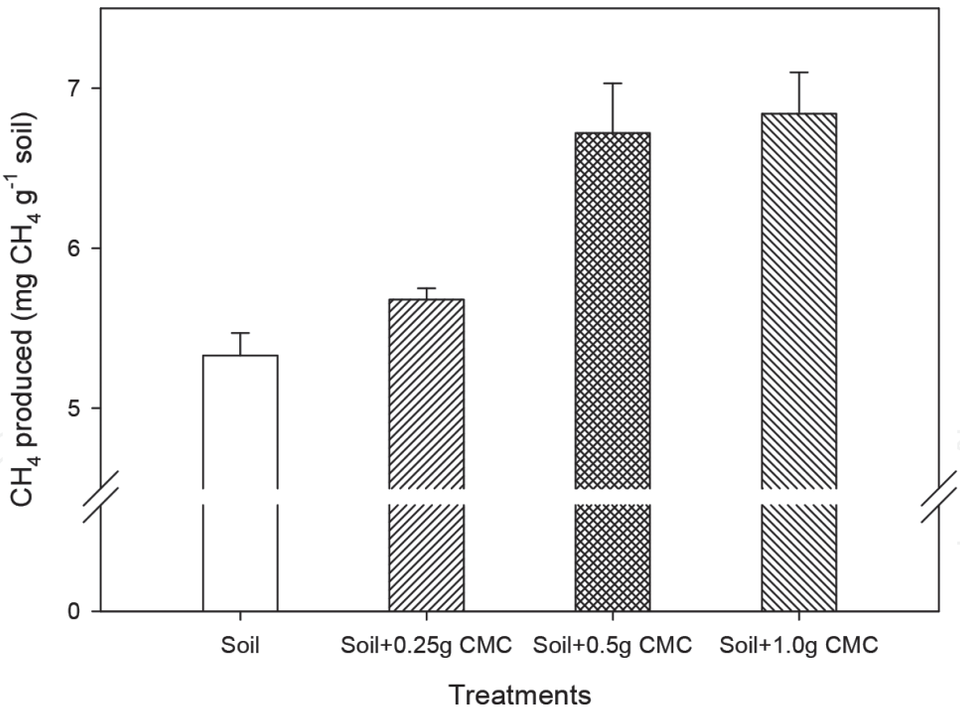

Figure 2. Changes in CH_4 production as affected by CMC application.

decomposable compounds like cellulose and hemicelluloses compounds as compared to their initial substrates. Lower cellulose contains leads to fewer cellulolytic microbial populations in soil and that generates lower amount of labile organic C compounds in soil. Therefore, reduced availability of precursor carbonaceous compounds is responsible for lower CH_4

