Methane Mitigation from Paddy Field Ecosystem: Role of Aquatic Earthworms and Farming Practices

水田生態系からのメタン放出緩和：水生ミミズの機能と農法の関係

A THESIS

BY

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A THESIS

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DEDICATED

TO

MY FAMILY

Methane Mitigation from Paddy Field Ecosystem: Role of Aquatic Earthworms and Farming Practices

水田生態系からのメタン放出緩和：水生ミミズの機能と農法の関係

Paddy field is a major source of methane (CH_4) emission where methanogens and methanotrophs both are involved in biogeochemical CH_4 cycle. Farming practices such as fertilization and tillage are very important for proper growth of rice plants having impacts on CH_4 emission from paddy. Feeding activities of aquatic earthworms in paddy soil add oxygen in lower soil layers by introducing oxygen rich water to those layers, and farming practice may influence densities of aquatic earthworms in paddy soil. Thus, it was hypothesized that aquatic earthworm's activities may accelerate oxidation of methane in paddy field and farming methods influence methane emission via earthworm densities. Therefore, this study was conducted to identify effects of integrated farming practices to reduce the use of agro-chemicals to conserve soil biodiversity and to reduce the burden of CH_4 emission by integrated farming practices and aquatic earthworms.

At first, a 2x2 factorial field experiment was conducted where two levels of tillage (tillage and no-tillage) and two different fertilizers (chemical fertilizer and organic fertilizer), was applied with four replications to determine the role of aquatic earthworms on methane emission from paddy field in 2010-2011 at Kamakura, Japan. The highest earthworm density was found in no tillage-organic fertilizer plot and organic fertilizer was found to have marginal significant effect on earthworm density in paddy field. For measuring methane flux, gas samples were collected by closed chamber method once in a month. Highest methane flux was during the month of July. The

negative effect of earthworms on methane flux was found in July, and the flux was half of no-worm condition when the worm density was more than 11,000/m^2. Thus, proper farming practices could influence favorable environment for aquatic earthworm's activity that might contribute in methane mitigation in paddy field ecosystem.

To identify effect of different farming practices on earthworm and CH_4 emission from different paddy field ecosystems and to investigate the effect of aquatic earthworms on methanotrophs, another field experiment was done in Nara, Japan on May-September, 2012. Soil and gas samples were collected from three different managements: i) conventional paddy turned from organic, ii) no-tillage paddy with weed mulch and iii) conventional paddy. Earthworm density was lower in no-tillage paddy field compared to conventional paddy fields due to absence of standing irrigation water in no-tillage plot and at the same time, no-tillage farming practice significantly lowered CH_4 emission from paddy field by enhancing biomass of methanotrophs. But in this study, relation between aquatic earthworms and CH_4 emission was poorly understood.

After this, a microcosm-experiment was conducted in two steps to identify effects of fertilizer management on activities of aquatic earthworms and CH_4 flux from paddy soil and to explore the effects of earthworms on the activity of methanotrophs. In the first experiment, two chemical fertilizers: urea and ammonium sulfate and control soil without fertilizer were incubated for 4 weeks. Urea was found to increase CH_4 flux significantly. In contrast, ammonium sulfate depressed CH_4 flux significantly from the soil. In the second experiment, two levels of aquatic earthworms, *Branchiura sowerbyi* (earthworm and no-earthworm) and two levels of urea (urea and no-urea) were prepared. Application of urea increased the earthworm density in soil. The earthworms were found to have positive effect in reducing CH_4 emission from soil. Results of phospholipid fatty acid analysis showed that aquatic earthworms contribute to increase biomass of methanotrophs in soil. Although the application of urea causes higher CH_4 emission over

ammonium sulfate application, the aquatic earthworms can decrease CH_4 emission from paddy soil fertilized by urea. But in this study, effect of ammonium sulfate on earthworm density was not discussed.

To know the role of aquatic earthworms on CH_4 flux from paddy soil under $(NH_4)_2SO_4$ treatment, another macrocosm experiment was set. Gas samples were collected once in a month starting from June, 2016 to August, 2016. Immediately after application of fertilizer, N_2O flux was increased. Methane flux was lower in $(NH_4)_2SO_4$ treated soil and with increase in earthworm density CH_4 flux was decreased. It was found that with increase of biomass of methanotrophs, CH_4 flux was decreased and with higher earthworm density, biomass of methanotrophs increased.

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Chapter: I

Introduction

Global warming is one of the most important environmental problems, which may affect all living things on earth. Greenhouse gases (GHGs) resulting from human activities are the most significant driver of climate change since mid of the last century. GHGs include carbon dioxide (CO_2), methane (CH_4), nitrous oxide (N_2O), fluorinated gases which contain fluorine including hydrofluorocarbons, perfluorocarbons and sulfur hexafluoride. The atmospheric concentration of CO_2 was 393.1 parts per million (ppm), CH_4 was 1819 parts per billion (ppb) and N_2O was 325.1 parts per billion (ppb) in 2012 (World Meteorological Organization, 2012). The lifetime of CH_4 in atmosphere is about 12 years whereas lifetime of N_2O in atmosphere is about 121 years and of fluorinated gases is a few weeks to thousands of years (IPCC, 2013). But the lifetime of CO_2 is poorly described, as this gas is not destroyed by time. Rather it moves among different parts of the ocean–atmosphere–land system. Some of the excess CO_2 is absorbed quickly and some remain in the atmosphere for thousands of years. Though atmospheric concentration of CO_2 is higher than CH_4 and N_2O but one molecule of CH_4 traps 28 times higher heat as does CO_2 and N_2O can trap 265 times heat compared to CO_2 (IPCC, 2013). Therefore, CH_4 and N_2O contribute to an increase of 0.6-0.7^{o}C in global surface temperature (Trenberth et al., 2007).

Methane is released in atmosphere from several sources, both natural and anthropogenic. Anthropogenic sources include enteric fermentation, animal and human wastes, termite, rice paddies, biomass burning and landfills. Anthropogenic and natural sources contribute 375 Tg CH_4/ year and 160 Tg CH_4/year respectively. Rice cultivation alone emits 11% of total anthropogenic methane (Smith *et al*., 2007).

Rice, a semi-aquatic species which is mostly grown under flooded lowland paddy fields (Kögel-Knabner et al., 2010) is one of the world's most important cereals. Periodic short-term flooding cycle over long period of time induces special soil characteristics which include anaerobic status, different bacterial communities, soil oxidation-reduction potential etc. (Yao *et al*., 1999, Lüdemann *et al*., 2000, Kögel-Knabner *et al*., 2010). Paddy field management develops pedogenic horizons and those are specific to paddy soils (Fig. 1). The uppermost horizon is developed of a thin layer of standing water which habitat of bacteria and lower layer of this horizon is an oxic or partly oxic zone. The thickness of this zone ranges from several mm during flooding to several cm after rice plants are fully grown, roots start to release O_2 (Frenzel *et al*., 1992). In anthraquic horizon, free O_2 is absent.

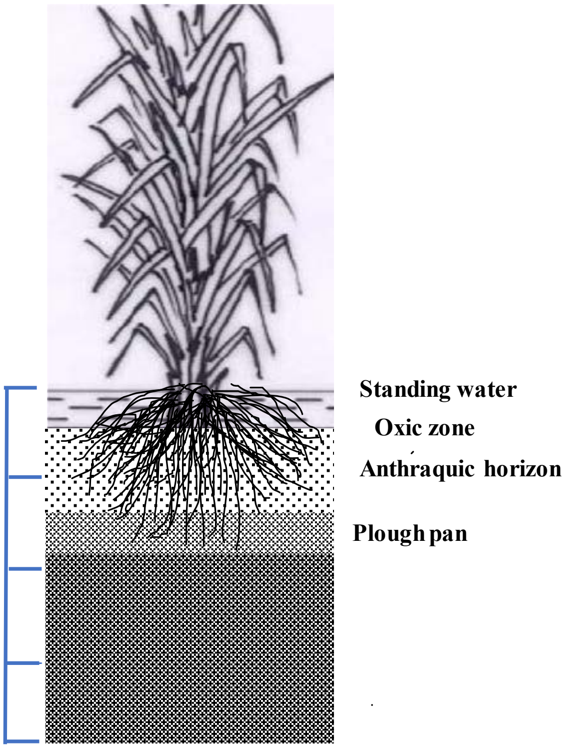


Figure 1: Typical horizon sequence of rice paddy soil. (Kögel-Knabner et al., 2010)

In Asia, about 136×10^6 ha area which is 88% of world's total was harvested to produce 556 Tg (Mt) rough rice, 90% of world's total production in 2004 (FAO, 2005). In Asia, rice lands can be divided into deepwater (3%), upland (7%), rain-fed lowland (33%) and irrigated (57%) (FAO, 2005). In deep water rice fields, flood water may raise more than 20 cm during the growing season. In rain-fed rice fields, precipitation controls flooding, during growing season rain-fed rice field may dry up or have flood water up to 50 cm. In irrigated rice fields, the floodwater can be controlled. Upland rice fields are not flooded and the topsoil does not become saturated at any period of growing season. Rice is generally cultivated in flooded paddy fields as the reductive conditions may flow out harmful substances to rice growth except the case in upland fields.

Generally, 10-20 cm deep water exists in irrigated paddy field about all over the cropping period except the drying period. For subsequent rice production, paddy fields are flooded which cuts off O_2 supply from atmosphere and microbial activities turns from aerobic to facultative and to anaerobic fermentation of organic matter (Fig. 2). The sequence includes aerobic respiration, nitrification, denitrification, then Mn^{4+} reduction, Fe^{3+} reduction, SO_4^{2-} reduction and at last methanogenesis which is the most important terminal process in C mineralization (Yao *et al*., 1999). Methane is produced as an end product after anaerobic fermentation of organic matter in paddy field by methanogenic archea. The major methanogenic substrates are H_2+CO_2, formate, acetate, methyl alcohol and methyl amine. The dominant reactions of methanogenesis in soil are: the reduction of CO_2 using H_2 and the transmethylation of acetate (Takai *et al*., 1970). During methanogenesis process, carbon substrates are supplied to soil from soil organic matter, sloughed tissues of rice plants, applied organic matter and root exudates (Kimura *et al*., 2004; Watanabe *et al*., 1999). Methanotrophs consume part of the produced CH_4 under oxidative condition around rhizosphere of rice plants and also in a thin layer where soil interferes with surface water. The rest of the produced CH_4 can be released to atmosphere from paddy field by various ways such as diffusion, ebullition from soil and by diffusion and mass flow through a continuous intercellular gas space system between rhizosphere and leaf of rice plant. Inubushi *et al*., (1989) reported that 90% of total CH_4 from paddy field is released through rice plant and ebullition and diffusion from soil contributes 10% and <1%, respectively. It indicates that a high amount of CH_4 is emitted from rice plant. Global estimates of methane emission rate from paddy fields range from 20 to 100 Tg/year (IPCC, 1992). The physiological structure of rice plant favors greater emission of CH_4. Intercellular channel that made of aerenchyma between leaf parts and rhizosphere acts as path of gas exchange between root zone and atmosphere through stomata in leaf and micropores in leaf sheath. In submerged paddy field CH_4 diffuse in root and transported to above part through intercellular channel and released to atmosphere through stomata and micropore (Nouchi *et al*.,1990).

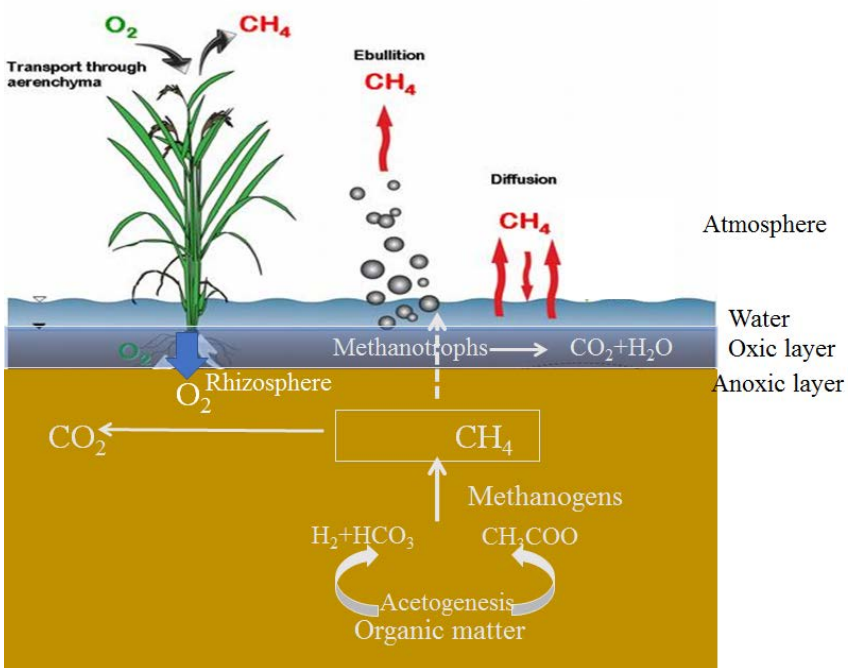


Figure 2: Mechanisms of CH_4 emissions from irrigated rice field (Dubey, 2005)

In irrigated rice fields, during drying period, CH_4 emission is decreased due to more oxidation and low production and at the same time, N_2O emission may be stimulated due to lesser reduction to N_2. Nitrogenous fertilizer is the source of N_2O in paddy field environment. After extended flooding, paddy fields develop a unique soil profile (Xing *et al*., 2009). During rice growing season, oxidized and reduced layers are developed in cultivated layer. After application, ammonium N containing fertilizer is got nitrified at the oxidized layer of paddy field i.e., NO_3^- is formed at water-soil interface and moves to lower reduced layer and got denitrified there and thus N_2O is produced. Not only in upper flooded layer, denitrification takes place in underground saturated soil layer also (Xing *et al*., 2002).

Rice cultivation is associated with application of agrochemicals such as chemical fertilizers and less input of organic fertilizers. Input of chemical fertilizers is being increased day by day. Both chemical and organic fertilizers have strong influences on CH_4 emission from paddy field soil (Yagi and Minami, 1990). Organic fertilizer is well known as it conserves soil fertility and in Japan, organic fertilizer has been recommended together with chemical fertilizer as it releases organic nitrogen very slowly during decomposition (Minamikawa *et al*., 2006). Rice residues such as rice straw are very popular organic fertilizer. Many researchers have found that these rice residues strongly stimulate CH_4 emission (Yagi and Minami, 1990). Rice straw is a good source of C and CH_4 generating bacteria, which are activated with additional available C source (Wang *et al*., 1992).

Chemical fertilizer such as nitrogenous fertilizer is fundamental to crops affecting growth and yield. Sulfate containing fertilizers are known to suppress CH_4 emission as there is a competition of sulfate-reducing bacteria with methanogenic archaea for substrates, hydrogen and acetate (Hori *et al*., 1990). In many studies, it has been demonstrated that ammonium sulfate decrease CH_4 emission (Banik *et al*., 1996) but was found to enhance N_2O emission because of nitrogen addition in NH_4^+ form (Kumar *et al*., 2000) which is accounted for only 2.5% of total amount of consumed nitrogenous fertilizers globally in 2002 whereas urea is the most consumed nitrogenous fertilizer in the world which is accounted for 47.4% in 2002 (FAO, 2005). However, the effects of urea on CH_4 emission are context dependent. Some studies revealed that CH_4 emission has been increased by increased rate of application of urea where increase in soil pH caused by hydrolysis of urea (Wang *et al*., 1992) and methanotrophic bacteria were inhibited by ammonium (Dubey *et al*., 2003). On the other hand, some studies showed that ammonium enhanced oxidation of CH_4 by methanotrophic bacteria (Bodelier *et al*., 2000). Slow releasing fertilizer such as urea super granule (USG) is known to mitigate N_2O emission by inhibiting NH_4^+ oxidation (Majumdar *et al*., 2000).

Water regime of soil is very important for gas exchange between soil and atmosphere and has a direct impact on the processes involved in CH_4 and N_2O emission. Water management involving midseason drainage and intermittent irrigation are very well known in irrigated paddy fields in Japan. It is done to control excess tillering and to supply rice roots with molecular O_2 for preventing sulfide toxicity (Kanno *et al*., 1997). Water management is one of the most effective options to decrease CH_4 emission as it inhibits the development of soil reductive conditions. A single mid-season drainage can reduce 50% seasonal CH_4 emission rate and percolating water also can carry organic solutes and dissolved gases into the subsoil and there leached CH_4 can be oxidized before releasing to atmosphere (Kimura *et al*., 1992). Wassmann *et al*., (2000) reported that field drying during mid tillering stage decreased CH_4 emission around 15-80% compared to continuous flooding and there was no significant effect on grain yield. On the other hand, drainage is responsible for emission of N_2O (Chen *et al*., 1997) via parallel nitrification and denitrification (Suratno *et al*., 1998). NH_4^+ of soil may be accumulated due to slow nitrification of applied or soil NH_4^+ and also from mineralization of soil organic matter (Majumdar, 2003). In this process, nitrification can produce significant amount of N_2O during drying cycle. Denitrification process can emit N_2O from NO_3^- during drainage (Arah *et al*., 1989). On the other hand, if flooding is continued to suppress N_2O emission, CH_4 emission will be increased considerably (Hou *et al*., 2000).

Different field management practices also have direct and indirect impacts on CH_4 emission in paddy field. Recently, no-tillage farming practice is being increased in some East Asian countries as no-tillage farming practice improves soil environment for crop production and preserves soil structure and enhances percolation in paddy soils (Hossain *et al*., 2000; Ota *et al*., 2002). Globally, organic carbon storage of soil is approximately 1500 Pg (Song *et al*., 2016). After awareness about greenhouse gas effect was increased, soil organic carbon has been reported to be changed easily by field management practices such as fertilization and tillage (Benbi and Senapati, 2010). Therefore, implementing various tillage practices is no longer restricted to increase yield but it has been extended to enhance soil carbon preservation and preventing greenhouse gas effects (Bajracharya *et al*., 1997; Koga and Tsuji, 2009). Long-term no-tillage farming practice can lead to excessive compactness of soil surface (Hama and Anderson, 2005) along with weed spread (Turley *et al*., 2003). No-tillage farming practice in rice cultivation reduces fraction volume of large pores and ultimately decreases more than 50% CH_4 emission (Hanaki *et al*., 2002). Li *et al*. (2011) also stated that when bulk density is increased in no tillage treated paddy soil; CH_4 emission to atmosphere can be blocked and CH_4 produced in soil might be kept for long time, which may increase probability of CH_4 oxidation by methanotrophs. On the other hand, conventional tillage highly disturbs soil surface, alters soil properties and biochemical processes.

Aquatic earthworms are a major group of invertebrate fauna in the paddy field ecosystem, and are well known to maintain soil quality (Simpson *et al*., 1993). More particularly, aquatic earthworms in paddy soils have the potential to improve soil health and increase soil fertility and plant production (Yokota and Kaneko, 2002). In flooded soil, aquatic earthworms (maximum length: 4–5 cm) mix soil and move water by burrowing and passaging soil through their gut. The bioturbation activities of aquatic earthworms may enhance oxidation by increasing the depth of the oxidized layer at the soil surface. Thus, aquatic earthworms may play a role to mitigate CH_4 emissions from flooded paddy soil and may affect methane-consuming microbes, which play a vital role in global warming because they are a biological sink of CH_4. Different environmental factors and management practices are well known to affect CH_4 emissions from paddy soil, but little is known about the effects of aquatic earthworms on CH_4 emissions. Aquatic earthworms are ‘conveyor belt’-type feeders (Rhoads, 1974) such as the head of aquatic earthworms is buried in the soil for feeding while its tail remains on the surface for respiration. Through this bioturbation activity, aquatic earthworms might be able to introduce O_2-rich water into the lower soil layer, and it also produces a layer of faecal pellets on the soil surface. This soil habitat modification led to an increase in the biomass of methanotrophs, which accelerated the oxidation of CH_4 produced by methanogens.

Therefore, the aim of this study was:

Co-benefit of integrated farming practices to reduce the use of agro-chemicals to conserve soil biodiversity and to reduce the burden of CH_4 emission by integrated farming practices and aquatic earthworms.

Chapter: II

Role of aquatic earthworms on methane emission from paddy field influenced by different farming practices

2.1 Introduction

Global warming is a kind of environmental problem which may affect all living things on Earth. Methane (CH_4) is one of the most efficient greenhouse gases, and its contribution to the greenhouse effect is almost half of that of carbon dioxide (CO_2) (Gavin, 2004). The atmospheric concentration of CH_4 is 1.8 ppm, much lower than that of CO_2 (399 ppm) (Blasing, 2016). However, the heat trapping capacity of CH_4 is about 25 times that of CO_2 (IPCC, 2007), which makes it harmful to the environment. A recent report by Blasing (2016) showed that the atmospheric CH_4 concentration has more than doubled over the last 200 years.

Methane is released in atmosphere from several sources, both natural and anthropogenic. Anthropogenic sources include enteric fermentation, animal and human wastes, rice paddies, biomass burning, and landfills. Anthropogenic and natural sources contribute 375 Tg CH_4/year and 160 Tg CH_4/year. Flooded rice cultivation alone emits 11% of total anthropogenic methane (Smith *et al.*, 2007)).

Rice is one of the world's most important cereals. In Asia, about 136×10^6 ha area which is 88% of world's total was harvested to produce 556 Tg (Mt) rough rice, 90% of world's total production in 2004 (FAO, 2005). In Asia, rice lands can be divided into deepwater (3%), upland (7%), rainfed lowland (33%) and irrigated (57%) (FAO, 2005). In deep water rice fields, flood water may raise more than 50 cm during the growing season. In rainfed rice fields, precipitation controls flooding; during growing season rainfed rice field may dry up or have flood water up to

50 cm. In irrigated rice fields, the flood water can be controlled. Upland rice fields are not flooded and the topsoil does not become saturated at any period of growing season.

Generally, 10-20 cm water exists in the paddy field about all over the cropping period and in flooded paddy field oxygen supply is reduced from the atmosphere to the soil. As a result, methanogens fermented organic matter in anaerobic condition and produces CH_4 as end product (Fig. 4). The major methanogenic substrates are H_2+CO_2, formate, acetate, methyl alcohol and methyl amine. The dominant reactions of methanogenesis in soil are: the reduction of CO_2 using H_2 and the transmethylation of acetate;

$$\text{Methanogenesis: } CO_2 + 4H_2 \rightarrow 2H_2O + CH_4$$

$$CH_3COOH \rightarrow CO_2 + CH_4$$

During methanogenesis process, carbon substrate is supplied to soil from soil organic matter, sloughed tissues of rice plants, applied organic matter and exudates (Kimura *et al*., 2004; Watanabe *et al*., 1999). Methanotrophs consume part of the produced CH_4 under oxidative condition around rhizosphere of rice plants and in a thin layer where soil interferes with surface water. The rest of the produced CH_4 can be released to atmosphere from paddy field by various ways such as diffusion, ebullition from soil and by diffusion and mass flow through a continuous intercellular gas space system between rhizosphere and leaf of rice plant. Inubushi *et al*., (1989) reported that 90% of total CH_4 from paddy field is released through rice plant and ebullition and diffusion from soil contributes 10% and <1%, respectively. It indicates that a high amount CH_4 is emitted from rice plant. The physiological structure of rice plant favors greater emission of CH_4. Intercellular channel that made of aerenchyma between leaf parts and rhizosphere acts as path of gas exchange between root zone and atmosphere through stomata in leaf and micropores in leaf sheath. In submerged paddy field, CH_4 diffuse in root and is transported to above part through intercellular channel and is released to atmosphere through stomata and micropore (Nouchi *et al.*,1990).

From the last century world population is being increased very rapidly day by day. World population continues to increase by about 85 million people a year (Wassmann *et al.*, 2000). At the same time, the demand for rice is being increased with time due to the rapid population growth as rice is the only major grain crop which is grown exclusively as food. To meet this demand, cropping intensity has been increased with practicing rice-rice/rice-rice-rice cropping pattern in developing countries. Thus, the world's harvested rice area increased during the past 40 years by 41% and rough (still in the hull) rice production by 304% (Neue, 1993). Rice production in Asia has become double than 25 years ago, but the cultivation area for rice has increased only 17%. Average yields have increased 72%, which is only slightly more than the 67% growth in population (IRRI 1991). The rice production has been increased in irrigated rice and favorable rain-fed rice area. By 2020, the world will need to produce 350 million tons more rice per year to feed an anticipated 3 billion more people. Consequently, the rate of methane emission is being increased from paddy field.

Aquatic earthworms affect physical, chemical and microbiological properties of soil (Grant and Seegers, 1985) as aquatic earthworms have influence on soil properties. They play an important role in the dynamics of the paddy field ecosystem as they can change biological and chemical properties in the soil and water (Kikuchi *et al.* 1977). In paddy field, aquatic earthworms usually remain near the soil surface and mix the soil and move water very actively not only by borrowing but also by passage of soil through their gut. Bioturbation activities of aquatic earthworms may increase oxidation by increasing oxidized surface of soil layer. By this process earthworms may play role to mitigate methane emission from flooded paddy field.

$$\text{Methane oxidation: } CH_4 + 2O_2 \rightarrow CO_2 + 2H_2O$$

Both chemical and organic fertilizers strongly affect CH_4 emissions from paddy field soil (Yagi and Minami, 1990). Organic fertilizer is well known to conserve soil fertility. In Japan, it has been recommended to combine organic fertilizer with chemical fertilizer, as the former releases organic nitrogen very slowly during decomposition (Minamikawa and Sakai, 2006). Chemical fertilizers such as nitrogen fertilizers are fundamentally important for crop growth and yield. Urea and ammonium sulfate are two of the most commonly used inorganic nitrogen fertilizers for lowland rice (Fageria *et al*., 2003). Sulfate-containing fertilizers suppress CH_4 emissions because they result in competition between sulfate-reducing bacteria and methanogenic archaea for substrates like hydrogen and acetate (Hori *et al*., 1990). Several studies have demonstrated that ammonium sulfate decreases CH_4 emissions from soil (Schutz *et al*., 1989, Hori *et al*., 1990, Banik *et al*., 1996). However, the effects of urea on CH_4 emissions from soil are context-dependent. In some studies, CH_4 emissions increased with increasing urea application rates, soil pH increased because of urea hydrolysis (Wang *et al*., 1992), and ammonium inhibited methanotrophs (Dubey *et al*., 2003). In other studies, ammonium enhanced oxidation of CH_4 and the activities of methanotrophs (Bodelier *et al*., 2000).

Different field management practices also have direct and indirect impacts on CH_4 emission in paddy field. Recently, no-tillage farming practice is being increased in some East Asian countries as no-tillage farming practice improves soil environment for crop production and preserves soil structure and enhances percolation in paddy soils (Hossain *et al*., 2000; Ota *et al*., 2002). Long-term no-tillage farming practice can lead to excessive compactness of soil surface (Hama and Anderson, 2005) along with weed spread (Turley *et al*., 2003). No-tillage farming practice in rice cultivation reduces fraction volume of large pores and ultimately decreases more than 50% CH_4 emission (Hanaki *et al*., 2002). Li *et al*. (2011) also stated that when bulk density is increased in no tillage treated paddy soil; CH_4 emission to atmosphere can be blocked and CH_4 produced in soil might be kept for long time, which may increase probability of CH_4 oxidation by methanotrophs. On the other hand, conventional tillage highly disturbs soil surface, alters soil properties and biochemical processes.

Therefore, the aim of this study was

1) To identify the effects of different farming practices on earthworm density in paddy field ecosystems and
2) To know the role of earthworms on methane flux in paddy field ecosystem under different farming practices.

2.2 Materials and methods

Location of study area

The study was conducted through field study and laboratory study. Field study was conducted at paddy field of Kamakura Central Park, Kanagawa Prefecture during May to October, 2010 and May to October, 2011. The field was located at 35°20′ N, 139°31′E, 33m above sea level**.**

Photo 1: Location of field study (Kamakura Central Park)

An important characteristic of the study site was that was an organic paddy field and the paddy fields were being maintained traditionally. In this paddy field, neither any kind of pesticides and herbicides nor chemical fertilizers were used since 2004. It was a rain fed paddy field and the hill stream was being used as irrigation water and this hill stream was being flowed from higher elevation paddy fields to lower elevation paddy fields.

Field study of 2010

Field preparation:

Field study was started from the month of May and continued until October. Field study was started on 15th May by pre-treatment sampling. Sixteen soil samples were collected by a core sampler having 28.26cm^2 surface area (height 11 cm and diameter 6 cm). Four treatments were selected in a 2x2 factorial design with four replications. The treatments were i) tillage with chemical fertilizer (T-C), ii) tillage with organic fertilizer (T-O), iii) no tillage with chemical fertilizer (N-C) and iv) no tillage with organic fertilizer (N-O). The area of study field was 24 m^2. The entire area was divided into four blocks with PVC plastic board and each block was divided into four plots having 1.5 m^2 area each (Fig. 3).

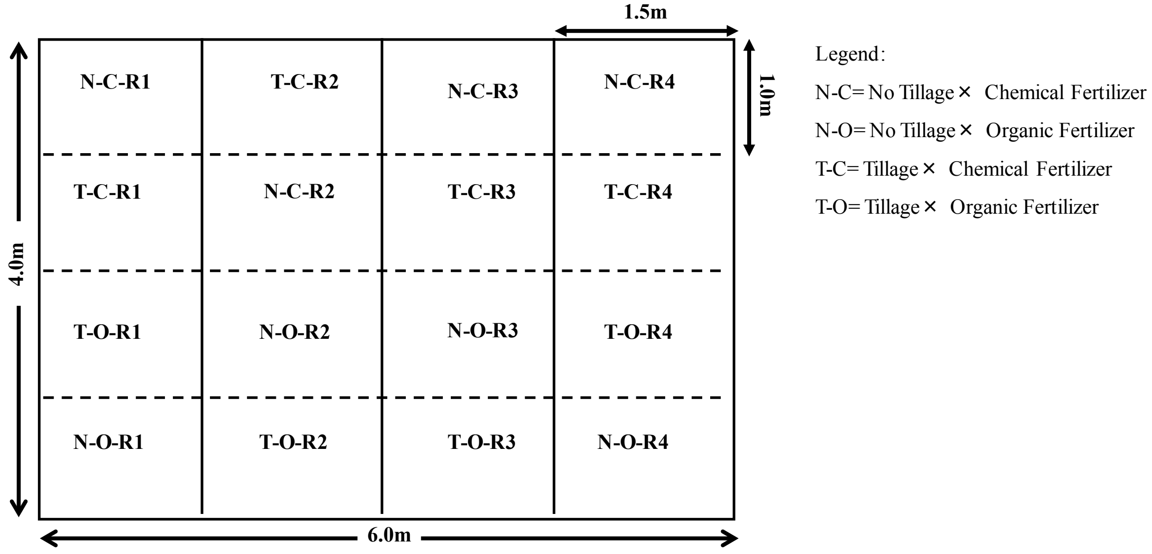


Figure 3: Field layout with treatments

Puddling and fertilizer application

On 29th May, treatments were imposed. For application of tillage, eight plots were puddled with hands and after puddling the plots were leveled by a leveler. In no-tillage treated plots the soils were kept undisturbed. Chemical and organic fertilizers were applied as fertilizer treatments by broadcasting method. Fertilizer doses are mentioned in the following table 1 :

Table 1: Name of fertilizer and rate of application in 2010

Kind of fertilizers	Name	N:P:K	Application rate (g/plot)	Applied N (kg/ha)
Organic fertilizer	Yuki Aguretto 666 Tokugo	6:6:6	117.50	47
Chemical fertilizer	Suito Haigo 100	10:10:10	70.58	47

In the study area, only organic fertilizer was being used for long time and N application rate was 47 kg/ha. So, during fertilizer application of this study N application rate was kept same in both organic and chemical fertilizer and following this N rate the fertilizer application rate was calculated.

Transplantation

On 5th June, rice seedlings were transplanted to paddy field from seed bed. For transplantation, only healthy seedlings were selected very carefully. During transplantation, hill to hill distance was kept 20 cm×30 cm. In each hill, three seedlings were planted.

Weeding

Weeds are plants which are grown in an undesired place and generally they are called enemy of crops, because they have very aggressive growth in crop field and start to compete with crop plants.

Thus, weeding is an important management practice for cropping season. During the cropping period of this study, two times on July and August weeds were cleaned by hands from all plots.

Harvesting

Field study of 2010 was completed by harvesting. Harvesting was done on 11th October. After harvesting six hills of rice plants from the middle of each plot were carried to laboratory for further measurements. In laboratory, the following parameters were measured:

I) Plant height
II) No. of filled grains per panicle
III) Weight of 1000 grains

Soil sampling

Soil samples were collected once in a month starting from May to September to measure the density of earthworms. Soil samples were taken from each of sixteen plots without any biasness with the core sampler having 6 cm diameter, 11 cm depth and 28.26 cm^2 surface area. Soil samples were put in plastic bins and brought back to laboratory for density measurement. Each soil sample was divided into two based on weight. Half of soil samples were used for measuring density of earthworms and rest of the half was used for further soil analysis. Tap water was kept in buckets for overnight to drop down the ions and that water was used on the next day for earthworm density measurement. A 500 µm mesh size sieve was used for sieving of earthworms. The collected earthworms were incubated in the incubator at 20^oC temperature under 14 hours light and 10 hours dark condition.

Water quality of paddy field

Some environmental parameters of the standing water in paddy field were measured by portable devices. pH was measured by D-21, HORIBA, electrical conductivity and oxidation reduction potential by D-54T, Horiba, and dissolve oxygen was measured by DO24P, DKK-TOA starting from May to September on sampling date.

Preparation of soil for soil analysis

Plant debris, other macrofauna and stones were removed from the soil and soil samples were weighed. Then soil samples were air dried in room temperature for one week. After air drying big soil clods were broken and put in oven for drying at 45ºC for 48 hours. After oven drying the soil samples were again weighed and preserved in air tight packets.

Total C and N analysis

Understanding the health of the soil in which crops are growing is a fundamental in ensuring healthy yields. Carbon is important because of its energy content in the form of species such as carbohydrates, whereas nitrogen is essential for growth. With the same purpose, CN ratio was analyzed in this study with the soil of the month of May. For this analysis, NC Analyzer (SUMIGRAPH NC-95A and SHIMADZU GC-8A) was used. About 160 mg dried soils were taken for this analysis.

Laboratory study of 2010

Earthworms

This laboratory study was done to measure methane flux from paddy soil. For this laboratory study, only one specific species of earthworm, *Branchiura sowerbyi* was used because this species was one of the most dominant species in paddy field (Yachi *et al*., 2012). This species is very vulnerable to autotomy of the posterior part of the body which may prevent any *in vivo* identification (Ducrot *et al*., 2007). Therefore, autotomized earthworms were discarded for this study to ensure that only *Branchiura sowerbyi* species was present.

Photo 2: *Branchiura sowerbyi*

Incubation

The entire laboratory study was the combination of two experiments.

Experiment 1:

Preparation

Three factors were considered on methane emission and the factors were I) soil, II) water and III) earthworms. Two levels of soil (HS: 100g soil, LS: 70g soil), two levels of water (HW: 30ml and LW: 16ml) and two levels of earthworms (W: 6 earthworms and NW: no earthworms) were used with four replications. Thirty-two glass bottles were prepared for incubation. Soil, water and earthworms were introduced in the glass bottles as mentioned above. Preparation was finished with addition of 0.1g rice husk powder as organic matter in all glass bottles. Then the glass bottles were kept open for incubation at 20°C temperature and in dark condition for 28 days.

Photo 3: Prepared glass bottles during incubation

Methane measurement

Methane concentration was measured by gas chromatograph (GC-2014, SHIMADZU) at 7 days interval. Before one hour of measurement, glass bottles were being closed with plastic leads having rubber septa. For measurement 0.5 ml gas samples were taken by a 1 ml gas-tight syringe through rubber septa from the head space of the glass bottles. Every 7 days after measuring methane concentration, water in the glass bottles were changed with deionised water. Methane flux per area was calculated by the acceleration of methane concentration with time.

Experiment 2:

Preparation

During this experiment, two factors were considered on methane flux: I) earthworms and II) organic matter. Two levels of earthworms (W: 6 earthworms and NW: no earthworms) and two levels of organic matter (OM: 0.1g organic matter and NO: no organic matter) were considered in this experiment with four replications. Rice husk powder was used as organic matter. Sixteen glass bottles were prepared and same amount of soil and water were put into each glass bottles with fixed amount of organic matter to the organic matter treated glass bottles. Before introducing earthworms, the glass bottles were kept in the incubator at 20°C temperature for the proper decomposition of organic matter. After 7 days earthworms were introduced into the glass bottles.

Methane measurement

During this experiment the same Gas Chromatograph of experiment 1 was used. Incubation of earthworms was lasted for 28 days. Gas samples collection and methane measurement were started just after introducing earthworms in glass bottles. This time also at 7 days interval methane measurement was continued. Every 7 days after measuring concentration, water of glass bottles was changed with deionised water and after measurement the glass bottles were again kept open and methane flux per area was calculated by the acceleration of methane concentration with time.

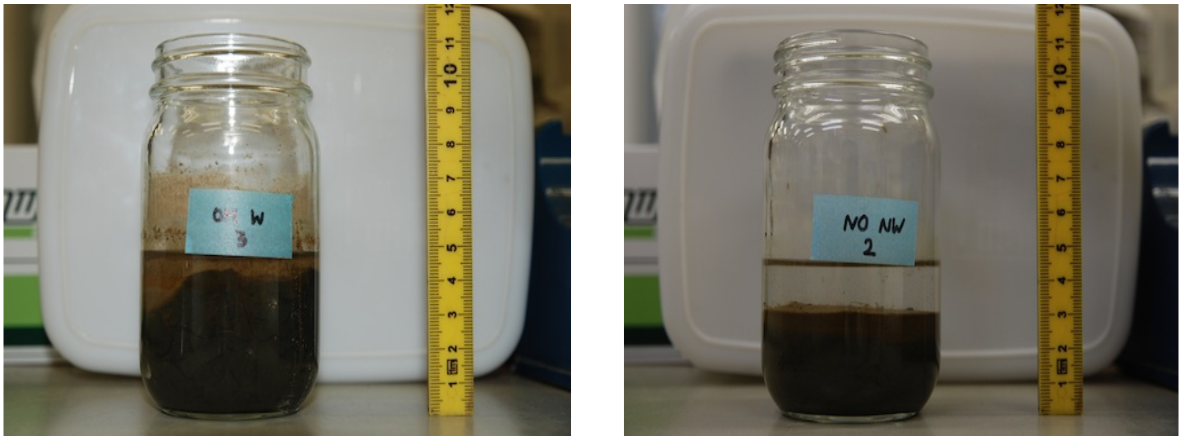


Photo 4: Change of soil surface after 28 days of incubation. With earthworms (left) and without earthworms (right)

Field study 2011

Field preparation

Field study of 2011 was started on May 18 by field preparation of paddy field and continued until October. At first, sixteen plots were divided according to the treatments. As the total area of study field was 24 m^2, the area of each plot was 1.5m^2. In 2011, four treatments were selected in a 2×2 factorial design with four replications. The treatments were: i) tillage with chemical fertilizer (T-C), ii) tillage with organic fertilizer (T-O), iii) no tillage with chemical fertilizer (N-C) and iv) no tillage with organic fertilizer (N-O). The area of study field was 24 m^2. The entire area was divided into four blocks with PVC plastic board and each block was divided into four plots having 1.5 m^2 area each. During field preparation walking path was prepared so that the soil surface cannot be disturbed.

Puddling and fertilizer application

On 28th of May all of four treatments were applied to the study field. Plots with tillage treatment was puddled by hands and leveled by hand leveler and plots which were to be treated as no-tillage were kept totally undisturbed. After this both chemical and organic fertilizers were applied on plots by broadcasting method. In 2011, fertilizers were applied twice on May and August. Rates of fertilizer application are given in following table 2:

Table 2: Name of fertilizer and rate of application in 2011

Month	Kind of fertilizer	Name	N:P:K	Application rate (g/plot)	N (kg/ha)
May	Organic fertilizer	Yuki Aguretto 666 Tokugo	6:6:6	117.5	47
	Chemical fertilizer	Suito Haigo100	10:10:10	70.6	47
August	Organic fertilizer	Yuki Yasaino Hiryo	8:8:8	88.1	47
	Chemical fertilizer	Suito Haigo100	10:10:10	70.6	47

Transplantation

On 4th June, healthy rice seedlings were collected from seedbeds and transplanted to the main field. Three seedlings were planted per hill and hill to hill distance was kept 20 cm×30 cm.

Weeding

Weeding was done on July, August and September. Weeds were removed from all the plots by hands.

Harvesting

Rice plants were harvested on October 11. After harvesting six hills from the middle of each of sixteen plots were carried to laboratory for further measurement. The following parameters were measured by using the cut rice plants in laboratory:

I) Plant height (cm)
II) No. of tillers per hill
III) Weight of 1000 grains (g)

Soil sampling

Soil samples were collected to measure the density of earthworms per square meter area. Soil sample collection was started from the month of May before application of treatments and continued until the month of October once in a month. Soil was collected by a core sampler having 6 cm diameter, 11 cm depth and 28.26 m^2 surface area. After collection soil samples were carried to laboratory and divided into two based on weight. Half of soil was used for soil analysis and another half was used for density measurement. For density measurement, one 500µm mesh-sized sieve was used for sieving of earthworms. For collection of earthworms tap water was used was kept in bucket overnight before use. After collection of earthworms those were kept in incubator at 20^{o}C temperature.

Species identification

Species composition was identified using the method of Yachi *et al*. (2012). After sorting, alive earthworms were washed with distilled water and filmed with a 10 × magnification digital microscope. Then dehydrated specimens were mounted on slides with Canada balsam. Chaetal and genital observations were done by an optical microscope (BX50-33 PHD, Olympus co., Tokyo, Japan). Identification of aquatic earthworms was done following literatures (Brinkhurst and Jamieson, 1971; Ohtaka, 1994 and Kathman and Brinkhurst, 1999).

Preparation of soil for analysis

All plant debris, macrofauna and stones were removed from the soil and all big clods were broken. Then soil samples were weighed and kept in room temperature for air drying for about a week. After being air dried the soil samples were transferred to oven for drying at 45^{o}C for 48 hours. Then soil samples were weighed again and preserved in air tight packets.

Gas sample collection and methane flux measurement

In 2011, gas samples were collected from each of sixteen plots of paddy field to measure methane flux by closed chamber method. Chambers having 0.6 m length, 0.5 m height and 0.3 m width were used for gas sample collection in this field study. Gas samples were collected once in a month starting from the month of May to October. First gas sampling was done before application of treatments and last gas sampling was done one week after harvesting. Gas samples were collected by a 60 ml plastic syringe. First sample was taken at 0 minute that means just after placing of gas chamber on the plots. Then second sample at 10 minute and the last sample at 20 minute were taken. Gas samples were carried to laboratory by 45 ml glass bottles. During sampling, air temperature of inside of chamber was measured by placing Thermo Recorder (RT- 13, ESPEC MIC CORP.).

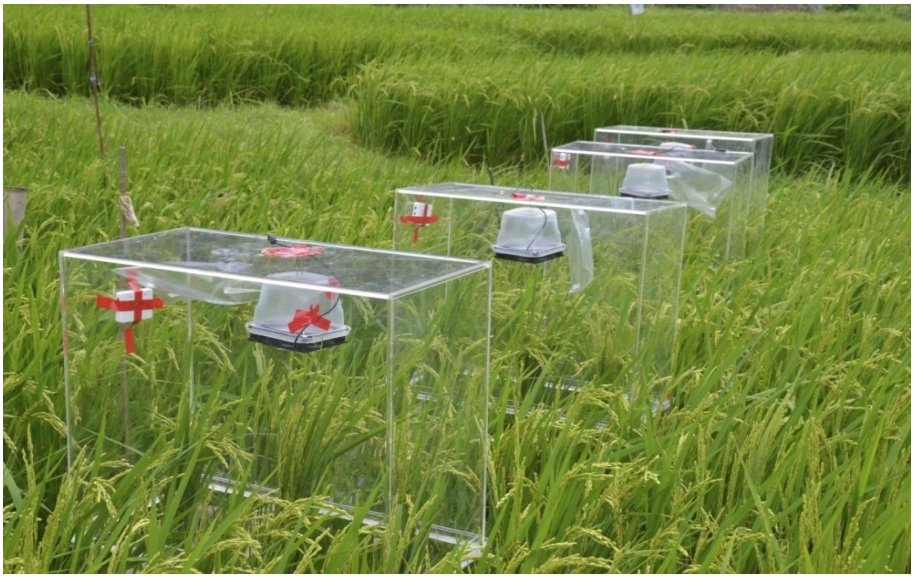


Photo 5: Chambers for gas sample collection

Methane concentration from gas samples was measured by Gas Chromatograph (GC-2014, SHIMADZU). Then methane flux per area per hour was calculated by the acceleration of methane concentration with time.

Water quality

Some environmental parameters were measured by portable devices. pH of paddy field water was measured by D-21, HORIBA, electrical conductivity and oxidation reduction potential by D-54T, Horiba, and dissolve oxygen was measured by DO24P, DKK-TOA on the month of June, July, September and October on sampling date. As on the month of August there was no water in paddy field, it was not possible to measure these parameters.

Soil temperature

Soil temperature is an important factor for the activities and the growth rates of microorganisms. Therefore, during this study temperature loggers (Thermocron, OnSolution Pty Ltd.) were placed at 0 cm, 5 cm and 10 cm depth from the month of June and every month the loggers were replaced.

Precipitation

As the study site was a rain fed paddy field, we had to depend on rainfall for irrigation water source. Water is necessary in the field for proper activities of aquatic earthworms and formation of methane in paddy field also depends on the flooding of paddy field. For these reasons rainfall observer (RF-3 T & D Corporation) was set near to the study area on the month of June and removed on October.

Total C and N analysis

Total of paddy field soil was analyzed by CN corder (MACRO CORDER JM1000CN) using dried soil of every month.

Particle distribution of soil

Earthworm activities may have effects on soil particle size and tillage treatment also may change particle size. To identify the effects of earthworm activities and tillage on particle size distribution, the soil particle was analyzed by Laser Diffraction Particle Size analyzer (LDPSA) Shimadzu SALD-3000. For this analysis, soil samples of June and September of 2011 were used and 0.65 mg representative soil sample was taken without biasness. Three ml H_2O_2 was added with soil and was placed in an oven at 101°C for 10 minutes to promote decomposition of organic matter. Then 27 ml pure water was added with the soil solution and was placed in a desiccator to cool down. After the soil solution became cool, 0.6 ml hexane was added to the solution and the solution was stirred by electrical stirrer for 1 minute. Then the solution was poured into the analyzer and analysis was started.

Statistical analysis

The data were compiled and tabulated in proper form for statistical analysis. The heterogeneity and normality of data were assessed by Bartlett's test and Shapiro-Wilk test respectively. The main and interaction effects of tillage, fertilizers and aquatic earthworms were determined by two-way and three-way analysis of variance (ANOVA) with generalized linear model (GLM). Data were analyzed by R 3.2.3 for Microsoft Windows (R Development Core Team, 2015).

2.3 Results

Water quality

pH range during our field study was 6.87-8.48 in 2010 and 7.03 -9.29 in 2011 i.e. the paddy field of study site was mostly alkaline. On average, electrical conductivity was found 29.34S/m during field study of 2010. The highest value was found 46.4 mS/m and the lowest was 21 mS/m. During field study of 2011, the average electrical conductivity value was measured 29.53 mS/m where the highest value was 39.2 mS/m and the lowest value was 20 mS/m. In 2010, the highest value of DO was measured 8.34 mg/L on the month of September and the lowest value was 2.05 mg/L. On average, the DO value was found 5.1 mg/L. In 2011, the highest DO value was found 7.07 mg/L during July and the lowest DO value was found 2.18 mg/L. Average DO value was calculated 5.2 mg/L. During our field study, the ORP was found -166 to -374 mV in 2010 and -158 to -376 mV in 2011. These ORP values were suitable for production of methane in study area.

Table 3: Environmental data (pH and ORP for soil, conductivity and DO for water) of study site in 2010

	May	June	July	August	September
pH	6.87	7.07	8.48	7.42	7.23
Conductivity (mS/m)	24.1	21	23.4	46.4	31.8
DO (mg/L)	4.88	2.05	7.07	3.64	8.34
ORP (mV)		-182	-266	-292	-274

Table 4: Environmental data (pH and ORP for soil, conductivity and DO for water) of study site in 2011

	June	July	August	September	October
pH	7.29	7.23	-	7.29	7.03
Conductivity (mS/m)	39.2	23	-	20	35.9
DO (mg/L)	7.03	7.07	-	7.18	4.54
ORP (mV)	-276	-288	-	-258	-285

Soil temperature

The highest surface temperature was 37ºC on July and the lowest surface temperature was 20ºC at the end of September (Fig. 4). At 5 cm depth, the highest soil temperature was found 36.5ºC on July and the lowest was recorded 17ºC on October and at 10 cm depth the highest soil temperature was measured 35ºC on August and the lowest temperature was recorded 17.5ºC on October. From the end of September, the surface temperature started to decrease. Soil temperature at 5 cm and 10 cm depth started to decrease very sharply from the mid-September.

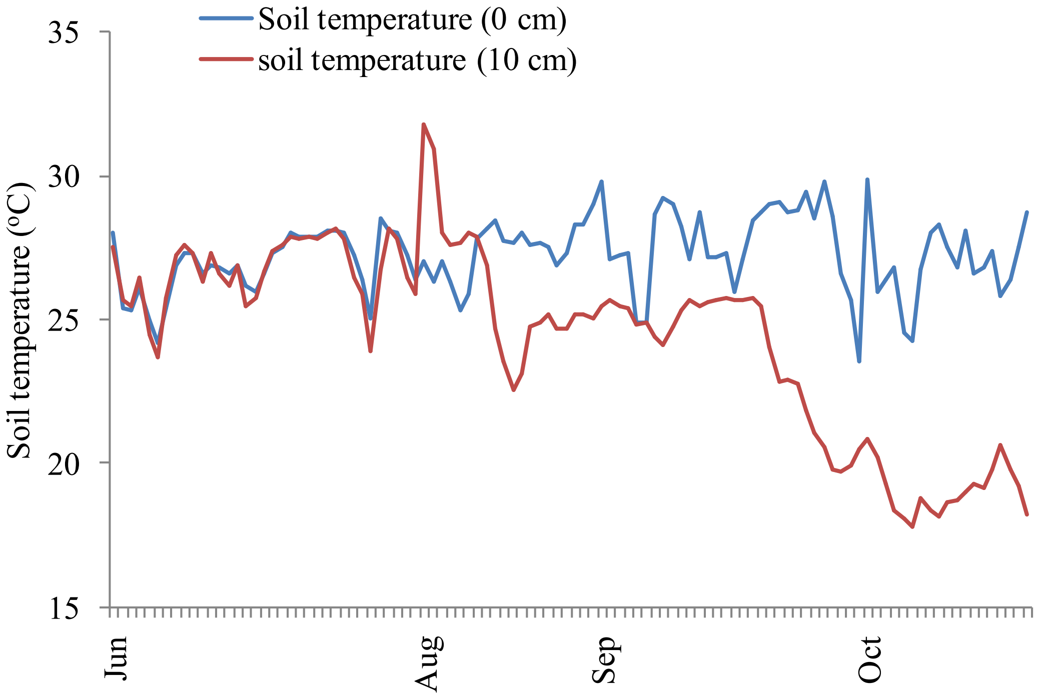


Figure 4: Soil temperature at different depth of soil of study field in 2011

Rainfall

As the study field was a rainfed paddy field, only the rain water was source of irrigation. So, rainfall was very important factor for this study. Rainfall was measured from the month of June to October, 2011. The total rainfall was recorded 668 mm during study period. The highest rainfall was recorded 250.5 mm on the month of September and the lowest rainfall was found 136 mm on the month of July (Fig. 5). Before sampling on August, there was no rainfall for many days and the temperature was also high at that time. As a result, during sampling on August the paddy field was found dry.