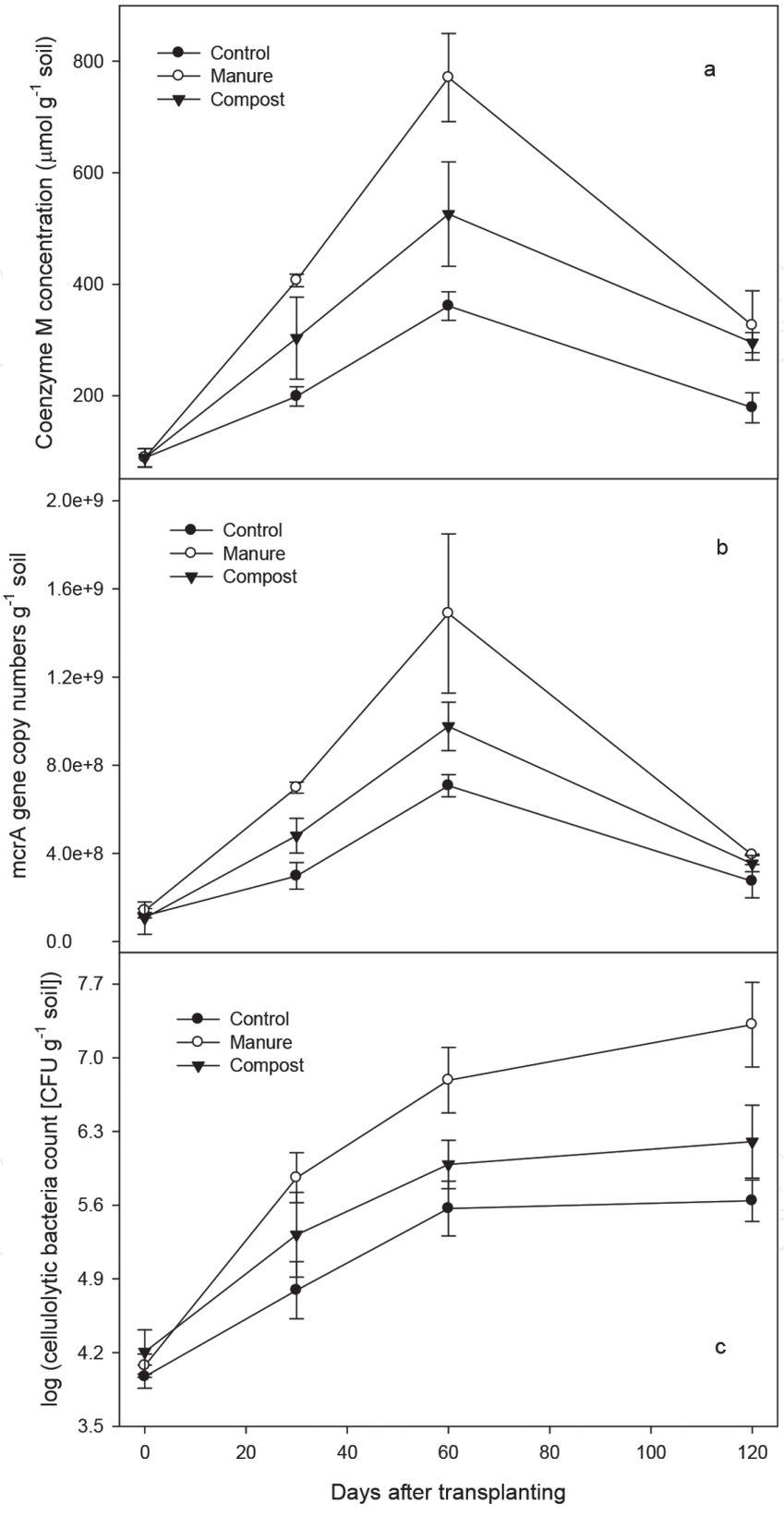

Figure 3. Coenzyme M concentrations (a), mcrA gene copy numbers, (b) and cellulolytic bacterial populations, (c) as affected by manure and compost applications in rice paddy soils.

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emission from compost treated rice paddy soil. Therefore, degree of stabilization or humification of applied organic substrates is inversely related to the flux of CH_4 emission, which may be reduced by ~20% by application of compost instead of air-dried cattle manure (**Figure 3**).

5. Biomarkers of methanogens: quantification

In many anoxic environments, methanogenesis is the predominant terminal electron accepting process involved in organic matter mineralization and that process is catalyzed by methanogenic *Archaea*. This group of microorganisms represents a unique but phylogenetically diverse group of prokaryotes [30]. The most widely used method for measuring the rate of methanogenesis is the quantification of CH_4 flux in a specific system within unit time interval [17]. Though this analysis is closely related to the metabolic functions of methanogenic community; it does not directly quantify methanogens [31]. One of the most convincing processes of quantifying methanogens is the direct determination of methanogens numbers on specific culture. However, isolating methanogenic *Archaea* remains a fastidious process because of the slow growth of these *Archaea* and also for their extreme intolerance to oxygen [32]. Growth of methanogens is also restricted to the availability of specific organic substrates and metal ions to complete their metabolic process [33]. Culturable microorganisms isolated by specific enriched medium can only detect a small portion (2–5%) of the total microbial community in soil.

Still researchers often prepared one specific culture medium for each one of the various methanogenic *Archaea* species to fulfill their specific requirements [34]. However, there are reports of versatile media like SAB medium, which is capable of supporting growth of wide spectrum of methanogens [33]. Hence, due to these limitations and difficulties, methanogens are preferably quantified by measuring their biomarkers.