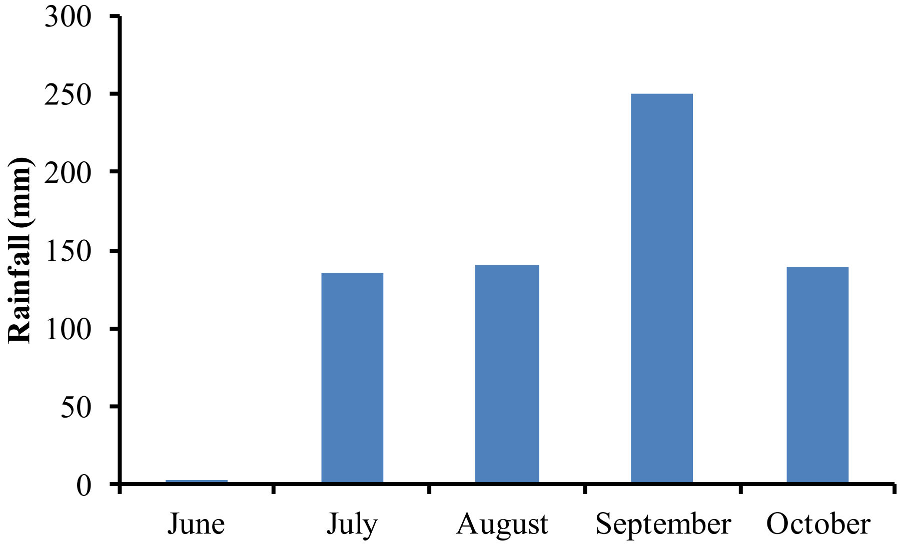


Figure 5: Monthly total precipitation of the study area

Total C and N paddy field soil

On May CN ratio was highest in no tillage plot with organic fertilizer and the lowest was in tillage- chemical fertilizer plot (Fig. 6). On the next month, the cumulative CN ratio increased and the highest CN ratio was found in tillage- organic fertilizer plot and this trend was continued until October and the lowest was calculated in no tillage- organic fertilizer plot. CN ration of no tillage- chemical fertilizer plot started to decrease from June and the trend was continued until October. On the following month, the lowest was in tillage- chemical fertilizer plot. On the month of August, the lowest CN ration was in no tillage- chemical fertilizer plot and until October from this plot the lowest CN ratio was found.

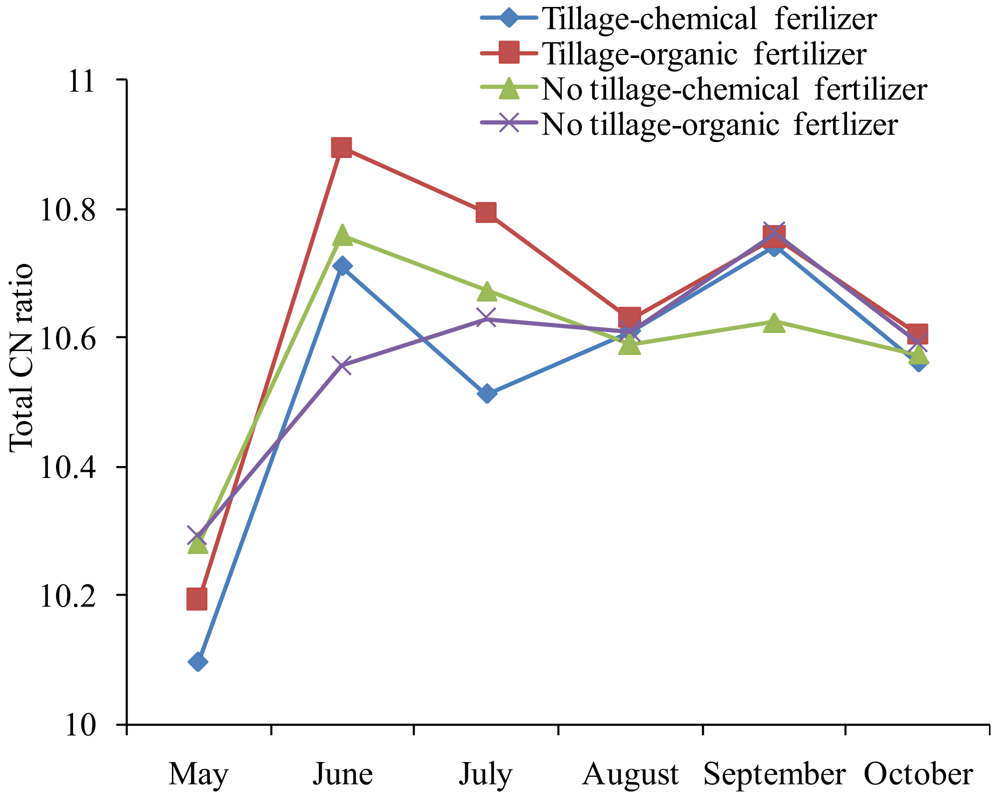


Figure 6: Temporal changes of CN ratio of paddy field soil in 2011

Particle distribution of soil

Percentage of sand, silt and clay particle of paddy field soil was about 40%, 58% and 2% respectively (Fig. 7 and 8).

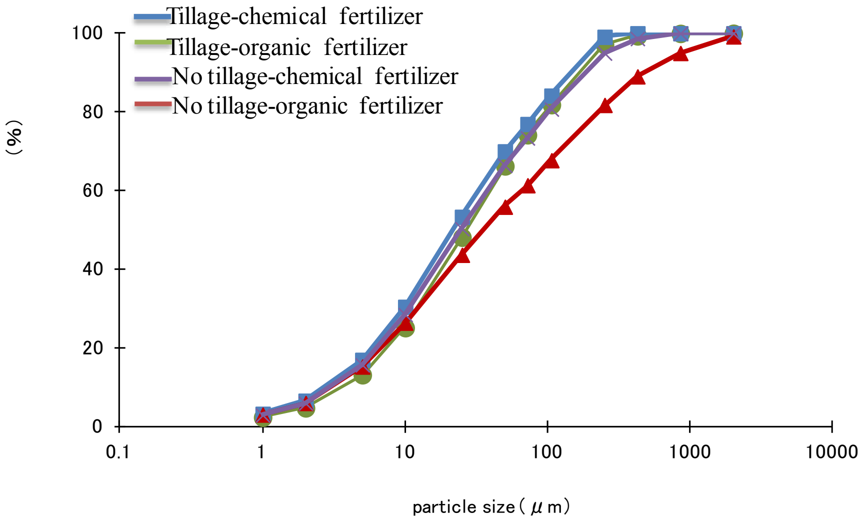


Figure 7: Particle distribution of paddy field soil (June, 2011)

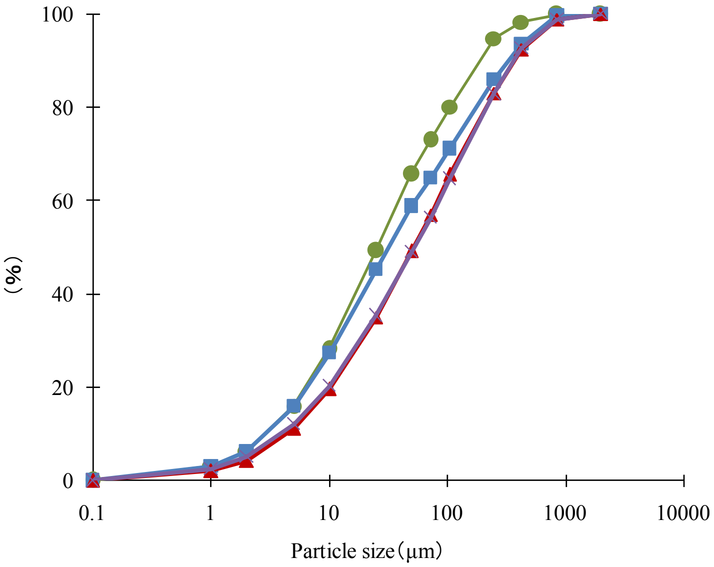


Figure 8: Particle distribution of paddy field soil (September)

Earthworm density

Earthworm density was measured from May to September during field study of 2010. During pre-treatment sampling the highest earthworm density was found in tillage-organic fertilizer plot and the lowest was found in no tillage-chemical fertilizer plot (Fig. 9). After application of treatment, cumulative earthworm density decreased due to disturbance during treatment application. On July, again the cumulative earthworm density started to increase and the highest density was found in no-tillage- organic fertilizer plot and the lowest was found in tillage-chemical fertilizer plot as the diffusion rate of organic fertilizer is lower than chemical fertilizer. On August, again the highest earthworm density was found in no tillage-organic fertilizer plot but the lowest earthworm density was found in tillage-chemical fertilizer plot. The cumulative earthworm density was increased compared to previous month due to seasonal variation. On September, again the cumulative earthworm density started to decrease. On September, also the highest earthworm density was got from no tillage-organic fertilizer plot and the lowest earthworm density was got from no tillage-chemical fertilizer plot.

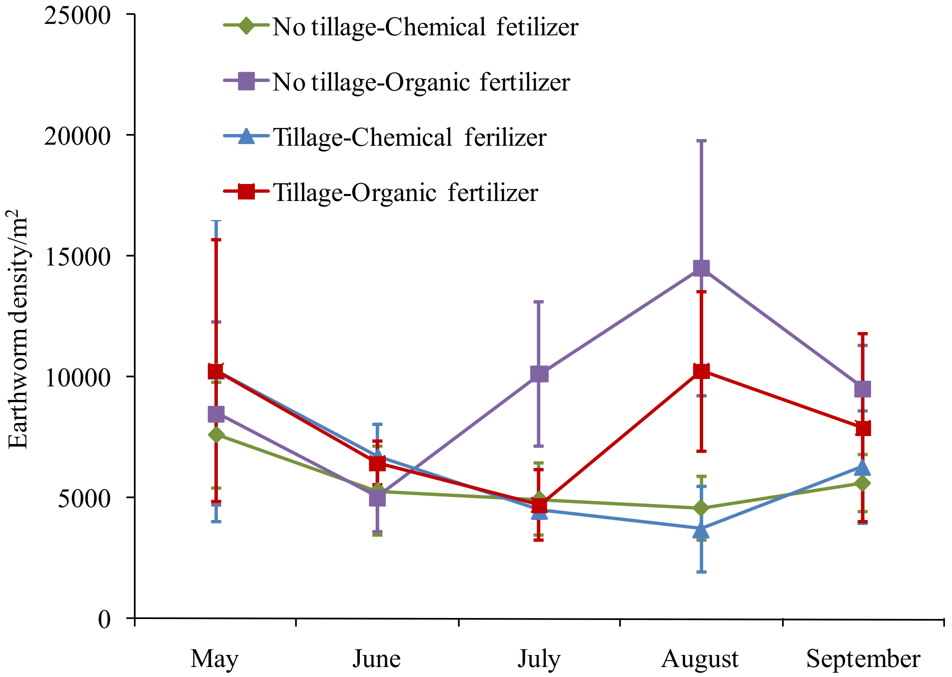


Figure 9: Temporal density of earthworm in study field during 2010

Table 5: ANOVA table for farm management practices on earthworm abundance

Month	Tillage		Fertilizer		Tillage: Fertilizer	
	F value	P value	F value	P value	F value	P value
May	0.22	0.65	0.01	0.93	0.01	0.92
June	1.02	0.33	0.04	0.85	0.00	1.00
July	3.21	0.09	2.87	0.12	2.55	0.13
August	2.06	0.18	3.82	0.07	1.83	0.20
September	0.19	0.67	0.98	0.34	0.19	0.67

P value>0.05 indicates insignificant effect

During the field study of 2011, earthworm density was measured starting from May to October. On May before application of treatments, the highest earthworm density was in no tillage-organic fertilizer plot and the lowest was in no tillage-chemical fertilizer plot (Fig. 10). On June after application of treatments cumulative earthworm density was increased and the highest and lowest earthworm density trends were similar with previous month. On July, the cumulative earthworm density decreased but the trends of the highest and lowest earthworm density were similar with previous months. On August, the cumulative earthworm density became very low. But at that time the highest earthworm density was found in no tillage-organic fertilizer plot and also in tillage-organic fertilizer plot and the lowest earthworm density was found in tillage-chemical fertilizer plot. On September, the cumulative earthworm density increased and the highest and lowest earthworm density was measured in no tillage-organic fertilizer plot and in no tillage-chemical fertilizer plot respectively. On October, the cumulative earthworm density increased compared to September. But suddenly the highest earthworm density was found in tillage-chemical fertilizer plot and the lowest was in tillage-organic fertilizer plot.

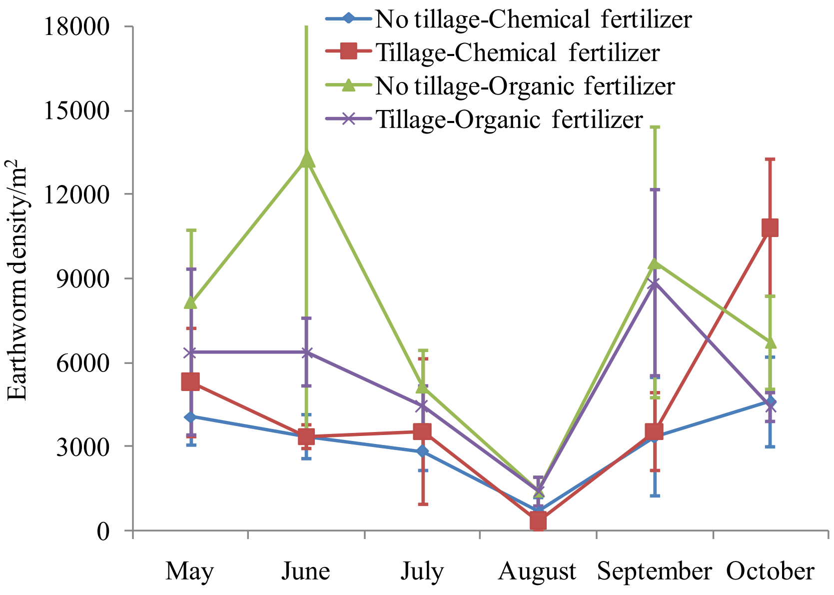


Figure 10: Temporal density of earthworms in study field during 2011

Table 6: ANOVA table for effect of farm management practices on earthworm abundance

Month	Tillage		Fertilizer		Tillage: Fertilizer	
	F value	P value	F value	P value	F value	P value
May	0.01	0.90	1.30	0.27	0.44	0.51
June	0.41	0.53	1.71	0.21	0.51	0.48
July	0.00	1.00	1.06	0.32	0.21	0.65
August	0.14	0.71	3.57	0.08	0.14	0.71
September	0.01	0.93	3.26	0.09	0.01	0.89
October	1.31	0.27	1.56	0.23	6.27	0.02*

P value>0.05 indicates insignificant effect

Table 7: Species composition of aquatic earthworms of paddy field in 2011 (Yachi *et al*., 2012)

Taxon	Months			
	June	July	August	September
Tubificinae				
Limnodrilus hoffmeisteri	30 (3)	16 (13)	2 (2)	5 (4)
Limnodrilus udekemianus	7 (0)	0	1 (0)	2 (1)
Limnodrilus claparedianus	0	0	0	0
Aulodrilus japonicas	0	0	0	0
Embolocephalus yamaguchii	0	0	0	0
Rhyacodrilinae				
Bothrioneurum vejdovskyanum	0	1 (0)	0	0
Branchiura sowerbyi	3 (0)	1 (1)	11 (4)	21 (3)
Naidinae				
Dero digitata	2 (1)	1 (0)	0	0
Unknown	8	10	9	3

(Figures in parenthesis are matures)

Methane flux in laboratory experiment

During laboratory study of 2010, effects of different treatments were measured on methane flux. In experiment 1, after one week of incubation, the highest methane flux was calculated in LS-HW-NW treatment and the lowest was in HS-LW-NW (Fig. 11). After two weeks of incubation the cumulative methane flux was became more than double of previous week. The highest methane flux was in LS-LW-W and the lowest was in LS-HW-W. At third week, the cumulative methane flux again decreased and at that time the highest methane flux was calculated in LS-HW-NW treatment and the lowest was in HS-LW-NW. At the last week, the cumulative methane flux became very low and the highest and lowest methane flux was found from the same treatments of previous week.

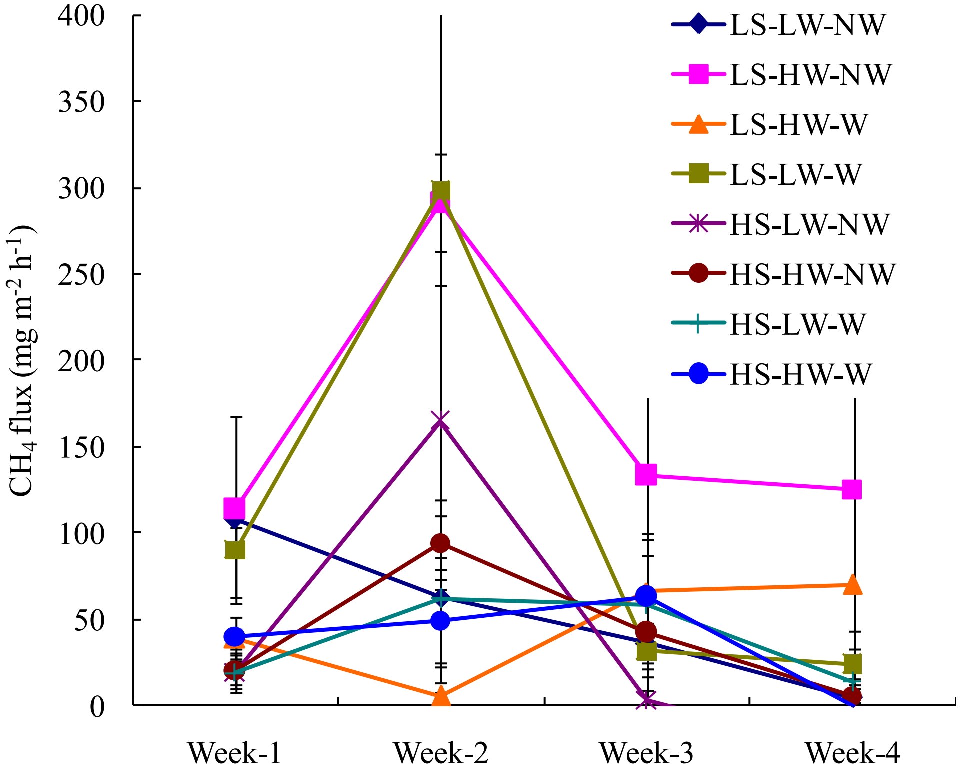


Figure 11: Influence of different treatments on methane flux (experiment 1)

Table 8: ANOVA table for effect of soil level, water level and presence of earthworms on methane flux (experiment 1)

Factors	F value	P value
Soil	4.53	0.04*
Water	0.21	0.65
Earthworm	0.42	0.52
Soil: Water	0.21	0.65
Soil: Earthworm	0.33	0.57
Water: Earthworm	3.08	0.09
Soil: Water: Earthworm	2.97	0.09

P value>0.05 indicates insignificant effect

In experiment 2, before introduction of earthworms the highest methane flux was in OM-W treatment and the lowest was in NO-NW treatment (Fig. 12). After one week of incubation and after introduction of earthworms the cumulative methane flux was increased and the highest methane flux was in OM-W bottles and the lowest was in NO-NW bottles. After two weeks of incubation, the cumulative methane flux was dropped down but the highest producer of methane was OM-NW and this time the lowest methane flux was in NO-W. After three weeks of incubation, the highest methane flux was in OM-W bottles and the lowest was in NO-W bottles. At the last week, also the highest methane flux was in OM-W and the lowest was in NO-NW bottles.

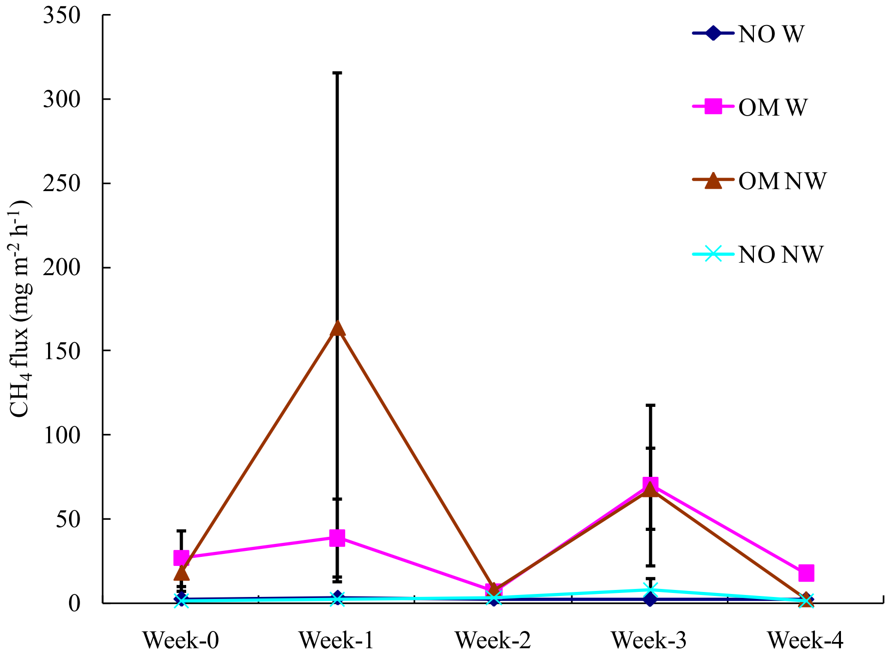


Figure 12: Influence of different treatments on methane flux (experiment 2)

Table 9: ANOVA table for effect of influence of earthworms with added organic matter on methane flux (experiment 2)

Factors	F value	P value
Organic matter	3.89	0.07
Earthworm	0.27	0.61
Organic matter: Earthworm	0.24	0.63

P value>0.05 indicates insignificant effect

Methane flux from field study

On May, before application of treatments, the highest methane flux was found from no tillage-organic fertilizer plot and the lowest was found from tillage-organic fertilizer plot (Fig. 13). After application of treatments, on June the cumulative methane started to increase and the highest methane flux was in no tillage-organic fertilizer plot where the lowest methane flux was recorded in tillage-organic fertilizer plot. On July, the increase of cumulative methane flux was continued due to the seasonal variation. That time highest methane flux was got from tillage-chemical fertilizer plot and the lowest was got from no tillage-organic fertilizer plot. On August the cumulative methane flux was dropped down and at that time, the highest methane flux was in no tillage-chemical fertilizer plot and the lowest methane flux was in no tillage-organic fertilizer plot. From September, the cumulative methane flux started to decrease with the decrease of temperature (Fig. 14). On September, the highest methane flux was found in no tillage-organic fertilizer plot and the lowest was in no tillage-chemical fertilizer plot. On October, the highest methane flux was in tillage-organic fertilizer plot and the lowest was in no tillage-chemical fertilizer plot. The highest cumulative CH_4 emission of 150 days was in no tillage-chemical fertilizer plots (31 g m^{-2}) and the lowest was in no tillage-organic fertilizer plot (24 g m^{-2}).

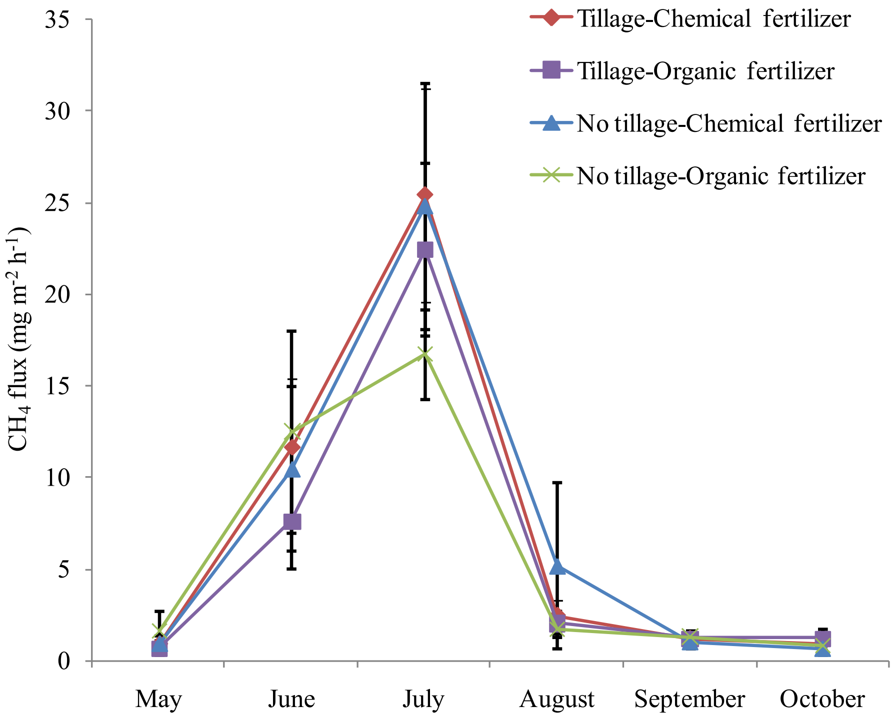


Figure 13: Influence of different treatments on methane flux from paddy field

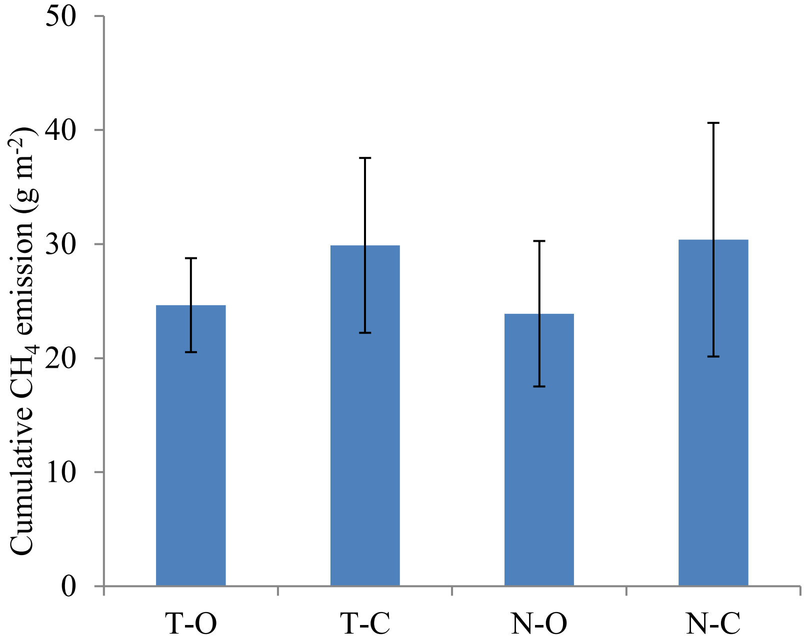


Figure 14: Influence of different treatments on CH_4 emission (vertical bars indicate S.E.)