Biomarkers are compounds that have a biological specificity in the sense that they are produced only by a limited group of organisms [35]. A variety of compounds such as fatty acids and ether lipids are used in microbial ecology and related fields like organic geochemistry to detect groups of organisms or their remains in natural or artificial ecosystems [36, 37].

5.1. Methyl coenzyme M reductase A (mcrA) gene

The mcrA gene is responsible for synthesizing methyl coenzyme M reductase enzyme, which is involved in the production of CH_4 during the final stage of methanogenesis. Culture-dependent and culture-independent techniques targeting 16S rRNA and methyl coenzyme M reductase (*mcrA*) genes have been used to assess the phylogenetic diversity of methanogens assemblages [38].

5.2. DNA extraction and quantification

The DNA may be extracted from natural and/or enriched samples using any suitable kit following manufacturers' protocol. The quality of the extracted DNA is observed in an agarose gel.

The extracted DNA is amplified by PCR in a final volume of 25 μl containing 2 μl of undiluted template DNA, 1 μl each of forward and reverse primers (10 mM) and 12.5 μl of Taq polymerase enzyme [39]. For detecting the presence of methanogens, a forward primer with 32-mer and a reverse primer with 23-mer were developed after testing against 23 species of methanogen representing all five recognized orders of this group of *Archaea* [40]. The two oligonucleotide primers were a forward primer, 5′-GGTGGTGTMGGATTCACACARTAYGCWACAGC-3′ and a reverse primers, 5′-TTCATTGCRTAGTTWGGRTAGTT-3′. The methanogen diversity in a sample may be studied by analyzing amplified DNA (or PCR product of extracted DNA) through denatured gradient gel electrophoresis (DGGE) [41].

Total population of methanogens can be determined from extracted DNA by quantitative PCR (qPCR) or real-time PCR (RT-PCR) using PCR efficiency, 110.5%; slope of the standard curve, −3.093; y-intercept, 5.134 and correlation coefficient, 0.9949 [31]. The C_t for the no template control was 24.03 and >26.5 for all the no-reverse transcriptase control. The qPCR results (mcrA gene copy numbers ng^{-1} DNA) of extracted DNA show significant correlation with specific methanogenic activities against H_2 and CO_2 gases. Steinberg and Regan [42] developed the TaqMan qPCR probe assay for successfully determining the environmental abundance of different phylogenetic groups of methanogens, including several groups with few or no cultivated members.

5.3. 2-mercaptoethane sulphonate (coenzyme M)

Methane production in soil is a complex enzyme-mediated multi-step process and methanogens reduce simple carbonaceous compounds like CO_2 and H_2, formate, methanol, methykamines and/ or acetate into CH_4 gas [43]. In the penultimate step of methanogenesis, coenzyme M (Co-M), 2-mercaptoethane sulphonate, is methylated and generated methyl Co-M is reduced by methanogens to CH_4 gas involving previously-mentioned methyl Co-M reductase enzyme [44]. Therefore, irrespective of the preference towards initial carbonaceous compounds, Co-M could be considered as the precursor of CH_4 formation [45]. The whole methanogenesis is intracellular and Co-M is synthesized inside methanogen cells [46]. The conversion factor (0.39 ± 0.07 fmol $cell^{-1}$) of Co-M to methanogens could be used for quantitative estimation of methanogen abundance and methanogenic activity in soil.

5.4. Coenzyme M quantification

Pramanik and Kim [47] developed a HPLC-based technique for quantifying Co-M in soil. Pure Co-M is detected at 270 nm wavelength using UV detector and a mixture of acetonitrile and 0.05 M trichloroacetic acid (TCA) solution at flow rate of 0.5 ml min^{-1} is used as mobile phase during this analysis.

Coenzyme M is an intracellular compound of methanogens; hence, rupturing cells of methanogens is mandatory prior to extraction. The lysis buffer was prepared by mixing Tris-HCl solution (pH 8.0), ethylenediaminetetraacetic acid (EDTA) solution (pH 8.0) and NaCl solution. The Co-M was extracted from fresh soil using lysis buffer through consecutive

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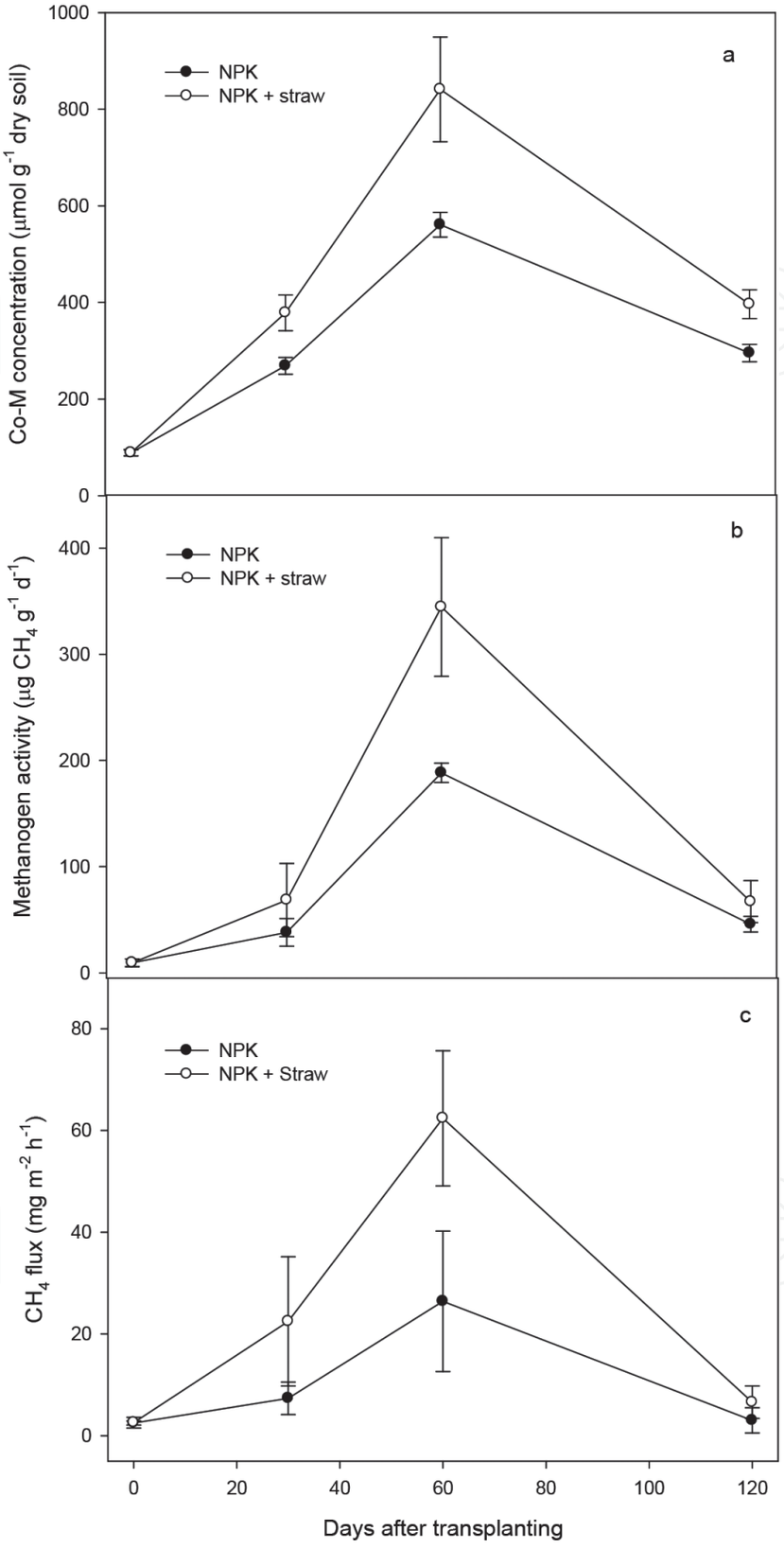