Table 10: ANOVA table for effect of tillage, fertilizer and earthworms on methane flux

Months	Tillage		Fertilizer		Earthworms	
	F value	P value	F value	P value	F value	P value
May	0.35	0.56	0.21	0.66	0.65	0.44
June	0.09	0.77	0.22	0.64	0.09	0.77
July	0.44	0.52	1.35	0.27	0.12	0.72
August	1.51	0.25	4.46	0.06.	23.97	0.001**
September	0.06	0.81	0.38	0.55	17.27	0.003**
October	0.87	0.37	0.40	0.54	0.68	0.43

P value>0.05 indicates insignificant value

Effect of water depth on methane flux

During field study, methane flux showed clear trend to increase with the increase of water depth and water depth significantly increased methane flux in paddy field as anaerobic condition is developed with increase of water depth (Fig. 15).

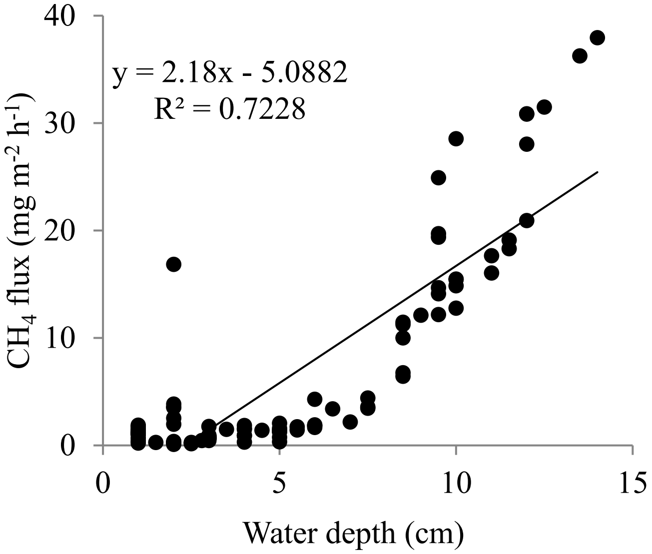


Figure 15: Change of methane flux with water depth

Effect of earthworm density on methane flux

During field study, except June and July on other months methane flux was very lower in all the plots as water depth was higher on June and July (Fig. 16). On July, aquatic earthworms showed clear trend to reduce CH_4 flux and aquatic earthworms reduced around 40% CH_4. On August, aquatic earthworms were found to reduce CH_4 flux significantly whereas on the following month aquatic earthworms increased CH_4 flux significantly in paddy field.

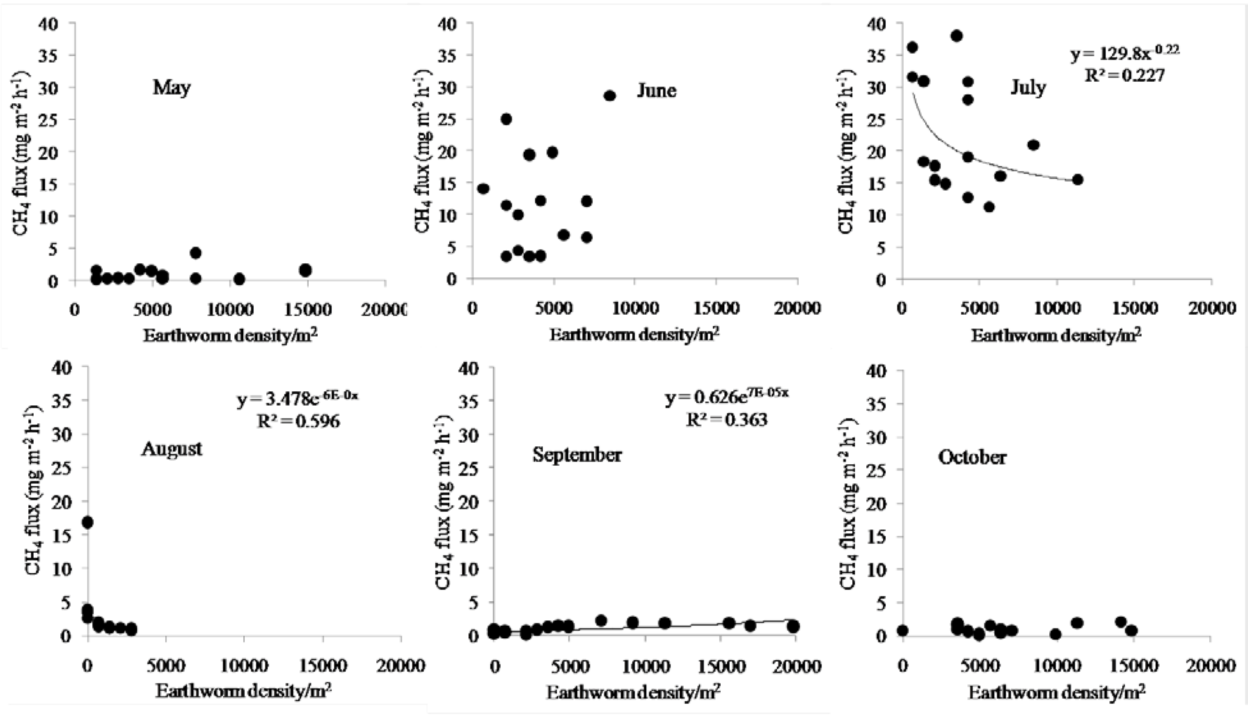


Figure 16: Correlation between earthworm density and CH_4 flux from May to October, 2011

Rice yield

Table 11: Yield of rice in 2010

Treatments	Plant height (cm)	No. of filled grains per panicle	Weight of 1000 grains (g)
Tillage-chemical fertilizer	85.8	108	27.2
No tillage-chemical fertilizer	91.3	142	26.2
Tillage-organic fertilizer	86.0	122	25.4
No tillage-organic fertilizer	93.0	93	25.2

Table 12: Yield of rice in 2011

Treatments	Plant height (cm)	No. of tillers per hill	Weight of 1000 grains (g)
Tillage-chemical fertilizer	78	29	26.9
No tillage-chemical fertilizer	74	15	26.8
Tillage-organic fertilizer	79	23	27.1
No tillage-organic fertilizer	81	24	27.0

2.4 Discussion

Water quality

pH is one of the important factors among soil properties for growth, establishment and diversity of microorganisms which have generally been reported to prefer neutral to slightly alkaline pH for optimum growth (Singh, 1961, Kaushik, 1994). Acidic conditions are therefore kind of the stressed environments for microorganisms and microorganisms may be absent at pH values below 4 or 5. The paddy field of study site was mostly alkaline.

The most influential water quality parameter on crop production is the salinity hazard which is measured by electrical conductivity value reflecting salt concentration (Agarwal *et al.*, 1982). Irrigation water with high electrical conductivity value results in salinity hazard. Salinity causes plants to lose energy for extracting pure water from saline water and lack of energy results in reduced crop growth and yield. The recommended electrical conductivity value for irrigation water in Japan is <30 mS/m. The quality of irrigation water for this study was doubtful as the highest EC value crossed the recommended value. The recommended DO for paddy field irrigation water is 5 mg/L (Hanya and Ogura, 1985). The suitability of irrigation water is doubtful as the highest DO values were more than 5 mg/L in 2010 and also on 2011.

Oxidation-reduction refers to two processes that occur simultaneously. Oxidation-reduction potential (ORP) is an important factor for methane production from submerged condition. After flooding, ORP starts to decrease gradually and Patrick (1981) mentioned that the ORP must be lower than approximately -150 mV to produce methane in soil. The evolution of methane from flooded paddy field may not commence until the ORP falls below -150 mV (Yamane and Sato, 1964). The ORP values of study site were indicating possibility of methane production from paddy field.

Relationship between water depth and CH_4 flux

During this study, CH_4 flux showed clear trend to increase with water depth as anaerobic condition is developed with increase of water depth. On June and July water depth was higher and with higher water depth CH_4 flux was also higher and the value was 12.3 mg m^{-2} hr^{-1} and 22.3 mg m^{-2} hr^{-1} respectively. During gas sampling on August, paddy field water was dried out due to lack of rainwater and paddy soil received oxygen and CH_4 flux dropped down to 2.81 mg m^{-2} hr^{-1}. Itoh *et al*. (2011) reported that prolonged mid-season drainage reduces seasonal CH_4 emission from paddy field. Yagi *et al*. (1997) and Sass *et al*. (1992) also recommended the same management. Cai *et al*. (1997) indicated that intermittent irrigation in paddy field significantly depresses CH_4 emission. On September and October water depth raised but the CH_4 flux did not raise as temperature started to fall down from September.

Effect of farming practices on CH_4 flux

Methane emission from no tillage treated plots was comparatively lower than total released methane from tillage treated plot. Hanaki *et al*. (2002) reported that no-tillage farming practice in rice cultivation can decrease CH_4 emission by reducing fraction volume of large pores. Li *et al*. (2011) also stated that when bulk density is increased in no tillage treated paddy soil; CH_4 emission to atmosphere can be blocked and CH_4 produced in soil might be kept for long time, which may increase probability of CH_4 oxidation by methanotrophs. Total released CH_4 from organic fertilizer treated plot was lower than that from chemical fertilizer treated plot. But Neue (1993) found in his study that organic fertilizer increased CH_4 emission from paddy field. In our study the paddy field was an organic farm and amount of applied organic fertilizer was 1.3 t ha^{-1}, which was lower than other paddy fields. In Japan, in conventional paddy fields, organic fertilizer application rate is around 6-12 t ha^{-1} (Yagi and Minami, 1990). Therefore, the amount of applied organic fertilizer in our study site was not enough to be responsible to increase CH_4 emission.

Effect of farming practices on aquatic earthworm density

In this study, the lowest earthworm density was observed in August when there was no water in paddy field. Sternert *et al*. (2009) also reported that the absence of irrigation water reduces the density of aquatic invertebrate, as those are not highly tolerant to drought. All over the study period except October, the highest earthworm density was observed from no tillage-organic fertilizer plot. This result agreed that no tillage farming practice can increase aquatic earthworm density in paddy field (Yokota and Kaneko, 2002) and according to Xiang *et al*. (2006) chemical fertilizer reduces earthworm population in paddy field. No significant effect of tillage on earthworm abundance was found during this field study.

Relationship between aquatic earthworm and CH_4 *flux*

During our field study, the highest CH_4 flux was observed in July. The seasonal trend of CH_4 flux of this study is comparable to the measurements done by Yagi and Minami (1990), Kagotani *et al*. (1996), Naser *et al*. (2007). After statistical analysis, earthworms were found to mitigate CH_4 significantly from paddy field on August. But that time there was no water in the paddy field. Therefore, field condition was not favourable to form CH_4. On the following month, earthworms were found to increase CH_4 significantly from paddy field. Although both organic and chemical fertilizers were applied on August, 2 weeks earlier to sampling but due to the lack of irrigation water granular fertilizers were found unchanged during sampling on August. At the end of August there was rainfall and earthworm density was started to increase and organic fertilizer was started to be decomposed. The correlation between earthworm density and CH_4 flux of July showed CH_4 flux was reduced around 40% when aquatic earthworm density reached to 11,000/m^2. Aquatic earthworms may enhance biomass of methannotrophs and reduce CH_4 emission from paddy soil as methanotrophs oxidize produced CH_4 in paddy soil. Aquatic earthworms are known as 'conveyor belt' type feeders (Rhoads, 1974). During feeding, tail of aquatic earthworm remains on the soil surface for respiration. Through this bioturbation activity, aquatic earthworms enter O_2 rich water to lower level of soil and accelerate oxidation of CH_4, which is produced by methanogens. The biomass of methanotrophs was not measured. There are several possible reasons for the absence of clear relationship between earthworm density and CH_4 flux. 1) There were high variation in CH_4 emissions and earthworm densities and also there might be field heterogeneity of soil organic matter. 2) Flux chamber covers two rice stumps. The rice plant is a major path of soil CH_4 to the atmosphere. Therefore, oxidation of CH_4 on the soil surface itself may not strong enough to reduce total CH_4 emissions. Nevertheless, significant negative correlation between earthworm density and CH_4 flux on July, when the highest CH_4 emission was observed means possibility of CH_4 mitigation adopting organic no-tillage farming. Statistical analysis of GLM also indicated marginal negative effect of aquatic earthworms to reduce CH_4 emission from paddy field.

Table 13: Effects of water depth, soil temperature and density of aquatic earthworms on CH_4 flux by generalized linear model (GLM) which had smaller Akaike's an information criterion (AIC)

(P value<0.05 indicates significant value; 0.05-0.10=marginally significant)

Factors	P-value
Water depth	2e-16
Soil temperature	0.01
Aquatic earthworms	0.06

2.5 Summary

- In 2010, no tillage farming system marginally increased the earthworm density in paddy field.
- Organic fertilization also showed marginal significant effect to increase earthworm density in paddy field.
- In 2011, though any of farming practices showed did not show any significant effect on earthworm density, the cumulative earthworm density was higher in no tillage treated plots.
- Organic fertilizer was found to have marginal significant effect to increase earthworm density again in paddy field.
- The effects of earthworms on methane emission from paddy field were found context-dependent but cumulative methane flux was lower in no tillage plot treated with organic fertilizer where cumulative earthworm density was the highest.
- Organic fertilizer was found to increase methane flux marginally from paddy field.
- The results of this study suggest that long term study is necessary to get more clear idea about earthworms' activity on methane mitigation from paddy field and also for having idea about effects of farming practices on earthworm density.

Chapter III

Impacts of different farming practices on earthworm activities and methane emission in paddy fields of Nara Prefecture

3.1 Introduction

Global warming represents environmental problem which is involved with all living things in the earth. Carbon dioxide (CO_2), Methane (CH_4), and nitrous oxide (N_2O) are known as major greenhouse gases (GHGs). GHGs have own lifetime against tropospheric OH loss, therefore time scales are considered in the global warming potentials (GWPs) for different time intervals, i.e. 20, 100, or 500 years (IPCC, 2001). By volume, CH_4 is next to CO_2 but GWP of CH_4 is 34 times compared to CO_2 on 100 years time horizon (IPCC, 2013).

Agriculture is crucial for food but agricultural activities have impacts on the environment. Rice is one of the most important cereals in world and in Asia, around 136×10^6 ha area is under rice cultivation which is 88% of world's total to produce 556 Tg (Mt) rice which is 90% of world's total (FAO, 2005). Rice is cultivated in flooded paddy fields as reductive conditions help to flow out substances which are harmful to rice growth. Flooded paddy fields are considered as primary CH_4 emission source which contribute around 15-20% of total anthropogenic CH_4 emission (Aulakh *et al*., 2001).

In flooded paddy soils, molecular O_2 is trapped and quickly been consumed. As a result, sequential reduction of the soil oxidants (such as NO_3^-, Mn^{4+}, Fe^{3+}, SO_4^{2-}, and CO_2) progresses following the thermo-dynamic theory. Methane is produced as the final product of the reductive process by methanogens. The following reactions are dominant in methanogenesis process in submerged soil: reduction of CO_2 in presence of H_2 and the transmethylation of acetate (Takai, 1970 and Schutz *et al*., 1989). Methanotrophs consume part of the produced CH_4 under oxidative conditions in the rhizosphere zone of rice plants and in a thin layer of soil interfacing with the surface water. Most of the CH_4 is emitted to the atmosphere through aerenchyma of rice plants.

Different field management practices also have direct and indirect impacts on CH_4 emission in paddy field. Recently, no-tillage farming practice is being increased in some East Asian countries as no-tillage farming practice improves soil environment for crop production and preserves soil structure and enhances percolation in paddy soils (Hossain *et al*., 2000; Ota *et al*., 2002). Long-term no-tillage farming practice can lead to excessive compactness of soil surface (Hama and Anderson, 2005) along with weed spread (Turley *et al*., 2003). No-tillage farming practice in rice cultivation reduces fraction volume of large pores and ultimately decreases more than 50% CH_4 emission (Hanaki *et al*., 2002). Li *et al*. (2011) also stated that when bulk density is increased in no tillage treated paddy soil; CH_4 emission to atmosphere can be blocked and CH_4 produced in soil might be kept for long time, which may increase probability of CH_4 oxidation by methanotrophs. On the other hand, conventional tillage highly disturbs soil surface, alters soil properties and biochemical processes.

Aquatic earthworms belong to a dominant group of invertebrate fauna in the paddy field ecosystem, and this group is well known to maintain soil quality (Simpson *et al*., 1993). More particularly, aquatic earthworms in paddy soils have the potential to improve soil health and increase soil fertility and plant production (Yokota and Kaneko, 2002). In flooded soil, aquatic earthworms mix soil and move water by burrowing and passaging soil through their gut. The bioturbation activities of aquatic earthworms may enhance oxidation by increasing the depth of the oxidized layer at the soil surface. Thus, aquatic earthworms may play a role to mitigate CH_4 emissions from flooded paddy soil and may affect methane-consuming microbes, which play a vital role in global warming because they are a biological sink of CH_4. Research is needed to identify the role of aquatic earthworms in controlling CH_4 emissions. Different environmental factors and management practices are well known to affect CH_4 emissions from paddy soil, but little is known about the effects of aquatic earthworms on CH_4 emissions. No-tillage farming practice might enhance abundance of aquatic earthworms as in no-tillage system, soil surface disturbance is limited. But, according to Yachi *et al*., (2012) tillage may have minor effect on population of aquatic earthworms. Yokota and Kaneko (2002) showed no tillage farming practice along with legume mulch increased aquatic earthworm abundance but only no tillage without legume mulch there was no increase of aquatic earthworms.

Thus, this study was conducted with the following objectives:

1. To identify the effect of different farming practices on earthworm ecosystems in different paddy field environments.
2. To elucidate the effect of different farming practices on methane emission from different paddy field ecosystems.
3. To investigate the effect of aquatic earthworms on methanotrophs.

3.2 Materials and Methods

Land Preparation:

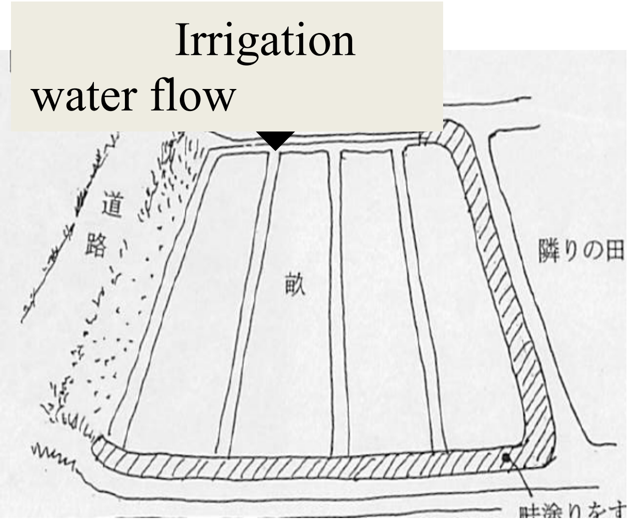
Field study was conducted in paddy fields of Makimuku, Nara city, Nara prefecture starting from May to September, 2012. Field study was held in three paddy fields. Among those two were conventional and another one was no-tillage paddy field (NT). Among two conventional paddy fields, one turned to conventional from organic (CVA) and another one was conventional (CVB).

Photo 6: Conventional paddy fields
(CVA at left and CVB at right)

The field preparation was started from late May by tilling the soil using machinery. Seedlings were transplanted in conventional fields on early June and 2-3 seedlings at 18 cm x 20 cm distances were planted per hill. In conventional paddy fields, chemical fertilizer (mixed fertilizer) was applied and herbicides were used for weeds. River water was used flood irrigation. Whereas, the no –tillage paddy field was kept untilled for last 33 years with weeds. In no-tillage field transplantation was done on late June and 1 seedling at 40 cm x 30 cm distances was planted per hill. No fertilizer was applied to no-tillage field and the weeds were cut manually and left on soil surface as mulch. A special feature of this no-tillage paddy field is there was a humus layer of around 5 cm above the soil layer. This layer provided the required nutrients to rice plants for proper growth instead of fertilizers.

Photo 7: No-tillage paddy field with weed mulch

Photo 8: Humus layer above soil layer in no-tillage paddy



In No-tillage field irrigation was applied 2-3 days after transplanting, then during mid-August and beginning of September. Irrigation water was not applied directly to the paddy surface but to the furrows constructed in paddy.

Soil sampling and aquatic earthworm collection:

Field study was started from May, 2012 and continued until September, 2012. During this time sampling was done three times i.e., once in two months (May, July and September). Soil sample was collected on July and September with a core sampler having 6 cm diameter, 11 cm depth and 28.26cm^2 surface areas. From each paddy field among three fields four samples were collected (in September, 3 samples). Soil samples were brought back to laboratory. Half of soil was used for measuring earthworm density and biomass and rest of the soil was used for further soil analysis. A 500 μm mesh sized sieve was used for sieving of earthworms and then density of earthworms was calculated.

Preparation of soil for analysis:

Plant debris, other macrofauna and stones were removed from the soil and soil samples were weighed. Some soil was stored at -25°C for microbial study and some soil was air dried in room temperature for one week. After air drying big soil clods were broken and put in oven for drying at 45°C for 48 hours. After oven drying the soil samples were again weighed and preserved in air tight packets.

Gas sample collection and methane flux measurement:

In each of paddy field from four sites gas samples were collected to measure methane flux by closed chamber method. Chambers having 0.6 m length, 0.5 m height and 0.3 m width were used for gas sample collection in this field study. The height of the chamber was changed to 1 m during sampling in September. Gas samples were collected once in two month starting from the month of May to September. First gas sampling was done before transplanting. Gas samples were collected by a 60 ml plastic syringe. First sample was taken at 0 minute that means just after placing of gas chamber on the plots. Then second sample at 10 minute and the last sample at 20 minute were taken. Gas samples were carried to laboratory by 45 ml glass bottles. During sampling, air temperature of inside of chamber was measured by placing Thermo Recorder (RT- 13, ESPEC MIC CORP.).

Methane concentration from gas samples was measured by Gas Chromatograph (GC-2014, SHIMADZU). Then methane flux was calculated by *flux* package in R (Jurasinski et al 2014).

Total C and N analysis:

Total C and N was of paddy soil of July was analyzed by CN corder (MACRO CORDER JM1000CN) using oven dried soil. Soil samples from no-tillage paddy were divided into two samples: upper 5 cm, the humus layer and lower soil sample.

Measurement of methanogenic bacterial community structure:

Phospholipid fatty acid (PLFA) analysis was conducted to assess biomass of methanotrophs in the second experiment. Lipid extraction was accomplished following a modified method of Frostegard *et al*., (1991) and Niwa *et al*., (2008). Lipids in 7-8 g fresh wet soil samples were extracted by theone-phase chloroform-methanol-phosphate buffer. After condensation of lipids, fractionation of phospholipid was carried out using silicic acid columns (BOND ELUT LRC-SI; Varian, Palo Alto, CA, USA) before separation of fatty acid methylesters from phospholipids following mild alkaline methanolysis. An internal standard, methyl non-adecanoate (19:0) was added to all samples. Fatty acid methylesters were identified by the Sherlock Microbial Identification System (MIDI, Newark, DE, USA). The fatty acids 18:1ω7c were used to estimate type II methane-oxidizing bacterial biomass, 16:1ω7c and 16:1ω5c were used to estimate type I methane-oxidizing bacterial biomass (Winden *et al*., 2010; Kip *et al*., 2011, Ruth *et al*., 2013; Zigah *et al*., 2015).

Statistical analysis

The heterogeneity and normality of data were determined with Bartlett's test and Shapiro-Wilk test, respectively. The main effects of tillage and aquatic earthworms were assessed by one-way analysis of variance (ANOVA). Statistical analyses were performed using R 3.2.3 for Microsoft Windows (R Development Core Team, 2015).

Results

Carbon and nitrogen content in paddy field

The percentage of C and N of conventional paddy soils and no-tillage lower soil were not significantly different (Fig. 17). But the percentage of C and N of upper layer of no-tillage plot significantly higher and it was 8 times higher than other plots.

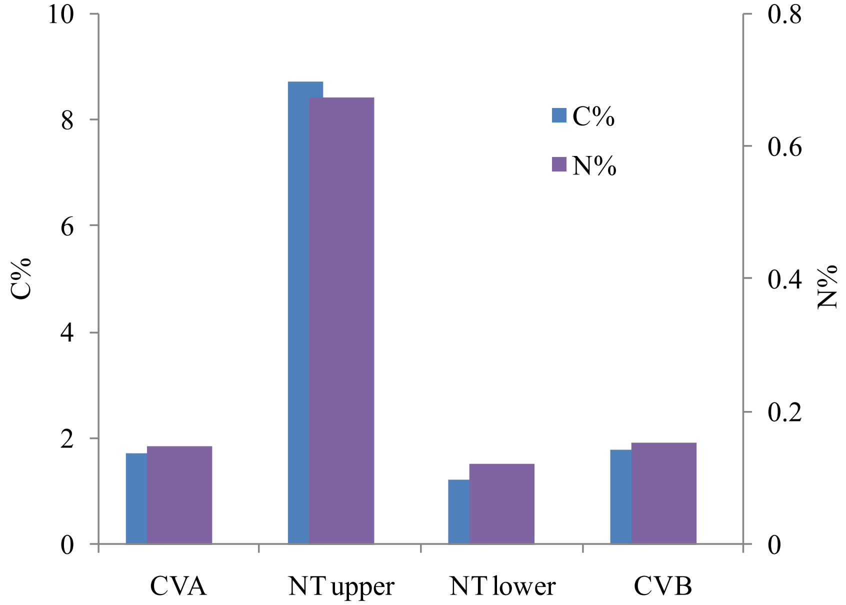


Figure 17: Percentages of C and N in different plots

Earthworm density

Earthworm density was measured on July and September. On July, the highest earthworm density was in CVB plot 2123/m^2 and the lowest earthworm density was found in NT plot 265/m^2 (Fig. 18). On September, the highest earthworm density was observed CVA plot 2123/m^2 and the lowest earthworm density was again in NT plot 236/m^2. Although there was no significant effect of tillage practices on earthworm density, NT plot had very few earthworms.

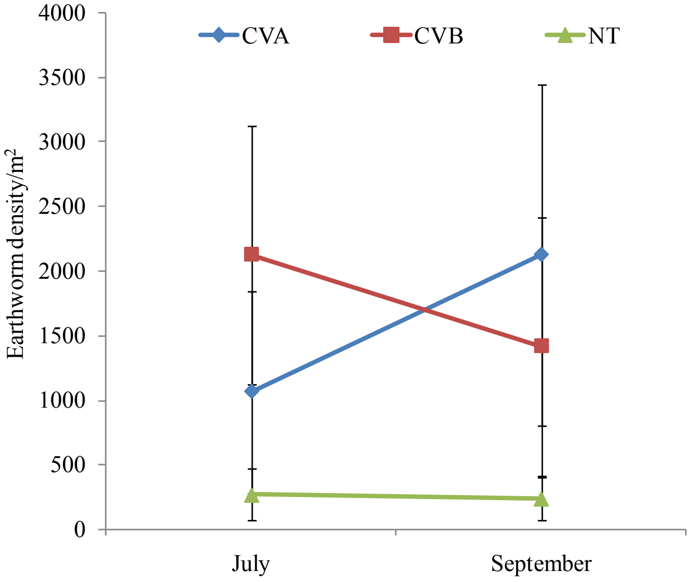


Figure 18: Temporal density of earthworm. Vertical bars show S.E. bars

Table 14: ANOVA (one-way) table for effects tillage practices on earthworm density (P>0.05 indicates insignificant effect)

Months	Conventional		No-tillage	
	F value	P value	F value	P value
July	0.09	0.76	2.54	0.14
September	1.47	0.27	0.70	0.43

Methane emission

The CH_4 flux was very low on May and on July CH_4 flux was increased in all plots (Fig. 19). On July, the highest CH_4 flux was 4.15 mg m^{-2} h^{-1} in CVA plot and in NT plot CH_4 flux was recorded very low 0.15 mg m^{-2} h^{-1}. On September, continuous increasing trend of CH_4 flux was seen. The highest CH_4 flux reached to 6.51 mg m^{-2} h^{-1} in CVA plot and the lowest CH_4 flux was recorded 1.25 mg m^{-2} h^{-1} in NT plot. After statistical analysis, none of tillage practices were found to affect

CH_4 flux on May. But on July, both conventional and no-tillage farming practices were found to affect CH_4 flux. Lastly, on September, no-tillage farming practice was found to reduce CH_4 flux from paddy field. The CH_4 emission of 116 days was highest in CVA plot 73.04 mg m^{-2} day^{-1} and in NT plot cumulative CH4 emission was very low 6.28 mg m^{-2} day^{-1} (Fig. 20).

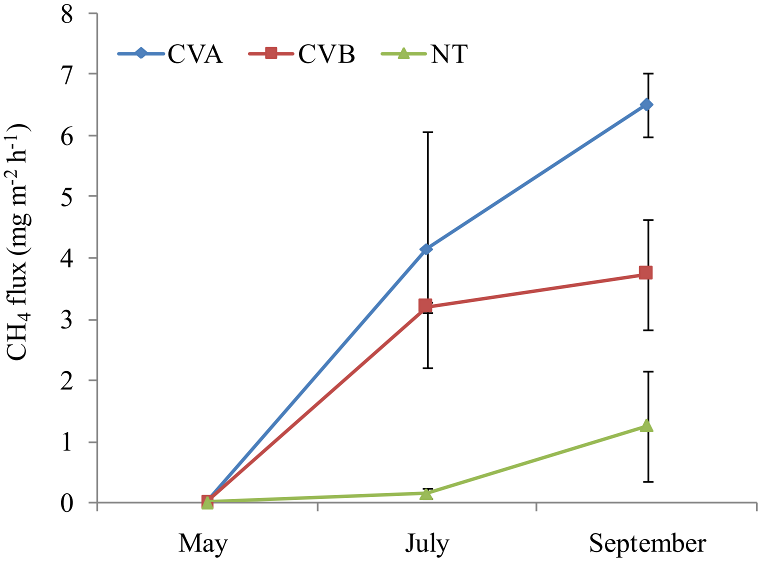


Figure 19: Influence of different treatments on CH_4 flux. Vertical bars show S.E.bars

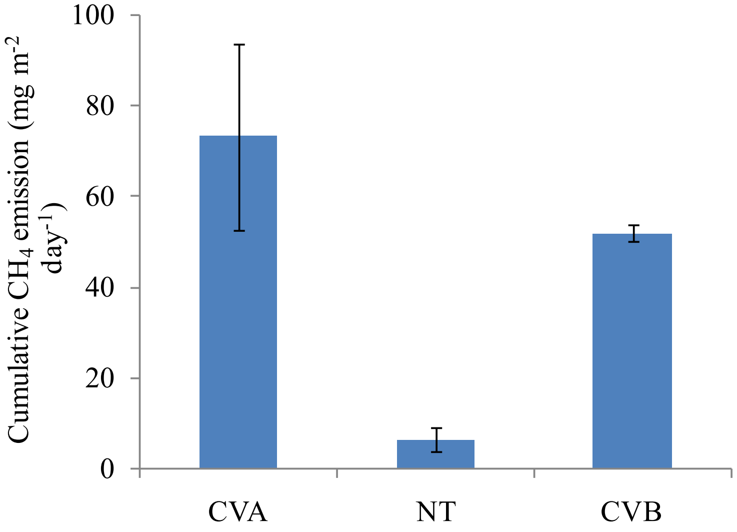


Figure 20: Influence of different treatments on CH_4 emission. Vertical bars show S.E. bars.

Table 15: ANOVA (one-way) table for effect of farming practices on CH4 flux (P>0.05 indicates insignificant effect)

Months	Conventional		No-tillage	
	F value	P value	F value	P value
May	0.24	0.63	0.62	0.44
July	9.85	0.01	11.45	0.008
September	3.75	0.10	19.99	0.004

Biomass of methanotrophs

Biomass of methanotrophs was highest in NT plot and lowest in CVA plot (Fig. 21). Biomass of methanotrophs in NT plot was 40 nmol/g and in CVA was 11 nmol/g. Biomass of fungi was also very high in NT plot and it was 2003 nmol/g and very low in CVB plot 8 nmol/g. After statistical analysis, NT farming practice was found to enhance biomass of methanotrophs (P<0.01).

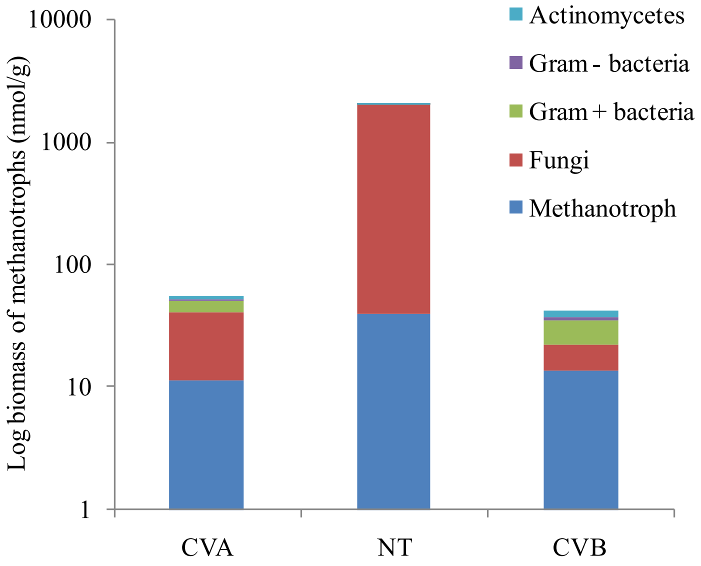


Figure 21: PLFA profile of microorganisms in paddy soil

Discussions

Effect of farming practices on earthworm density

Although it was hypothesized that no-tillage farming practice will enhance density of earthworms as no-tillage practice disturbs the soil surface less but in this no significant effect of farming practices on earthworm density was found. More particularly, in NT plot earthworm density was very low. Because, in NT plot limited irrigation water was applied in furrows and irrigation was done only after transplantation, middle of August and early September. Except these periods paddy field was mostly dry and Stenert *et al*., (2009) reported that absence of irrigation water reduces the density of aquatic invertebrate as those are not highly tolerant to drought. Therefore, low density of earthworms was found in NT paddy field.

Effect of farming practices on CH_4 emission and biomass of methanotrophs

In this field study, no-tillage farming practice reduced CH_4 emission and enhanced biomass of methanotrophs very clearly. In previous other studies also stated that no-tillage practice can reduce CH_4 emission by increasing bulk density of soil (Hanaki *et al*., 2002; Ali *et al*., 2009; Ahmad *et al*., 2009). But during this study, NT plot was not highly anaerobic. Highly anaerobic condition is only responsible to produce CH_4 from flooded paddy field. Cai *et al*. (1997) indicated that intermittent irrigation in paddy field significantly depresses CH_4 emission. In this study, not only no-tillage farming practice but also water management was responsible for mitigating CH_4 emission from paddy field.

Correlation among CH_4 emission, earthworm density and biomass of methanotrophs

As earthworm density was very low due to different management practices, no correlation was observed between earthworm density and CH_4 flux. But biomass of methanotrophs clearly reduced CH_4 flux (Fig. 22).

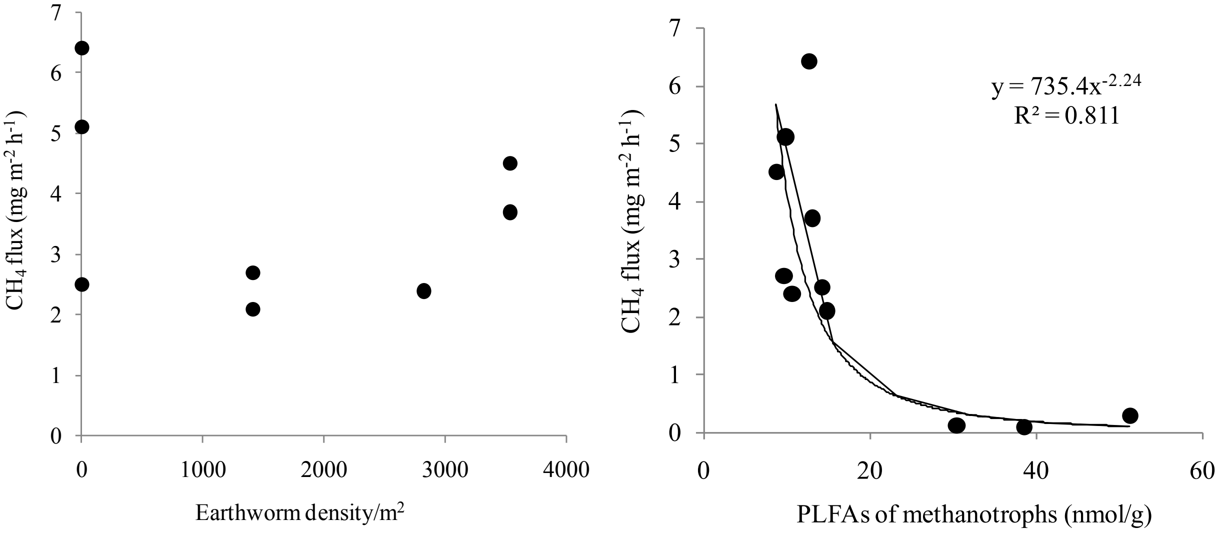


Figure 22: Correlation among earthworm density CH_4 flux and biomass of methanotrophs

3.5 Summary

Lower earthworm population was found from no tillage plot compared to conventional paddy fields due to lower moisture content of no tillage paddy field. Methane emission from no tillage paddy was considerably lower than conventional paddies and biomass of methanotrophs was higher noticeably in no tillage paddy field. No tillage farming practice along with water management was beneficial in terms of CH_4 emission from paddy field.

Chapter IV

Impacts of fertilization and aquatic earthworm activities on methane emission from paddy soil

4.1 Background

The Paris Agreement has set a target to limit global warming to well below a 2°C (UNFCCC, 2015). Diversified efforts and actions to reduce emissions of major greenhouse gases (GHGs) are critical to achieve this ambitious but important target. Methane (CH_4) is one of the most efficient GHGs, and its contribution to the greenhouse effect is almost half of that of carbon dioxide (CO_2) (Gavin, 2004). The atmospheric concentration of CH_4 is 1.8 ppm, much lower than that of CO_2 (399 ppm) (Blasing, 2016). However, the global warming potential of CH_4 is about 25 (IPCC, 2007), which makes it harmful to the environment. A recent report by Blasing (2016) showed that the atmospheric CH_4 concentration has more than doubled over the last 200 years. Methane in the atmosphere originates from both natural and anthropogenic sources, which contribute approximately 64% and 36% of atmospheric CH_4, respectively (Bousquet *et al*., 2006). Among the various CH_4 sources, flooded rice cultivation is responsible for 11% of total anthropogenic methane (Smith *et al*., 2007).

Rice is generally cultivated in flooded paddy fields as the reducing conditions decrease the amount of substances that are harmful to rice growth. Generally, there is 10-20 cm deep water in paddy fields during the whole cropping period, except for the drying period. Therefore, the oxygen supply from the atmosphere to the soil is limited during the cropping period. Thus, organic matter ferments in anaerobic conditions, and the activity of methanogens results in the production of CH_4 as an end product. The major methanogenic substrates are H_2+CO_2, formate, acetate, methyl alcohol, and methylamine. The dominant reactions of methanogenesis in soil are the reduction of CO_2 using H_2 and the transmethylation of acetate (Takai, 1970).

During methanogenesis, carbon substrates are supplied to soil from soil organic matter, sloughed tissues of rice plants, applied organic matter, and root exudates (Kimura *et al.*, 2004; Watanabe *et al.*, 1999). Methanotrophs consume some of the CH_4 produced under oxidative conditions in the rhizosphere of rice plants and also in the thin layer where the soil and surface water meet (Dubey, 2005). The rest of the produced CH_4 is released from paddy fields into the atmosphere via diffusion, ebullition from soil, and by diffusion and mass flow through the continuous intercellular gas space system between the rice rhizosphere and leaves. The physiological structure of rice plants favors greater emission of CH_4. The intercellular channels through the aerenchyma connecting the rhizosphere to the leaves function as a path of gas exchange between the root zone and atmosphere via stomata in the leaves and micropores in the leaf sheaths. In submerged paddy fields, CH_4 diffuses from the roots and is transported to higher parts of the plant via intercellular channels. It is then released into the atmosphere through stomata and micropores (Nouchi *et al.*, 1990).

During last two to three decades, farming practices and management have become more intensive in many rice-producing countries (Parsons *et al.*, 2010). Because of recent socio-economic changes, agricultural fields are being abandoned in Southeast and East Asian countries including Japan, Korea, and Vietnam (Kubo, 2013). Japan has a 3000-year history of rice cultivation. Most of the rice produced in Japan is grown using commercial herbicides and pesticides to reduce labor and working hours, since organic farming needs higher labor inputs (e.g., weeding). Although the herbicides used in paddy fields are far milder than before, they can be non-specific and kill non-target species (Simpson and Roger, 1995). Modern cultivation practices have increased the yield of rice by around 60.7% per unit area from 1946 to 2012 (MAFF, 1970-2003, 2004-2007, 2008-2010) but biodiversity in paddy fields has decreased. Environmentally friendly conservation management practices have the potential to enrich biodiversity. In Japan, there is a law to promote sustainable agricultural production practices (MAFF, 2014). Kitazawa *et al*. (2011) reported higher species richness and invertebrate diversity in organic rice fields in Japan, compared with conventionally managed fields.

Both chemical and organic fertilizers strongly affect CH_4 emissions from paddy field soil (Yagi and Minami, 1990). Organic fertilizer is well known to conserve soil fertility. In Japan, it has been recommended to combine organic fertilizer with chemical fertilizer, as the former releases organic nitrogen very slowly during decomposition (Minamikawa and Sakai, 2006). Chemical fertilizers such as nitrogen fertilizers are fundamentally important for crop growth and yield. Urea and ammonium sulfate are two of the most commonly used inorganic nitrogen fertilizers for lowland rice (Fageria *et al*., 2003). Sulfate-containing fertilizers suppress CH_4 emissions because they result in competition between sulfate-reducing bacteria and methanogenic archaea for substrates like hydrogen and acetate (Hori *et al*., 1990). Several studies have demonstrated that ammonium sulfate decreases CH_4 emissions from soil (Schutz *et al*., 1989, Hori *et al*., 1990, Banik *et al*., 1996). However, the effects of urea on CH_4 emissions from soil are context-dependent. In some studies, CH_4 emissions increased with increasing urea application rates, soil pH increased because of urea hydrolysis (Wang *et al*., 1992), and ammonium inhibited methanotrophs (Dubey *et al*., 2003). In other studies, ammonium enhanced oxidation of CH_4 and the activities of methanotrophs (Bodelier *et al*., 2000).

Aquatic earthworms are a major group of invertebrate fauna in the paddy field ecosystem, and are well known to maintain soil quality (Simpson *et al*., 1993). More particularly, aquatic earthworms in paddy soils have the potential to improve soil health and increase soil fertility and plant production (Yokota and Kaneko, 2002). In flooded soil, aquatic earthworms (maximum length: 4–5 cm) mix soil and move water by burrowing and passaging soil through their gut. The bioturbation activities of aquatic earthworms may enhance oxidation by increasing the depth of the oxidized layer at the soil surface. Thus, aquatic earthworms may play a role to mitigate CH_4 emissions from flooded paddy soil and may affect methane-consuming microbes, which play a vital role in global warming because they are a biological sink of CH_4. Research is needed to identify the role of aquatic earthworms in controlling CH_4 emissions. Different environmental factors and management practices are well known to affect CH_4 emissions from paddy soil, but little is known about the effects of aquatic earthworms on CH_4 emissions.

In this study, we investigated the effects of aquatic earthworms on the biomass of methanotrophs in paddy soil, and on CH_4 flux from paddy soil. We hypothesized that O_2 penetration through bioturbation by aquatic earthworms will enhance the biomass of methanotrophs, ultimately leading to a decrease in CH_4 emissions.

4.2 Materials and Methods

Fertilizer experiment

The laboratory study was conducted in 2015. Soil for this study was collected on 10th May, 2013, from a paddy field in Kamakura, Kanagawa (35°20′ N, 139°31′E, 56 m above sea level). The soil contained 40% sand, 58% silt, and 2% clay. The soil was sun-dried, plant debris and stones were removed, and then the soil was sieved to achieve a homogenous texture. The homogenous soil was stored in a plastic house until use. Four weeks before starting the study, water was added to soil to make the soil conditions anaerobic. Three treatments were established: i) soil + urea ($CO(NH_2)_2$), ii) soil + ammonium sulfate ($(NH_4)_2SO_4$), and iii) soil with no fertilizer (control), with four replicates per treatment. Each treatment consisted of 100 g soil (dry weight) and 120 ml pure water in a glass bottle (5 cm diameter, 11 cm height). For the soil + fertilizer treatments, granular urea (0.046 g urea; 46% N) or 0.104 g ammonium sulfate (20.5% N) equivalent to 90 kg N/ha was mixed with soil. Rice straw powder (2500 kg/ha) was added as organic matter to each soil treatment and the soil mixture was mixed gently using a spoon. The bottles containing the various soil treatments were placed in an incubator and kept at 25°C under a 14-h light/10-h dark photoperiod for 28 days.

Aquatic earthworm experiment

Water was added to dry paddy soil before adding aquatic earthworms. After 8 weeks of maintaining ananaerobic conditions in paddy soil, aquatic earthworms (*Branchiura sowerbyi*; Beddard, 1892) were collected by sieving the soil through a 500-µm mesh sieve. Then they were kept in deionized water for 48 h to excrete intestinal residues. Then, the live earthworms were placed on filter paper to remove extra water from the body, and each earthworm was weighed and biomass was recorded. Soil samples were prepared as described in section 2.1, using urea as the fertilizer. All the soil samples contained rice straw powder as described in section 2.1. Two factors were considered in two levels; i) presence or absence of aquatic earthworms (EW and NW, respectively), and ii) presence or absence of chemical fertilizer (UR and NU, respectively), with six replications per treatment (i.e. 24 bottles in total). The bottles containing soil were kept in an incubator at 25°C under a 14-h light/10-h dark photoperiod for 35 days. After 7 days of pre-incubation, 6 aquatic earthworms (2525/m^2) were added to each bottle. At the end of the incubation period, aquatic earthworms were collected from the soil in the bottles by sieving through a 500-µm mesh size sieve and the numbers and biomass of earthworms were recorded.

Gas sampling and methane measurement

Gas samples were collected once per week starting from Day 0. The first sample was collected 2 h after preparing soil. To eliminate the bubbling effect, bottles were placed in the sampling area 30 min before sampling. Then, the mouth of bottles was closed with plastic leads with rubber septa. The first gas sample was collected 10 min after closing the bottles, and then after 20 and 30 minutes with a plastic disposable 50 ml syringe. Gas samples were collected into 30-ml glass vials. Once per week, after gas sampling, the water in the glass bottles was replaced with fresh deionized water. The methane concentration was measured using a gas chromatograph (GC-2014, SHIMADZU, Japan) and CH_4 flux was calculated by using the *flux* package in R (Jurasinski *et al*., 2014).

Soil Sampling

Soil samples were collected after the final gas sampling and oven-dried at 40°C for 48 h for measuring total C and N.

Total C and N analysis

About 1500 mg dried soil from each treatment was used to measure total C and N using a CN Macro Corder (JM1000CN, J-Science Lab. Co. Ltd., Kyoto, Japan).

Soil pH and Eh

Once per week starting from Day 0 after gas sample collection, soil pH and Eh were measured around 1–5 cm depth. Soil pH was measured using a pH/COND Meter D-54 instrument (Horiba, Kyoto, Japan) and soil Eh was measured using a pH/ORP/DO Meter D-75 instrument (Horiba).

2.7. Analysis of biomass of methanotrophs

A phospholipid fatty acid (PLFA) analysis was conducted to determine the biomass of methanotrophs in the aquatic earthworm experiment. Lipids were extracted using a method modified from Frostegard *et al*., (1991) and Niwa *et al*., (2008). Lipids were extracted from 7–8 g fresh wet soil samples in a one-phase chloroform-methanol-phosphate buffer. After condensation of lipids, phospholipids were fractionated using a silicic acid column (BOND ELUT LRC-SI; Varian, Palo Alto, CA, USA) and then fatty acid methylesters were separated from phospholipids after mild alkaline methanolysis. An internal standard, methyl nonadecanoate (19:0), was added to all samples. Fatty acid methylesters were identified using the Sherlock Microbial Identification System (MIDI, Newark, DE, USA). The fatty acid 18:1ω7c was used to estimate the biomass of type II methane-oxidizing bacteria, and 16:1ω7c and 16:1ω5c fatty acids were used to estimate the biomass of type I methane-oxidizing bacteria (Winden *et al*., 2010; Kip *et al*., 2011; Ruth *et al*., 2013; Zigah *et al*., 2015).

Statistical analysis

The heterogeneity and normality of data were determined with Bartlett's test and Shapiro-Wilk test, respectively. When required, data were log-transformed to meet the assumptions of normality. The main and interaction effects of fertilizers and aquatic earthworms were assessed by one-way and two-way analysis of variance (ANOVA). Statistical analyses were performed using R 3.2.3 for Microsoft Windows (R Development Core Team, 2015).

4.3Results

Fertilization experiment

Soil environment

The properties of the soil in each treatment are shown in Tables 16 and 17. There was no significant difference in soil Eh, soil pH, or soil total C and N among the treatments. The soil Eh values were higher at day 0 but decreased over time as the soils became more anaerobic.

Table 16: Properties of the studied soil during fertilization experiment

	Eh (mV)			pH		
	Urea	$(NH_4)_2SO_4$	Control	Urea	$(NH_4)_2SO_4$	Control
Day 0	-47	-60	-51	7.14	7.19	7.2
Day 7	-218	-219	-219	7.23	7.27	7.29
Day 14	-244	-256	-253	7.54	7.54	7.56
Day 21	-275	-286	-284	7.55	7.56	7.55
Day 28	-270	-267	-260	7.47	7.46	7.48

Table 17: Total C and total N percentage of studied soil and cumulative CH_4 emission after 4 weeks of incubation during fertilization experiment

Treatments	Soil total C (%)	Soil total N (%)	CH_4 emission (g m^{-2} d^{-1})
Urea	1.85	0.18	21.65
$(NH_4)_2SO_4$	2.01	0.19	0.0045
Control	2.02	0.17	0.19

Chemical fertilization and CH_4 emission

During the 28-day incubation period, the CH_4 flux from soil was lowest at the start of the incubation period, and then increased rapidly in soils containing urea and ammonium sulfate. In soil containing urea, CH_4 flux started to increase after 5 days of incubation and increased until day 21, when the average CH_4 flux was 2091 mg m^{-2} h^{-1}. The CH_4 flux then rapidly declined (Fig. 23). In soil containing ammonium sulfate, CH_4 flux increased after 5 days of incubation. The CH_4 flux was highest at 14 days, and then it declined gradually. The effect of urea to accelerate CH_4 flux was highly significant. The cumulative CH_4 emission rate in the urea-treated soil was 21.7 g m^{-2} day^{-1}, compared with 0.0045 g m^{-2} day^{-1}in ammonium sulfate-treated soil (Table 17).

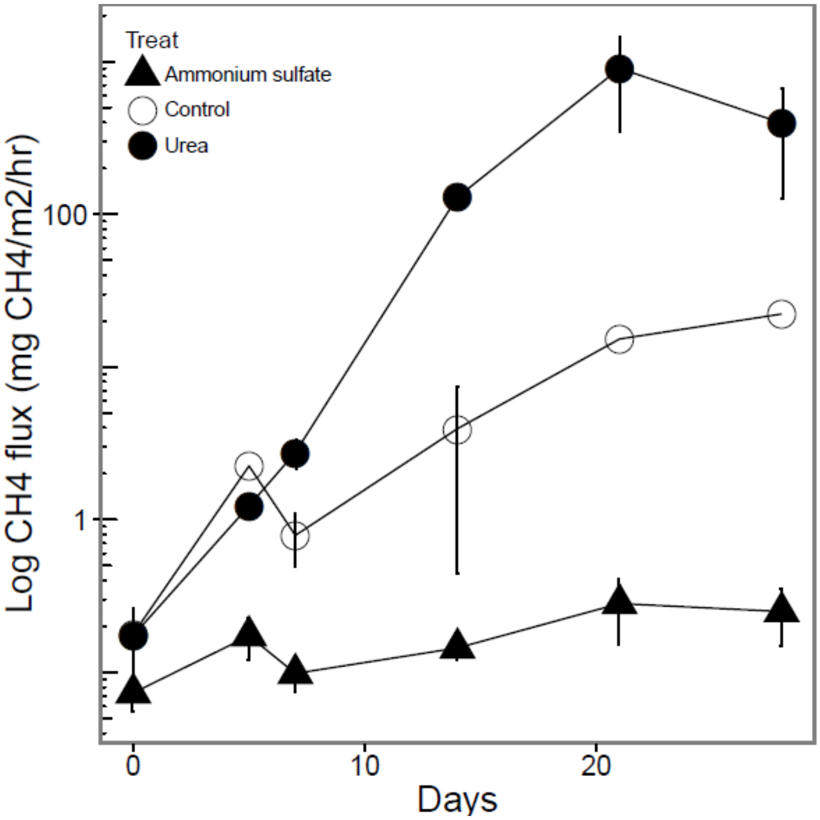


Figure 23: Influence of different treatments on CH_4 flux. Vertical bars indicate S.E. (n=4)

Table 18: ANOVA table for effect of fertilization on CH_4 flux. (P value<0.05 indicates significant value)

Days	Urea		Ammonium sulfate	
	F value	P value	F value	P value
Day 0	0.49	0.51	8.05	0.02
Day 7	45.51	0.0002	1.62	0.24
Day 14	171.57	0.0001	0.005	0.95
Day 21	9.8	0.01	0.00	0.01
Day 28	7.87	0.02	0.00	0.99

Aquatic earthworm experiment

Soil environment

The soil Eh value was higher at 1-cm depth than at 5-cm depth (Table 19). After 7 days of incubation, the soil Eh at 5-cm depth was very low remained low throughout the incubation period. The soil Eh values at 1-cm depth showed no noticeable difference throughout the incubation period. There were no significant differences in soil pH and soil total C and N among the treatments (Table 20).