Figure 4. Changes in coenzyme M concentration (a) and methanogen activity (b) and methane flux (c) from soil during rice cultivation (bars represent standard errors).

homogenization by vortex shaker and sonication [47]. The Co-M was precipitated from ethanolic solution of the supernatant and re-dissolved in distilled water prior to the HPLC analysis. The precision of this method to quantify Co-M in the soil matrix was >97%. The high recovery rate (90.3 ± 8.1%) indicated that Co-M is not adsorbed to the ionic sites of soil colloids and the measured values are very close to the actual Co-M content in soil (**Figure 4**).

Methanogen activity in soil is linearly correlated to the Co-M content (r = 0.857*) of soil and the mean conversion factor between these two parameters is 155.03 ± 14.20 μg CH_4 produced $mmol^{-1}$ Co-M d^{-1} [47]. Therefore, it could be stated that both *mcrA* gene copy numbers and the concentration of Co-M could be quantified as biomarkers of methanogens for precise estimation of methanogenic activity in submerged rice paddy soils.

6. Mitigation techniques of CH_4 emission

Rice cultivation under flooded condition is regarded as an important source of CH_4 emission in soil. Methane is produced during decomposition of organic matter under anaerobic condition and simple carbonaceous compounds are biochemically reduced by methanogenic *Archaea* to form CH_4 [48]. During this reduction process, an electron donor is required to transfer the electron and availability of such electron donors might control the flux of CH_4 emission in rice paddy soil. Iron (Fe) is a transition metal with partially filled d-orbital in its configuration. Lee et al. [48] observed that Fe in active form (ionic form) may accept electrons required for reductive methanogenesis process and that in turn decreased CH_4 emission flux from rice paddy soil. Methane emission from submerged soil may be reduced approximately 14.5% by application of byproduct of steel industry as silicate fertilizers. Those byproducts of steel industry provide adequate silicate ions, which are necessary for higher rice productivity [49] and also for inducing resistance to biotic and abiotic stress [50]. However, Fe present in silicate fertilizers absorbs part of free electrons generated in the system and that restricts the terminal electron transfer during methanogenesis. This property enabled to reduce CH_4 emission from conventional (chemical fertilizer treated) rice paddy soils. However, Fe-enriched silicate fertilizer is not a strong mitigating agent; in fact a contrasting effect of silicate fertilizers on CH_4 emission was observed in organic matter treated rice paddy soil.

6.1. Effect of EDTA on CH_4 emission

In the last step of methanogenesis, methyl coenzyme M reductase (MCR) enzyme is involved in the reduction of methyl Co-M to CH_4 and nickel (Ni) ion is involved as the cofactor F_{430} in MCR enzyme [44]. Hence, availability of Ni determined the concentration of cofactor F_{430}, which in turn controls the activity of MCR enzyme [51]. Therefore, the rate of CH_4 production is enhanced by addition of Ni-salts to rice paddy soil [52]. Transition metals like Ni form soluble complexes with EDTA and that increases the solubility of Ni in soil. However, Pramanik and Kim [53] revealed that methanogens could not assimilate Ni^{2+} ions from Ni-EDTA complexes and suffered starvation for Ni^{2+} ions when stoichiometric amount of Ni salt and EDTA

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were mixed in culture broth. Finazzo et al. [54] stated that Ni is present as Ni(I) in F_{430} and conversion of Ni(I) to Ni(II) provides necessary electron for reduction of methyl coenzyme to CH_4. The presence of Ni as Ni-EDTA complex might retard this electron transfer during methanogenesis. Application of Ni as Ni-EDTA complex limited bioaccumulation of Ni by methanogens and that lower Ni content in methanogen biomass significantly ($P \leq 0.05$) reduced the rate of CH_4 production [53].