Table 19: Soil Eh at different depth during earthworm experiment

Treatment	Eh at 1 cm depth (mV) Days						Eh at 5 cm depth (mV) Days						Cumulative CH4 emission ($g\ m^{-2}\ d^{-1}$)
	0	7	14	21	28	35	0	7	14	21	28	35	
EW UR	-38	-45	-51	-53	-57	-60	-41	-258	-240	-235	-252	-242	3.2
EW NU	-35	-45	-48	-50	-57	-62	-38	-252	-247	-232	-246	-237	4.38
NW UR	-35	-44	-54	-58	-64	-67	-37	-240	-230	-230	-254	-249	18.01
NW NU	-38	-48	-56	-62	-66	-73	-40	-244	-236	-228	-247	-245	17.98

Table 20: Soil properties during earthworm experiment

	pH				Soil total C (%)				Soil total N (%)			
Days	EW UR	EW NU	NW UR	NW NU	EW UR	EW NU	NW UR	NW NU	EW UR	EW NU	NW UR	NW NU
0	7.16	7.17	7.17	7.21	-	-	-	-	-	-	-	-
7	7.44	7.48	7.44	7.47	-	-	-	-	-	-	-	-
14	7.47	7.41	7.35	7.31	-	-	-	-	-	-	-	-
21	7.48	7.42	7.39	7.35	-	-	-	-	-	-	-	-
28	7.61	7.56	7.52	7.52	-	-	-	-	-	-	-	-
35	7.51	7.42	7.29	7.29	1.89	1.84	1.84	1.95	0.14	0.14	0.16	0.17

Relationship among aquatic earthworms, urea, and CH_4 emissions

During the 35-day incubation period, the CH_4 flux started to increase considerably after 7 days (Fig. 24). The CH_4 flux peaked at 28 days and then decreased. The effects of aquatic earthworms on CH_4 flux were evident from day 14 to the end of the incubation period. On day 14, CH_4 flux was lower in soil containing earthworms ($P<0.05$) than in soil without earthworms.

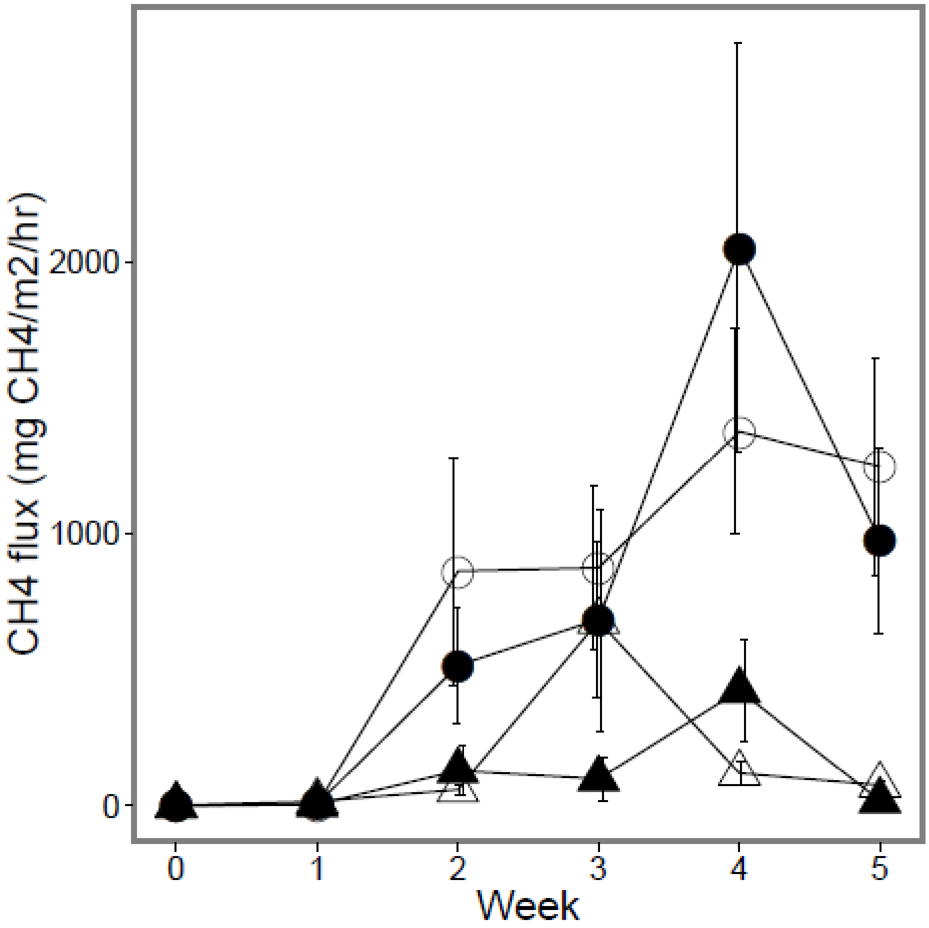


Figure 24: Influence of treatments on CH_4 flux. Filled circle indicates NW NU, open circle NW NU, filled triangle EW UR and open triangle EW NU respectively. Vertical bars indicate standard errors (n=6).

Table 21: ANOVA table for effect of urea fertilization and aquatic earthworms on CH_4 flux. (P value>0.05 indicates insignificant value; 0.05-0.10=marginally significant)

Days	Urea		Earthworm	
	F value	P value	F value	P value
Day 0	2.98	0.09	1.17	0.29
Day 7	0.89	0.35	1.64	0.21
Day 14	0.38	0.54	7.06	0.01
Day 21	1.97	0.17	2.03	0.16
Day 28	1.35	0.25	11.67	0.002
Day 35	0.43	0.51	18.17	0.0003

On day 28 when the CH_4 flux was highest, the effect of aquatic earthworms on CH_4 flux was highly significant. Aquatic earthworms also suppressed CH_4 flux significantly at the end of the incubation period. During the incubation period, there was no significant effect of urea on CH_4 flux, but the presence of urea increased the abundance of aquatic earthworms. The highest cumulative CH_4 emission was in NW-UR (18.0 g m^{-2} day^{-1}) and the lowest was in EW-UR (3.2 g m^{-2} day^{-1}; Table 19). Aquatic earthworms significantly mitigated CH_4 emissions after 35 days of incubation.

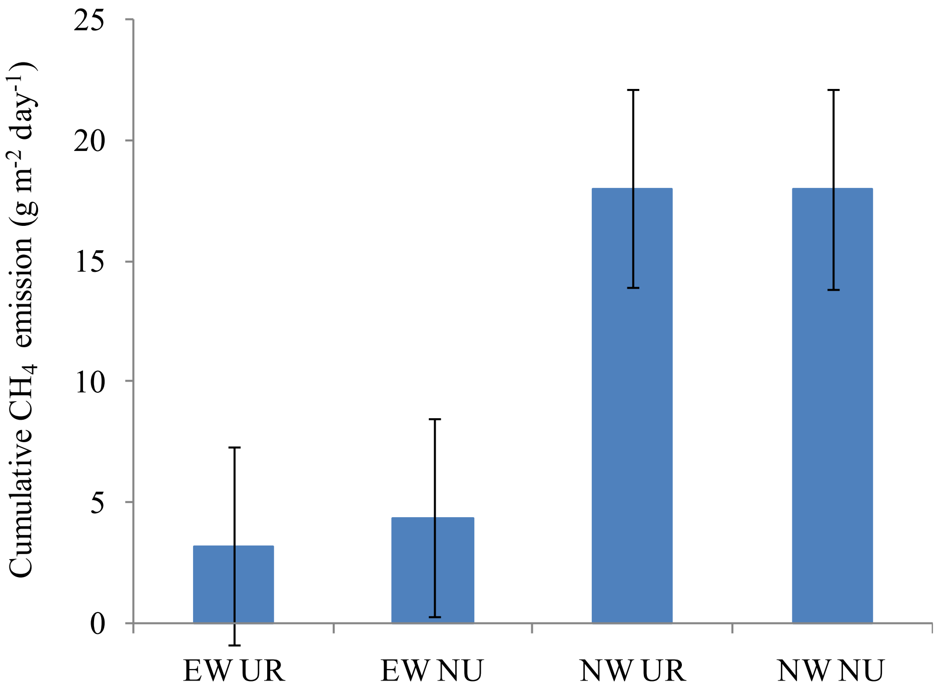


Figure 25: Influence of different treatments on cumulative CH_4 emission. Vertical bars indicate S.E.

With application of urea fertilizer, density of earthworms was increased to 5471 m^{-2} and the density of earthworms clearly mitigated CH_4 flux (Fig. 26).

When the aquatic earthworm density was 1684 m^{-2}, CH_4 flux was 116 mg m^{-2} h^{-1} but when the aquatic earthworm density was 5471 m^{-2}, CH_4 flux was only 5.32 mg m^{-2} h^{-1} on day 35 (Fig. 27). Increased aquatic earthworm density was related to decreased CH_4 flux.

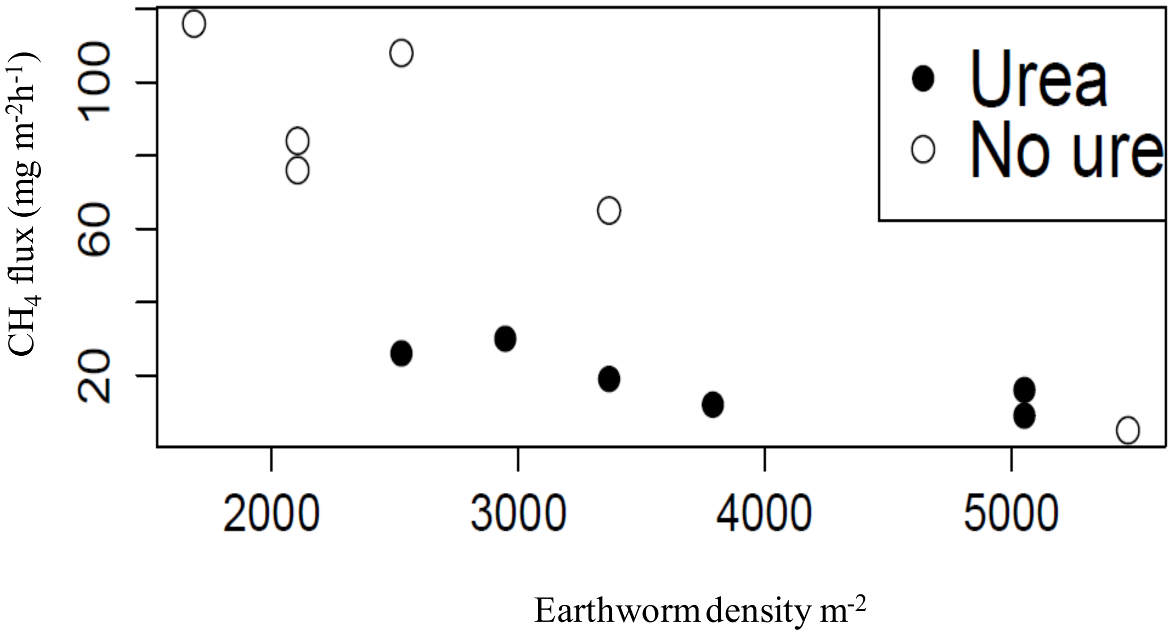


Figure 26: Relationship among earthworm density, urea application and CH_4 flux

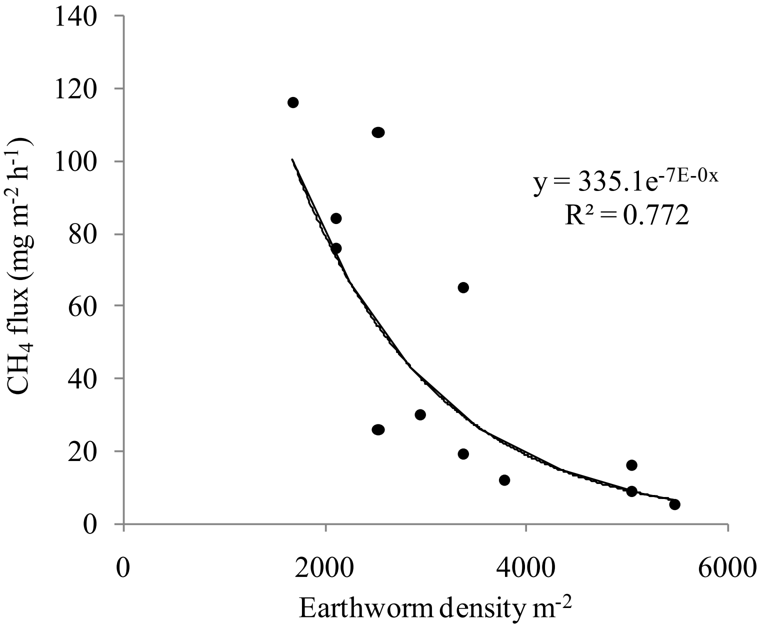


Figure 27: Correlation between earthworm density and CH_4 flux after 35 days of incubation in aquatic earthworm experiment

Aquatic earthworms and biomass of methanotrophs

The amount of PLFAs was compared among treatments to estimate the biomass of methanotrophs (Fig. 28). The highest biomass of methanotrophs, based on the PLFAs content, was detected in the EW-NU and EW-UR treatments (13.8 and 13.5 nmol g^{-1}, respectively) and the lowest biomass of PLFAs of methanotrophs was in the NW-NU treatment (5.61 nmol g^{-1}).

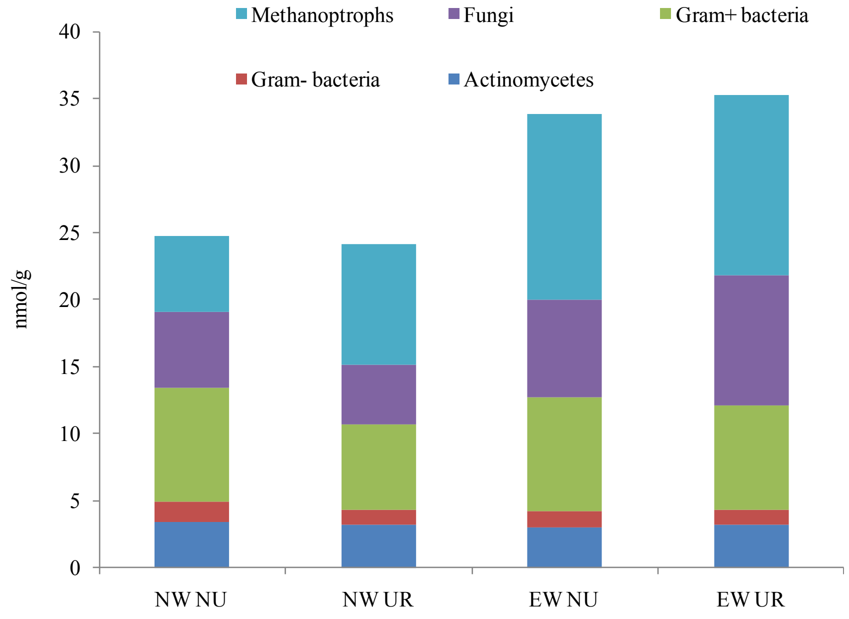


Figure 28: PLFA profile of microorganisms in paddy soil after 5 weeks of incubation

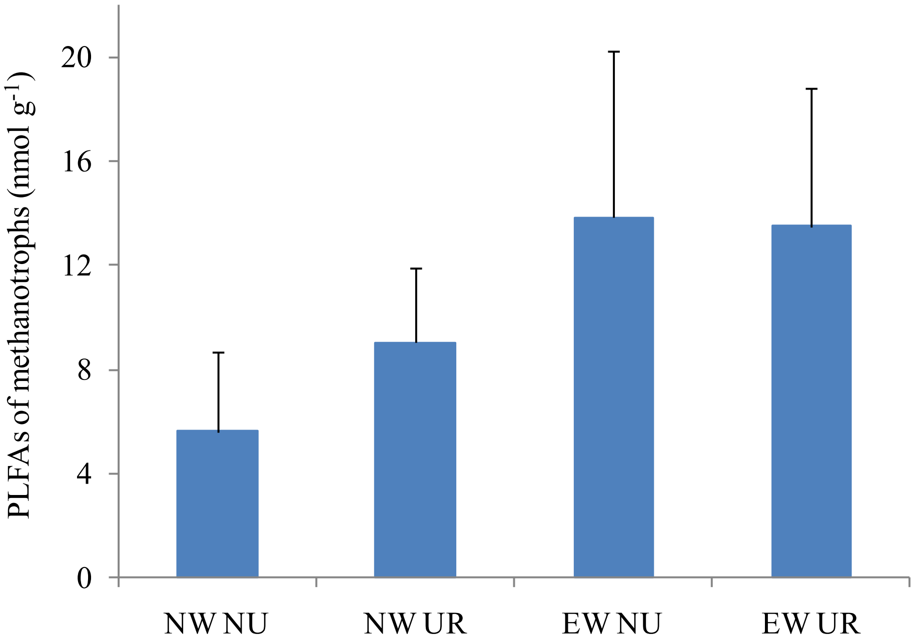


Figure 29: Biomass of methanotrophs in different treatments. Vertical bars show standard errors (n=6)

Table 22: ANOVA table for effect of urea and aquatic earthworms on biomass of methanotrophs. (P value>0.05 indicates insignificant value; 0.05-0.10=marginally significant)

Factors	F value	P value
Urea	0.67	0.42
Earthworms	11.12	0.003

The results of the ANOVA confirmed that the presence of aquatic earthworms increased the biomass of methanotrophs. Urea did not affect the biomass of methanotrophs. Methanotrophs considerably reduced the CH_4 flux from soil (Fig. 30) i.e., an increase in the biomass of methanotrophs was associated with a decrease in CH_4 flux. The presence of aquatic earthworms decreased CH_4 flux, and increased the biomass of methanotrophs (Fig. 31).

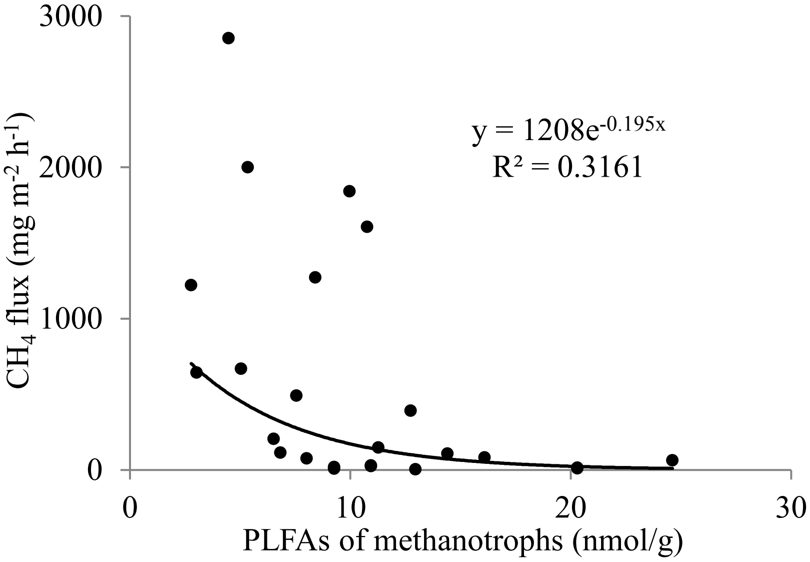


Figure 30: Correlation between biomass of methanotrophs and CH_4 flux

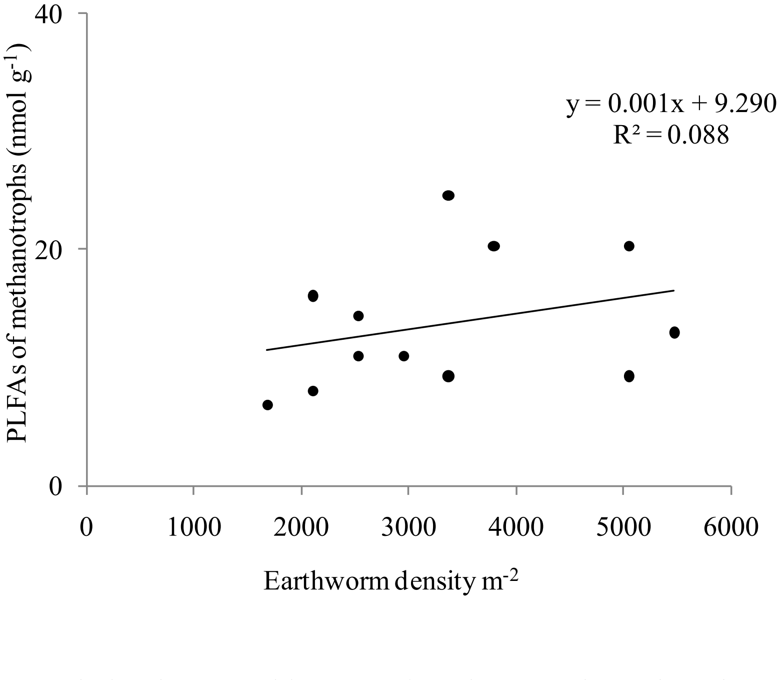


Figure 31: Correlation between biomass of methanotrophs and earthworm density

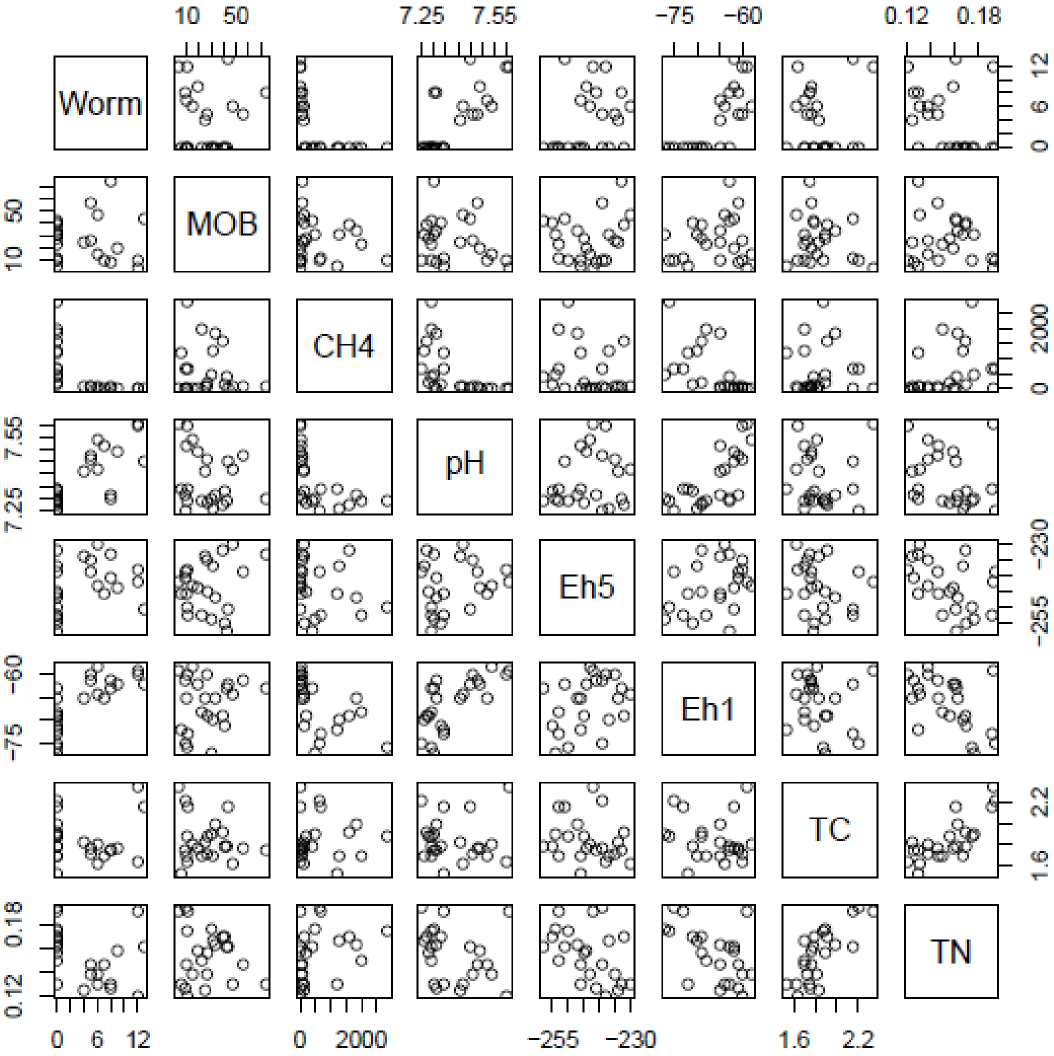


Figure 32: Combined effect of all environmental factors

4.4 Discussions

Effect of chemical fertilizers on CH_4 emissions

Our results showed that adding urea to soil resulted in higher CH_4 emissions from submerged soil. Similar results have been reported in previous studies (Banik *et al*., 1996, Dubey, 2003, Wang *et al*., 1992, Yang and Chang, 1997). A previous study reported a direct relationship between CH_4 production and lower Eh values/higher pH (Wang *et al*., 1992) in flooded paddy soil. After urea is applied to paddy soil, it is hydrolyzed by microbial ureases, resulting in an alkaline soil pH and lowers oil Eh. In the present study, CH_4 flux from ammonium sulfate-treated soil was lower than that from control soil. Previous studies have shown that sulfate-containing fertilizers can decrease CH_4 emissions (Banik *et al*., 1996, Wang *et al*., 1992) because of the competition between sulfate-reducing bacteria and methanogens for their common substrates (e.g.,hydrogen, acetate) (Hori *et al*., 1990, Westermann and Ahring, 1987). Urea was shown to increase the abundance of aquatic earthworms in this study. Mineral N has been shown to stimulate the aquatic earthworm population (Simpson *et al*., 1993). Also, higher N availability increases the growth of photosynthetic aquatic organisms, including microorganisms that are food sources for aquatic earthworms.

Role of aquatic earthworms in mitigating CH_4 emissions from paddy soil

In this study, aquatic earthworms increased the biomass of methanotrophs, and reduced CH_4 emissions from paddy soil. Methanotrophs intercept and then oxidize the CH_4 produced in paddy soil as it escapes into the atmosphere via aerobic interfaces (Dubey, 2005). Aquatic earthworms are ‘conveyor belt’-type feeders (Rhoads, 1974). For example, the head of *B. Sowerbyi* is buried in the soil for feeding while its tail remains on the surface for respiration. Through this bioturbation activity, *B. sowerbyi* is able to introduce O_2-rich water into the lower soil layer, and it also produces a layer of faecal pellets on the soil surface. This soil habitat modification led to an increase in the biomass of methanotrophs, which accelerated the oxidation of CH_4 produced by methanogens. However, contrasting results were reported for the tropical earthworm *Pheretima* sp. (John *et al*. 2015). This species increased CH_4 emissions from flooded soil by bioturbation where methanotrophs were not able to consume CH_4. Since the earthworm used in this study is an aquatic species, we assume that aquatic earthworms are more likely to decrease CH_4 emissions from flooded paddy soil.

Methane reduction effects as compared with water management

Standing water on the soil surface is the cause of CH_4 emissions as gas exchange between soil and atmosphere becomes limited. The fundamental requirement for methanogenesis to occur is that the soil should have sufficient humidity to develop a totally anaerobic environment. Intermittent irrigation and mid-season drainage are common water management practices in Japan to control tillering and to allow O_2 to reach roots of rice plants to prevent sulfide toxicity (Kanno *et al*., 1997). These water management practices are among the most effective ways to reduce CH_4 emissions because they prevent reducing conditions developing in the soil. One or several drainage systems were shown to reduce CH_4 emissions, compared with continuous flooding in field conditions (Nishimura *et al*., 2004) and pot experiments (Minamikawa *et al*., 2005). A single mid-season drainage decreased CH_4 emissions by 50% (Kimura *et al*., 1992). Therefore, water management, especially to control Eh, has been proposed to control CH_4 emissions (Minamikawa *et al*., 2005).

On the other hand, drainage increases N_2O emissions (Bronson *et al*., 1997, Chen *et al*., 1997) through nitrification and denitrification occurring simultaneously (Suratno *et al*., 1998). One molecule of N_2O can trap around 245 times as much heat as does CO_2 (IPCC, 2007); that is, the heat-trapping capacity of N_2O is higher than that of CH_4. However, if flooding is continued to control N_2O emissions, then CH_4 emissions increase. The results of our incubation study show that the presence of aquatic earthworms during continuous flooding reduced CH_4 emissions by more than 90% after increasing the biomass of methanotrophs via their bioturbation activity.

4.5 Summary

In the incubation experiment, soil containing urea emitted approximately 95% more CH_4 than did soil containing ammonium sulfate after 4 weeks of incubation. However, adding urea to soil increased the population of aquatic earthworms, which reduced CH_4 emissions by more than 90%. The results of these experiments showed that, in the presence of aquatic earthworms, urea fertilizer did not enhance CH_4 emissions. In these conditions, aquatic earthworms effectively controlled CH_4 emissions.

Chapter V

Role of ammonium sulfate and aquatic earthworms activities on methane from paddy field

5.1 Background

Global warming is unequivocal and unavoidable. Global warming is caused by greenhouse gases (GHGs) e.g., carbon dioxide (CO_2), nitrous oxide (N_2O) and methane (CH_4) etc. In recent days, anthropogenic emissions are not only responsible for continuous increase of GHGs levels, but also due to longer lifetime of GHGs which is happened by stability and decreases in the amount of atmospheric [OH-] (Montzka *et al*., 2011). Atmospheric concentration of CO_2 has already risen to 391 ppm by 2011 and this exceeded pre-industrial level by 40% and CO_2 concentration is predicted to increase 421 to 936 ppm within 2100 (Alexander *et al*., 2013). Deforestation management practices, poor agricultural management practices have direct and indirect influence to increase atmospheric concentration of GHGs (Houghton *et al*., 1995) whereas projected increases in atmospheric concentrations and global temperature will be harmful for future food security and crop yield (Han *et al*., 2016, Ray *et al*., 2015). Van Groenigen *et al*., (2013) reported that global warming by every 1°C rise of temperature will significantly decrease yields by 14.6%. Therefore, mitigation of GHGs emission by altering human activities will be a very important effort.

Methane (CH_4) is the second most important GHG after CO_2 which has 25 times higher global warming potential than CO_2 over a 100-year time scale (Shindell *et al*., 2009). On a global scale, agricultural activities account for 5.1-6.1 Gt CO_2-eq/yr of which is 10%- 12% of total GHGs emission from anthropogenic sources and CH_4 and N_2O emission has been increased by nearly 17% from 1990 to 2005 (IPCC, 2007).

Rice is a crucial food for more than 50% of the world's population and the current estimate of world rice production is 481.5 million tons (USDA, 2016) more than 90% of which is produced in Asia. Cultivation of rice done with a semi-aquatic ancestor and for this semi-aquatic ancestry, rice plants are very sensitive to water shortage, therefore, rice is grown under water stagnant lowland situation in paddy (Kögel-Knabner *et al.*, 2010). Paddy fields have special soil characteristics including anaerobic condition, soil oxidation-reduction potential (ORP), different bacterial community etc. which are produced by periodic short-term flooding cycle over long period (Yao *et al.*, 1999, Lüdemann *et al.*, 2000, Kögel-Knabner *et al.*, 2010). Flooded lowland paddy fields are one of the most important sources of GHGs emission. Methane is highly emitted from lowland paddies which contributes approximately 15-20% of total anthropogenic CH_4 emission (Aulakh *et al.*, 2001). Rice production consists of multiple cropping management practices such as tillage, fertilization and the effects of these management practices on carbon stock of soil have been reported in many studies (Baggs *et al.*, 2003; Zhang *et al.*, 2007; Ahmad *et al.*, 2009; Morell *et al.*, 2011).

Nitrogen fertilized paddy fields have been reported as source of N_2O emission by several authors (Smith *et al.*, 1982; Lindau *et al.*, 1990; Cai *et al.*, 1997). Although lowland paddy fields emit lower N_2O but still represent substantial source of atmospheric N_2O (Hasegawa *et al.*, 1998). In general, anaerobic and aerobic alteration promotes N_2O emission while CH_4 emission is reduced.

Nitrogenous fertilizers are fundamentally important for crop growth and yield. Sulfate-containing fertilizers suppress CH_4 emissions because they result in competition between sulfate-reducing bacteria and methanogenic archaea for substrates like hydrogen and acetate (Hori *et al.*, 1990). Several studies have demonstrated that ammonium sulfate decreases CH_4 emissions from soil (Schutz *et al*., 1989, Hori *et al*., 1990, Banik *et al*., 1996).

Aquatic earthworms are a major group of invertebrate fauna in the paddy field ecosystem, and are well known to maintain soil quality (Simpson *et al*., 1993). More particularly, aquatic earthworms in paddy soils have the potential to improve soil health and increase soil fertility and plant production (Yokota and Kaneko, 2002). In flooded soil, aquatic earthworms (maximum length: 4–5 cm) mix soil and move water by burrowing and passaging soil through their gut. The bioturbation activities of aquatic earthworms may enhance oxidation by increasing the depth of the oxidized layer at the soil surface. Thus, aquatic earthworms may play a role to mitigate CH_4 emissions from flooded paddy soil and may affect methane-consuming microbes, which play a vital role in global warming because they are a biological sink of CH_4. Research is needed to identify the role of aquatic earthworms in controlling CH_4 emissions. Different environmental factors and management practices are well known to affect CH_4 emissions from paddy soil, but little is known about the effects of aquatic earthworms on CH_4 emissions.

Therefore, this aims of this study were:

i. to identify effects of ammonium sulfate on CH_4, N_2O and CO_2 flux and aquatic earthworm density.

ii. to know the role of aquatic earthworms on CH_4, N_2O and CO_2 flux in paddy soil and on the activity of methanotrophs.

5.2 Materials and methods

Soil collection and preservation

Soil for this study was collected on 10th May, 2013 from a paddy field in Kamakura, Kanagawa (35°20′ N, 139°31′E, 56m above sea level). Approximately half of the collected soil was left in a plastic house for drying. After drying homogenous texture of soil was achieved by sieving through 2 mm sieve and then soil was stored in a plastic house under anaerobic condition. Plant debris and stones were removed from the wet soil, and then aquatic earthworms from another half wet soil were collected by sieving the soil through a 500 µm mesh sized sieve. Soils for lower layer were collected from YNU forest to use in lower part of microcosm.

Macrocosm preparation

Stratified paddy field model was constructed to grow rice plants. Paddy field models were prepared using poly vinyl chloride pipe. Macrocosms having 15.4 cm diameter and 40 cm height were used for this study. Soils collected from YNU forest were used for making the lower layer which was 13 cm in depth and where soil bulk density was 1.5 g/cm^3. For making plow layer and plow sole layer, paddy soil was used.

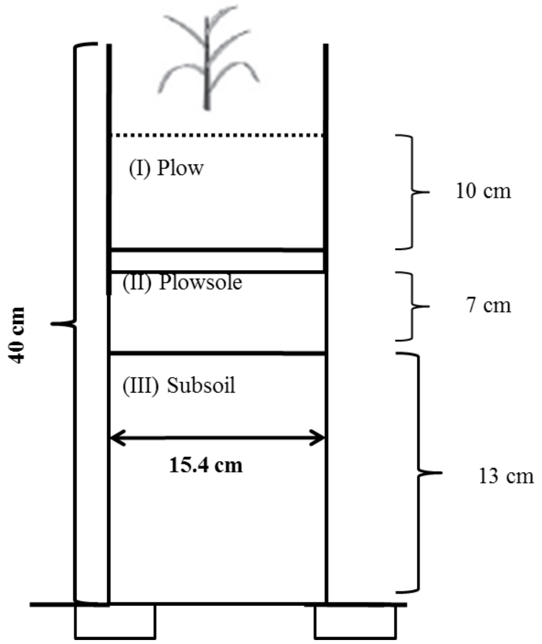


Figure 33: Stratified paddy field model

The depth of this layer was 17 cm and bulk density of soil was 0.95 g/cm^3. After putting soils, macrocosms were filled with water and 10 cm water level was maintained.

Soil Preparation and fertilizer application

Aquatic earthworms were collected by sieving the soil through a 500 µm sieve. Two factors were considered in two levels; i) aquatic earthworms (with earthworms: EW and no earthworm: NW), and ii) ammonium sulfate (with ammonium sulfate: AS and no ammonium sulfate: NA) in four replications. Thus sixteen macrocosms were prepared. Rice straw powder (800 g/m^2) was added in soil and was mixed gently with upper 10 cm soil. $(NH_4)_2SO_4$ (where NH4% is 20.5) equivalent to 90 kg N/ha was mixed with soil in the fertilizer treated macrocosm. After 1 week of fertilizer application aquatic earthworms were inoculated in earthworm treated macrocosm and the density of earthworms was 2525/m^2 . Before inoculation, earthworms were incubated in soil separately with organic matter as food source.

Seedling preparation and transplantation

Rice seeds of satojima variety which is recommended in Kanagawa prefecture were used for growing seedlings. Seeds were soaked in water for 3 days until germination of seeds was started. Then seeds were placed on humid soil for growing. After 3 weeks, healthy seedlings were collected and 2 seedlings per macrocosm were transplanted.

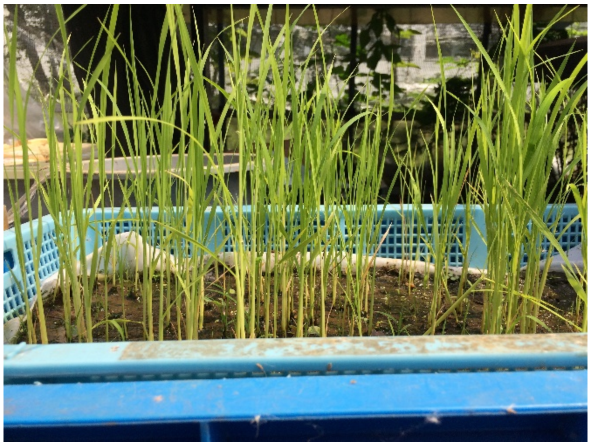


Photo 9: Rice seedling before transplantation

Gas sampling and measurement

Gas samples were collected by closed chamber method. Spherical chambers having 144 cm length and 21 cm diameter were used for gas sample collection. A 15 ml gas vials were used for gas sampling. Before sampling gas vials were vacuumed for 4-5 hrs by freeze drier. Gas samples were collected once in amonth starting from the day of fertilizer application on June and sampling was continued until August. First sample was taken at 0 minute that means just after placing of gas chamber on the macrocosms. Then second sample at 10 minutes third sample at 20 minutes and the last sample at 30 minutes were taken. Then methane (CH_4), carbon dioxide (CO_2) and nitrous oxide (N_2O) concentration will were measured by gas chromatograph and flux of each gas will be calculated by *flux* package in R (Jurasinski *et al*., 2014).

Photo 10: Gas sampling by closed chamber method

Soil sampling

Soil samples were collected two times. Once soil will be collected before application of any treatment to measure total carbon and total nitrogen and to analyse microbial community structure. And at last, after gas sampling on August, destructive soil sampling was done. Soil which was collected for measuring total C and total N was oven dried for 48 hours at 45°C temperature and then was preserved in air-tight plastic packets. Soil which was collected for microbial study was preserved in sterilized pots at -25°C temperature and soil which was collcted for measuring earthworm density was sieved through a 500 µm mesh size sieve.

Total C and N analysis

About 1500 mg of previously oven-dried soil was used from each treatment for measuring total C and N per treatment and was analyzed by CN corder (MACRO CORDER JM1000CN, J-SCIENCE LAB Co. Ltd., Japan).

Soil pH and Eh

Once in a month after collection of gas samples, soil pH and Eh were measured by portable devices. Soil pH was measured by pH/COND METER D-54, HORIBA, Japan and soil Eh was by pH/ORP/DO METER D-75, LAQUA act, HORIBA, Japan. Soil Eh was measured at 5 cm depth.

Analysis of methanotrophic bacterial biomass

Phospholipid fatty acid (PLFA) analysis was conducted to assess methane-oxidizing bacterial (MOB) biomass. Lipid extraction was accomplished following a modified method of Frostegard *et al*., (1991) and Niwa *et al*., (2008). Lipids in 7-8 g fresh wet soil samples was extracted by one-phase chloroform-methanol-phosphate buffer. After condensation of lipids, fractionation of phospholipid was carried out using silicic acid columns (BOND ELUT LRC-SI; Varian, Palo Alto, CA, USA) before separation of fatty acid methyl-esters from phospholipids following mild alkaline methanolysis. An internal standard, methyl non-adecanoate (19:0) was added to all samples. Fatty acid methyl-esters was identified by the Sherlock Microbial Identification System (MIDI, Newark, DE, USA). The fatty acids 18:1ω7c was used to estimate type II methane-oxidizing bacterial biomass, 16:1ω7c and 16:1ω5c were used to estimate type I methane-oxidizing bacterial biomass (Winden *et al*., 2010; Kip *et al*., 2011; Ruth *et al*., 2013; Zigah *et al*., 2015).

Statistical analysis

The heterogeneity and normality of data were determined with Bartlett's test and Shapiro-Wilk test, respectively. The main and interaction effects of fertilizers and aquatic earthworms were assessed by two-way analysis of variance (ANOVA). Statistical analyses were performed using R 3.2.3 for Microsoft Windows (R Development Core Team, 2015).

5.3 Results

Soil environment

The soil Eh value was higher at 1-cm depth than at 5-cm depth (Table 23). From July, the soil Eh at 5-cm depth was very low remained low throughout the study period. The soil Eh values at 1-cm depth showed no noticeable difference throughout the study period. There were no significant differences in soil pH and soil total C and N among the treatments (Table 24).

Table 23: Soil Eh at different depth of soil

Treatments	Eh at 5 cm depth (mV)			Eh at 1 cm depth (mV)		
	June	July	August	June	July	August
EW AS	-144	-259	-240	-45	-48	-51
EW NA	-152	-257	-241	-37	-41	-48
NW AS	-157	-251	-250	-39	-46	-49
NW NA	-156	-239	-228	-38	-44	-53

Table 24: Soil pH and soil total C and N content

Treatments	Soil pH			Soil C content (%)			Soil N content (%)		
	June	July	August	June	July	August	June	July	August
EW AS	7.44	7.44	7.42	1.79	-	1.89	0.16	-	0.18
EW NA	7.38	7.46	7.42	1.84	-	1.87	0.18	-	0.18
NW AS	7.44	7.5	7.43	1.85	-	1.91	0.17	-	0.19
NW NA	7.36	746	7.45	1.83	-	1.93	0.18	-	0.19

Relationship among aquatic earthworms, ammonium sulfate and CH_4 emissions

During the study period, the CH_4 flux started to increase considerably after June (Fig. 34). The CH_4 flux increased on July and reached at peak on August. There was no significance difference of CH_4 flux between earthworm treated and no earthworm macrocosm. After 2 months study, during destructive sampling earthworm population was also found from no-earthworm treated macrocosm. But CH_4 flux from ammonium sulfate treated macrocosm was lower than no ammonium sulfate macrocosm.

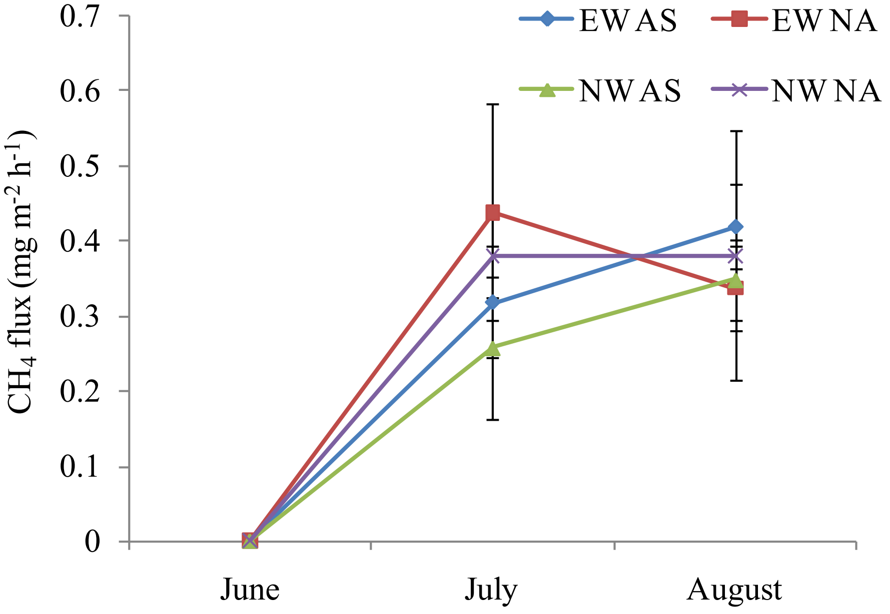


Figure 34: Influence of different treatments on CH_4 flux. Vertical bars indicate standard errors (n=4)

Table 25: ANOVA table for effect of ammonium sulfate fertilization and aquatic earthworms on CH_4 flux. (P value>0.05 indicates insignificant value)

Months	Ammonium sulfate		Earthworm	
	F value	P value	F value	P value
June	0.05	0.82	0.08	0.82
July	0.50	0.50	2.05	0.18
August	0.09	0.77	0.03	0.80

The highest cumulative CH_4 emission was in EW NA (26.09 mg m^{-2}) and the lowest was in NW AS (18.92 mg m^{-2}).

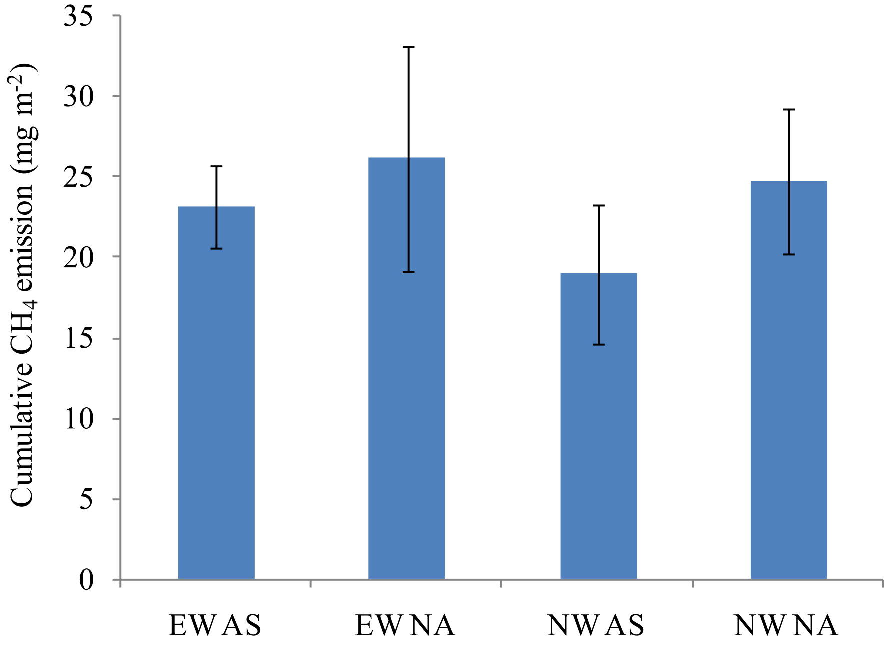


Figure 35: Influence of different treatments on CH_4 emission. Vertical bars indicate standard error (n=4)

When the aquatic earthworm density was 1237 m^{-2}, CH_4 flux was 0.15 mg m^{-2} h^{-1} but when the aquatic earthworm density was 215 m^{-2}, CH_4 flux was only 0.488 mg m^{-2} h^{-1} on August (Fig. 36). Increased aquatic earthworm density was related to decrease CH_4 flux.

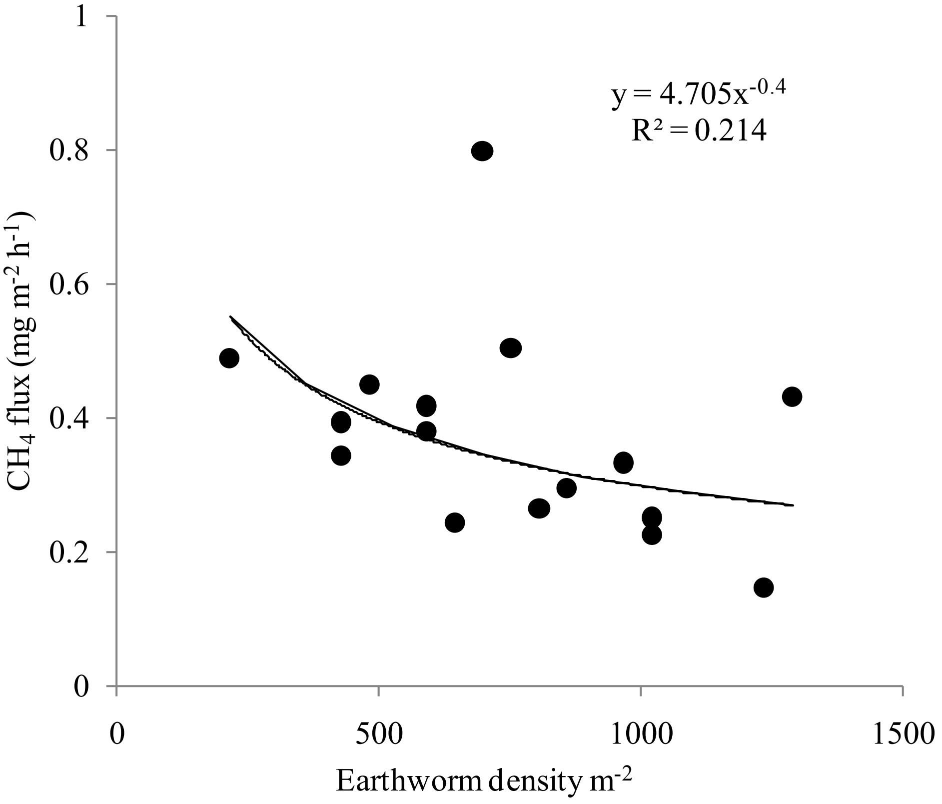


Figure 36: Correlation between earthworm density and CH_4 flux on August

Aquatic earthworms and biomass of methanotrophs

The amount of PLFAs was compared among treatments to estimate the biomass of methanotrophs (Fig. 37 and 38). The highest biomass of methanotrophs, based on the PLFAs content, was detected in the EW NA treatment (20.94 nmol g^{-1}) and the lowest biomass of PLFAs of methanotrophs was in the EW AS treatment (14.15 nmol g^{-1}).