Ethylenediaminetetraacetic acid (EDTA) is a strong chelating agent and often shows adverse effect on plants when applied in higher doses. Pramanik and Kim [55] established that EDTA application at smaller doses (up to 5.0 ppm) proportionately reduces the flux of CH_4 emission from rice paddy soils without suppressing crop productivity (**Figure 5**). However, EDTA application at higher doses may increase CH_4 emission and also adversely affected maturity and productivity of rice. Therefore, application of 5.0 ppm EDTA is possibly the most rational dose to mitigate CH_4 flux from rice paddy soil. The activity of methanogens in EDTA-treated soil was significantly lower than that of control treatment. However, activity of all the microorganisms (microbial respiration) in soil was initially decreased due to EDTA application during rice cultivation. Application of EDTA enhances availability of nutrients especially nitrogen content in soil solution and that in turn gradually boosts microbial activity in soil. After 30 days of rice cultivation, microbial respiration of EDTA-treated soils did not differ significantly from that of control soil. Application of EDTA leads up to 18.1% reduction in CH_4 emission flux during submerged rice cultivation.

Unlike Fe-enriched silicate fertilizers, EDTA is also effective to mitigate CH_4 emission from organic amended submerged soils. Application of EDTA did not suppress the rate of organic

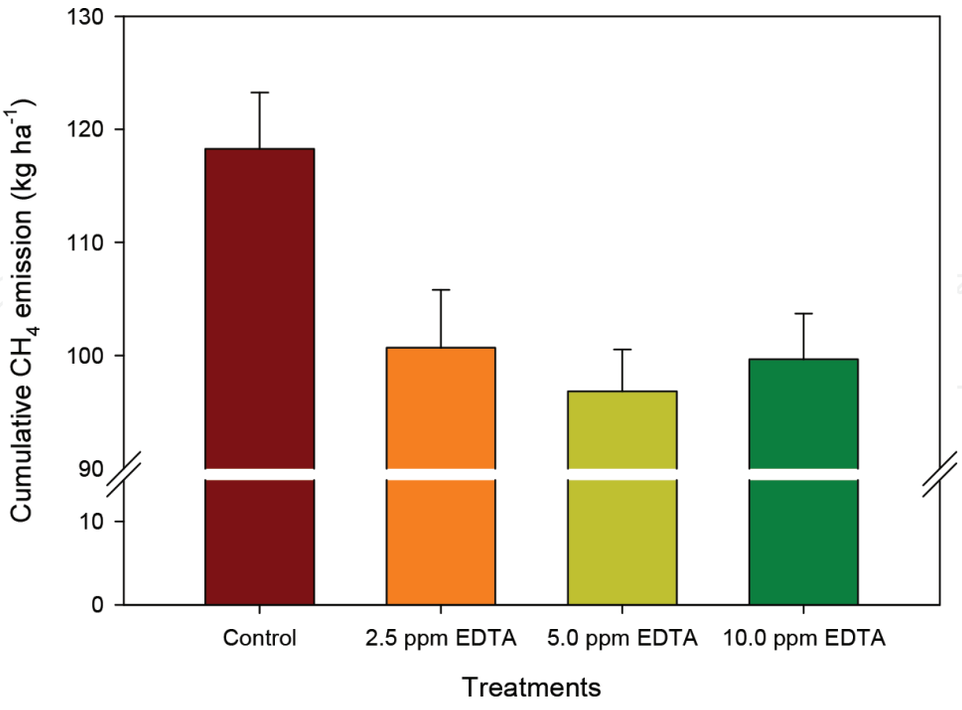

Figure 5. Cumulative CH_4 emission from different rice paddy soils.

matter mineralization; hence, the concentration of labile organic C compounds was not decreased due to EDTA application in soil. In spite of higher abundance of precursor materials (labile organic C compounds) in soil, EDTA application leads up to 22.5% reduction in CH_4 emission from straw incorporated rice paddy soils [56].

One noticeable drawback of EDTA application is that EDTA-treated soils have higher nitrate N content, which acts as the precursor for nitrous oxide formation under anaerobic reducing of submerged soil condition. Higher nitrate N content enhanced the flux of nitrous oxide, another greenhouse gas having 290 times higher global warming potential than equivalent amount of carbon dioxide. Study revealed that total global warming potential in 5.0 ppm EDTA-treated soil was 14.5% lower than that of control soil (not treated with EDTA) during rice cultivation.

Organic amendment increased C-to-N ratio, which in turn decreased the rate of mineralization and nitrate N content in soil. Therefore, the adverse effect of EDTA application could be minimized by organic amendment in soil. However, incorporation of organic matter in submerged rice paddy soil facilitated the risk of CH_4 emission to the atmosphere. Optimum combination of EDTA and compost may effectively reduce the net global warming potential due to CH_4 and N_2O emissions. It was observed that 5.0 Mg organic substrates ha^{-1} and 5.0 ppm EDTA combination had global warming potential 11186.17 ± 749.35 kg CO_2-equiv. ha^{-1}, which was 20.8% lower than that of only organic amended rice paddy soil (**Figure 6**).

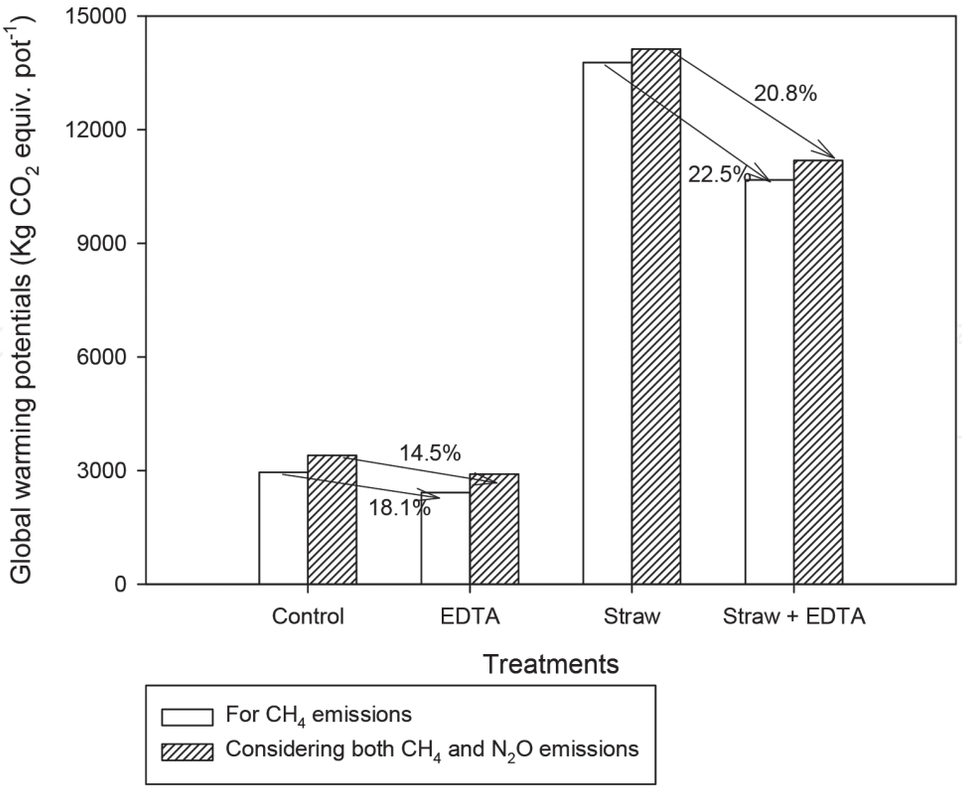

Figure 6. Global warming potentials due to methane (CH_4) and nitrous oxide (N_2O) emissions from submerged rice paddy soils.

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7. Conclusion

Soil of submerged rice fields is the most important anthropogenic source of CH_4 emission to the atmosphere. Methanogens, a group of strictly anaerobic microorganisms, convert labile organic C compounds into CH_4 gas and that emits from soil to the atmosphere. Activity of methanogens may be quantitatively studied by measuring mcrA gene and/ or coenzymeM biomarkers in soil.

Acknowledgements

This work was supported by Basis Science Research Program through the National Research Foundation of Korea (NRF) funded by the Ministry of Education (NRF-2015R1A6A1A03031413).

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