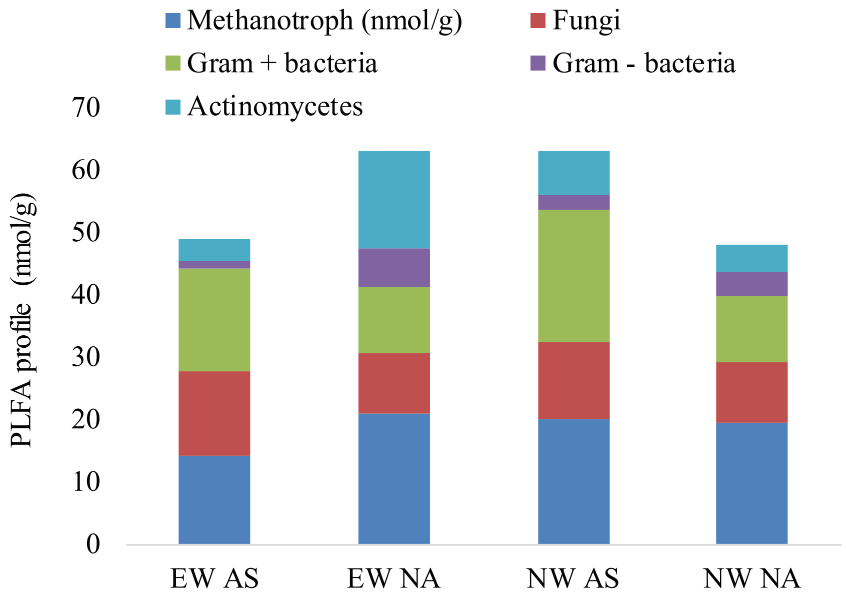


Figure 37: PLFA profile of studied soil on August

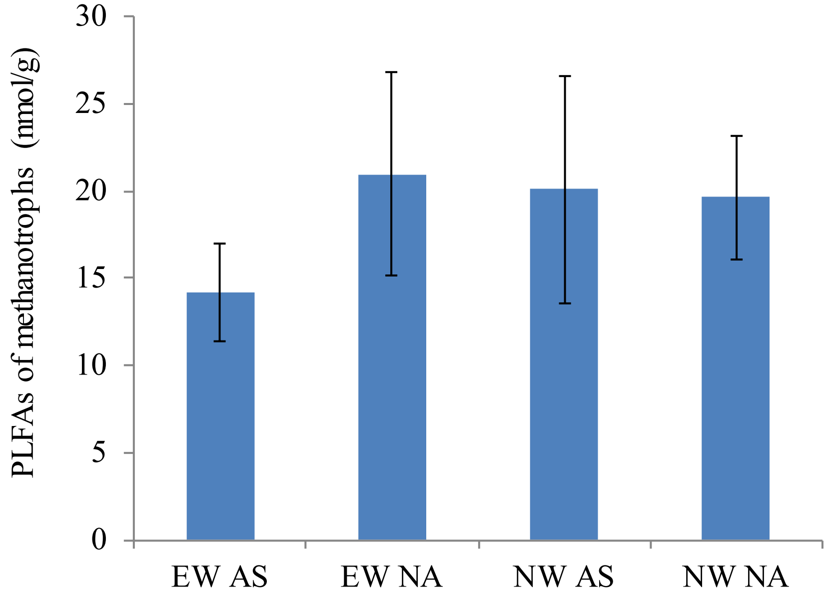


Figure 38: Biomass of methanotrophs in different treatments. Vertical bars show standard errors (n=4)

Table 26: ANOVA table for effect of ammonium sulfate and aquatic earthworms on biomass of methanotrophs. (P value>0.05 indicates insignificant value; 0.05-0.10=marginally significant)

Factors	F value	P value
Ammonium sulfate	0.59	0.38
Earthworms	2.48	0.15

There was no significant effect of fertilization and earthworms on biomass od methanotrophs as earthworm population was found from no earthworm treated soil. But methanotrophs considerably reduced the CH_4 flux from soil (Fig. 39) i.e., an increase in the biomass of methanotrophs was associated with a decrease in CH_4 flux. The presence of aquatic earthworms increased the biomass of methanotrophs (Fig. 40).

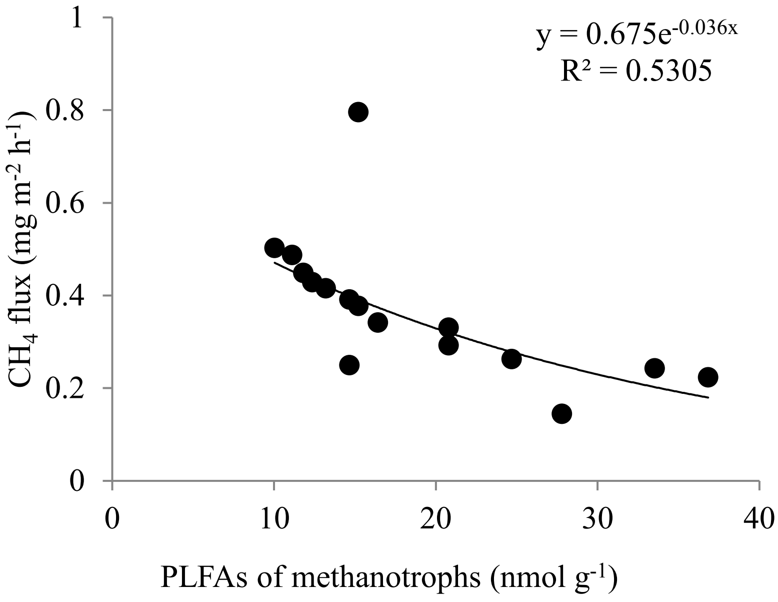


Figure 39: Correlation between biomass of methanotrophs and CH_4 flux in August

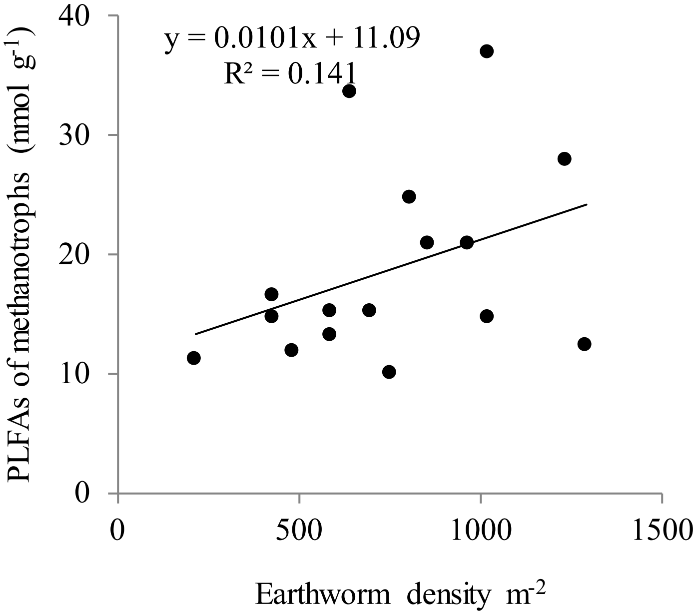


Figure 40: Correlation between biomass of methanotrophs and earthworm density in August

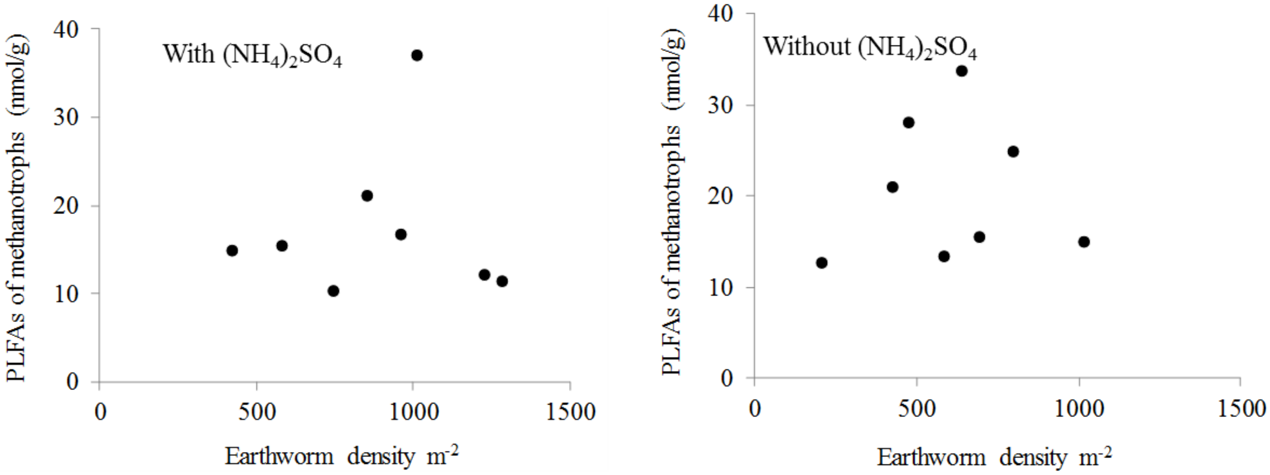


Figure 41: Correlation between earthworm density and biomass of methanotrophs in $(NH_4)_2SO_4$ and without $(NH_4)_2SO_4$ treatments

Relationship among aquatic earthworm, ammonium sulfate and CO_2 flux

After application of fertilizer and organic matter on June, CO_2 flux was found very high. On June, the highest CO_2 flux was recorded 2180 mg m^{-2} h^{-1} in NW NA treatment and the lowest CO_2 flux was observed 882 mg m^{-2} h^{-1} from NW AS treatment. On the following month CO_2 flux started to decrease and the decreasing trend continued until August.

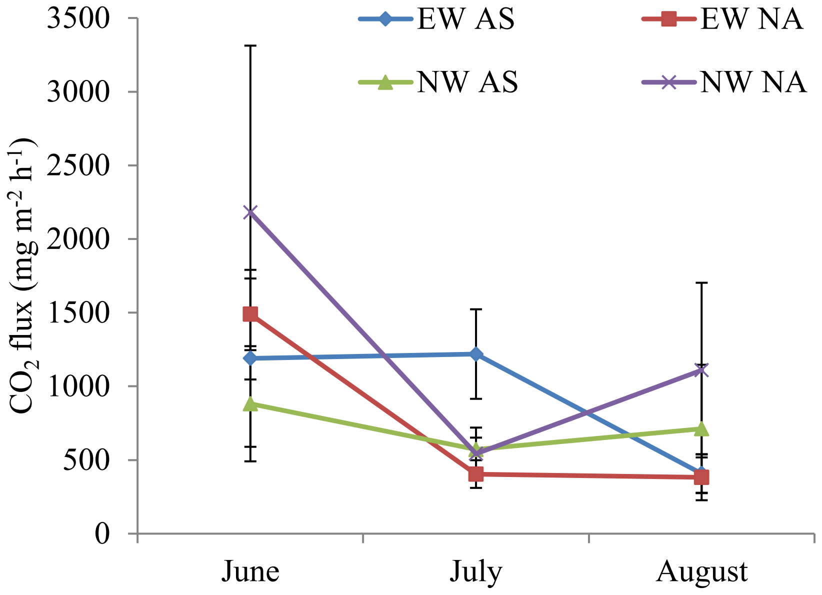


Figure 42: Influence of different treatments on CO_2 flux. Vertical bars indicate standard error (n=4)

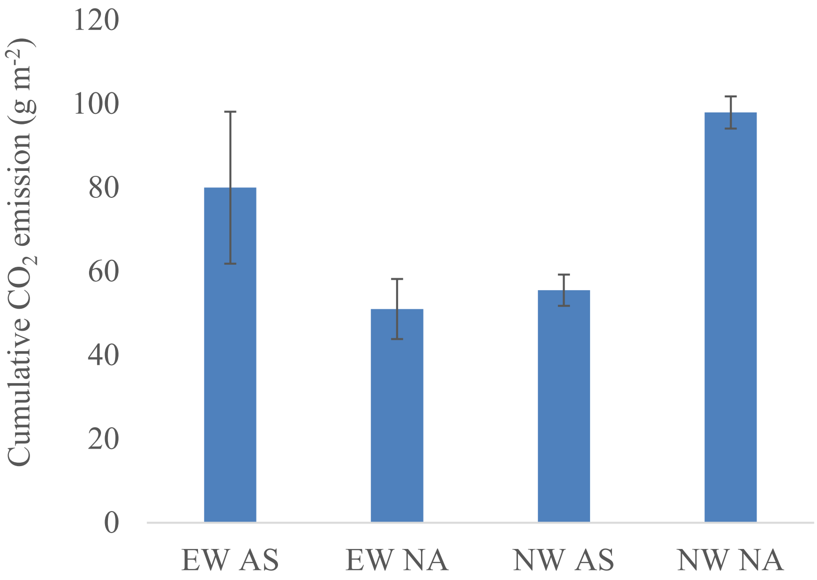


Figure 43: Influence of different treatments on cumulative CO_2 emission. Vertical bars indicate standard error (n=4)

On July, ammonium sulfate was found to increase CO_2 flux significantly and on August earthworms were found to mitigate CO_2 flux marginally.

Table 27: ANOVA table for effect of ammonium sulfate and aquatic earthworms on CO_2 flux. (P value>0.05 indicates insignificant value)

Months	Ammonium sulfate		Earthworm	
	F value	P value	F value	P value
June	1.83	0.20	0.11	0.75
July	7.02	0.02	2.56	0.14
August	1.13	0.31	3.49	0.09

Relationship among aquatic earthworm, ammonium sulfate and N_2O flux

After application of ammonium sulfate fertilizer on June, N_2O flux was increased and the highest N_2O flux was 0.07 mg m^{-2} h^{-1} observed in EW AS treatment and the lowest N_2O flux was found 0.01 mg m^{-2} h^{-1} from EW NA treatment. After this, N_2O flux started to decrease and on July, the highest N_2O flux was 0.03 mg m^{-2} h^{-1} in NW AS and the lowest N_2O flux was 0.01 mg m^{-2} h^{-1} in EW NA treatment. On August, N_2O flux was very low and reached to negligible level.

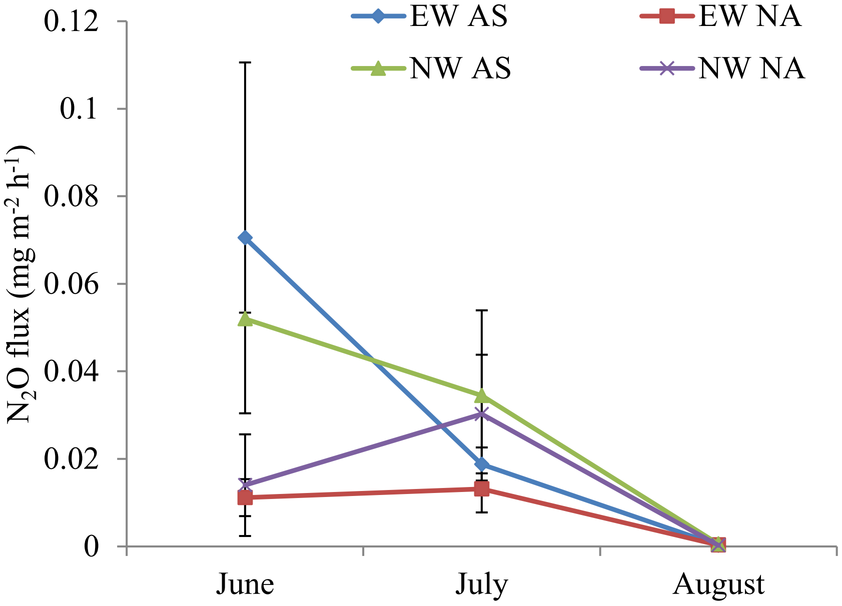


Figure 44: Influence of different treatments on N_2O flux. Vertical bars indicate standard error bars (n=4)

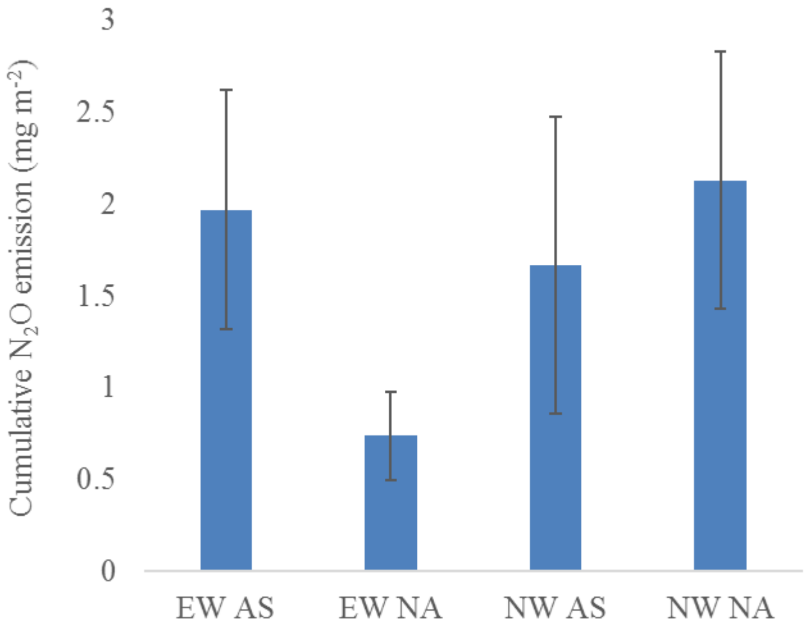


Figure 45: Influence of different treatments on cumulative N_2O emission. Vertical bars indicate S.E. (n=4)

On June, immediately after application of ammonium sulfate fertilizer, ammonium sulfate was found to have significant effect to increase N_2O flux. On July and August, there was no significant effect of treatments on N_2O flux.

Table 28: ANOVA table for effect of ammonium sulfate and aquatic earthworms on N_2O flux. (P value>0.05 indicates insignificant value)

Months	Ammonium sulfate		Earthworm	
	F value	P value	F value	P value
June	7.17	0.02	0.09	0.67
July	0.21	0.65	2.38	0.15
August	1.16	0.27	0.82	0.38

5.4 Discussions

Effect of fertilization on CH_4, CO_2 and N_2O flux

During this study, there was no significant effect of ammonium sulfate on CH_4 emission but cumulative CH_4 emission was lower in ammonium sulfate treatments. Previous studies have reported that sulfate-containing fertilizers decreased CH_4 emissions (Banik *et al*., 1996, Wang *et al*., 1992) because of the competition between sulfate-reducing bacteria and methanogens for their common substrates (e.g., hydrogen, acetate) (Hori *et al*., 1990, Westermann and Ahring, 1987). Minami (1994) described that SO_4^{2-} acts as proton acceptor in anaerobic condition and thus CH_4 emission is mitigated. Besides this, H_2S which is produced through SO_4^{2-} reduction may poison methanogenic bacteria and CH_4 production can be depressed.

Ammonium sulfate fertilizer was found to increase CO_2 flux on July. Application of nitrogenous fertilizer stimulates production of plant biomass and soil biological activity and thus, increases CO_2 flux (Dick, 1992). Wilson and Al-Kaisi (2008) and Iqbal *et al*., (2009) also reported the similar results.

On June, immediately after application of ammonium sulfate, a nitrogenous fertilizer, N_2O flux was increased significantly. Kumar *et al*., (2000) reported N_2O emission due to application of nitrogenous fertilizer as soil receives N as NH_4^+. Iqbal (2009) also reported that application of nitrogen fertilizer is the immediate source of N_2O by nitrification and denitrification processes in a strongly anaerobic condition. This mechanism may have increased N_2O flux after application of fertilizer. Nitrification and denitrification processes are taken place concurrently (Suratno *et al*., 1998).

Role of aquatic earthworms in mitigating CH_4 emissions

Although aquatic earthworms were inoculated in macrocosms which were selected only for earthworm treatments but after destructive sampling on August, earthworm population was found from no-earthworm treated macrocosm. In this study, aquatic earthworms increased the biomass of methanotrophs, and reduced CH_4 emissions. Methanotrophs intercept and then oxidize the CH_4 produced in paddy soil as it escapes into the atmosphere via aerobic interfaces (Dubey, 2005). Aquatic earthworms are 'conveyor belt'-type feeders (Rhoads, 1974). For example, the head of aquatic earthworms is buried in the soil for feeding while its tail remains on the surface for respiration. Through this bioturbation activity, aquatic earthworms are able to introduce O_2-rich water into the lower soil layer, and it also produces a layer of faecal pellets on the soil surface. This soil habitat modification led to an increase in the biomass of methanotrophs, which accelerated the oxidation of CH_4 produced by methanogens.

5.5 Summary

Although it was difficult to control aquatic earthworm population in flooded soil, but a negative trend of CH_4 flux was found with increase of population of aquatic earthworms after enhancing biomass of methanotrophs. Application of $(NH_4)_2SO_4$ as agro-chemical was beneficial for mitigation of CH_4 but immediately it increased N_2O flux. But with time N_2O flux was lowered to negligible level.

Chapter VI

General Discussion

Methane (CH_4) is a major greenhouse gas. The Intergovernmental Panel on Climate Change (IPCC) reported atmospheric CH_4 concentration in 2005 was 1774 ppb. Although paddy fields have been considered as important source of CH_4 since earlier of last century (Harrison and Aiyer, 1913), but the measurements of CH_4 flux from paddy fields have been reported in detail in early 1980s (Cicerone and Shetter 1981; Cicerone *et al*. 1983; Seiler *et al*. 1984; Holzapfel-Pschorn *et al*. 1985). The total global CH_4 emission is about 600 Tg (1 Tg= 10^{12} g) per year (Danier *et al*., 2002). This CH_4 is emitted both from natural and anthropogenic sources and only paddy field alone emits 11% of all anthropogenic sources (Smith *et al*., 2007).

There are different CH_4 producing factors in soil which have been discussed previously such as water regime, cultivar, mineral fertilizers, soil texture, tillage etc. In this study, some of these mitigating factors were applied in field scale and small scale. Additionally, the effect of aquatic earthworms, which belong to a major group of invertebrate fauna in paddy field ecosystem, on CH4 mitigation was analyzed.

Water regime

During field study in Kamakura in 2011, CH_4 flux showed clear trend to increase with water depth as anaerobic condition is developed with increase of water depth. On June and July water depth was higher and with higher water depth CH_4 flux was also higher and the value was 12.3 mg m^{-2} hr^{-1} and 22.3 mg m^{-2} hr^{-1} respectively. During the gas sampling on August, paddy field water was dried out due to lack of rainwater and paddy soil received oxygen and CH_4 flux dropped down to

2.81 mg m^{-2} hr^{-1}. Itoh *et al*. (2011) reported that prolonged mid-season drainage reduces seasonal CH_4 emission from paddy field. Yagi *et al*. (1997) and Sass *et al*. (1992) also recommended the same management. Similar trend was observer during field study in Nara in 2012, the paddy field (NT) which was not highly flooded emitted very low CH4. Highly anaerobic condition is only responsible to produce CH_4 from flooded paddy field. Cai *et al*. (1997) indicated that intermittent irrigation in paddy field significantly depresses CH_4 emission. In this study, not only no-tillage farming practice but also water management was responsible for mitigating CH_4 emission from paddy field. Cai *et al*. (1997) indicated that intermittent irrigation in paddy field significantly depresses CH_4 emission. Intermittent irrigation and mid-season drainage are common water management practices in Japan to control tillering and to allow O_2 to reach roots of rice plants to prevent sulfide toxicity (Kanno *et al*., 1997). These water management practices are among the most effective ways to reduce CH_4 emissions because they prevent reducing conditions developing in the soil. One or several drainage systems were shown to reduce CH_4 emissions, compared with continuous flooding in field conditions (Nishimura *et al*., 2004) and pot experiments (Minamikawa *et al*., 2005). A single mid-season drainage decreased CH_4 emissions by 50% (Kimura *et al*., 1992). Therefore, water management, especially to control Eh, has been proposed to control CH_4 emissions (Minamikawa *et al*., 2005). On the other hand, drainage increases N_2O emissions concurrently (Bronson *et al*., 1997, Chen *et al*., 1997) through nitrification and denitrification occurring simultaneously (Suratno *et al*., 1998). One molecule of N_2O can trap around 245 times as much heat as does CO_2 (IPCC, 2007); that is, the heat-trapping capacity of N_2O is higher than that of CH_4. However, if flooding is continued to control N_2O emissions, then CH_4 emissions increase.

According to Stenert *et al*., (2009) absence of irrigation water reduces the density of aquatic invertebrate as those are not highly tolerant to drought and similar result was observed in Kamakura and Nara field study.

Tillage practices

During field study in Kamakura, CH4 flux was comparatively lower on no tillage plot and during field study Nara, CH4 flux was considerably lower in no tillage paddy field. Hanaki *et al*. (2002) reported that no-tillage farming practice in rice cultivation can decrease CH_4 emission by reducing fraction volume of large pores. Li *et al*. (2011) also stated that when bulk density is increased in no tillage treated paddy soil; CH_4 emission to atmosphere can be blocked and CH_4 produced in soil might be kept for long time, which may increase probability of CH_4 oxidation by methanotrophs. This no tillage farming practice was found beneficial for earthworm population in Kamakura paddy field and this result agreed that no tillage farming practice can increase aquatic earthworm density in paddy field (Yokota and Kaneko, 2002) as soil surface disturbance is very limited. But in no tillage paddy field in Nara, earthworm population was very few as it was under water management.

Fertilization

During field study in Kamakura, total released CH_4 from organic fertilizer treated plot was lower than that from chemical fertilizer treated plot. But Neue (1993) found in his study that organic fertilizer increased CH_4 emission from paddy field. In that study the paddy field was an organic farm and amount of applied organic fertilizer was 1.3 t ha^{-1}, which was lower than other paddy fields. In Japan, in conventional paddy fields, organic fertilizer application rate is around 6-12 t ha^{-1} (Yagi and Minami, 1990). Therefore, the amount of applied organic fertilizer in our study site was not enough to be responsible to increase CH_4 emission. Besides this, aquatic earthworm population was also lower in chemical fertilizer treat plot and Xiang *et al*. (2006) already reported that chemical fertilizer reduces earthworm population in paddy field.

Another incubation study was done to identify effects of mineral fertilizers on CH4 emission and the results showed that adding urea to soil resulted in higher CH_4 emissions from submerged soil compared to $(NH_4)_2SO_4$. Similar results have been reported in previous studies (Banik *et al*., 1996, Dubey, 2003, Wang *et al*., 1992, Yang and Chang, 1997). A previous study reported a direct relationship between CH_4 production and lower Eh values/higher pH (Wang *et al*., 1992) in flooded paddy soil. After urea is applied to paddy soil, it is hydrolyzed by microbial ureases, resulting in an alkaline soil pH and lowers oil Eh. In the present study, CH_4 flux from ammonium sulfate-treated soil was lower than that from control soil. Previous studies have shown that sulfate-containing fertilizers can decrease CH_4 emissions (Banik *et al*., 1996, Wang *et al*., 1992) because of the competition between sulfate-reducing bacteria and methanogens for their common substrates (e.g.,hydrogen, acetate) (Hori *et al*., 1990, Westermann and Ahring, 1987). But ammonium sulfate application resulted in CO_2 and N_2O emission. Application of nitrogenous fertilizer stimulates production of plant biomass and soil biological activity and thus, increases CO_2 emission (Dick, 1992; Wilson and Al-Kaisi, 2008; Iqbal et al., 2009). Kumar et al., (2000) reported N_2O emission due to application of nitrogenous fertilizer as soil receives N as NH_4^+. Iqbal (2009) also reported that application of nitrogen fertilizer is the immediate source of N_2O by nitrification and denitrification processes in a strongly anaerobic condition. This mechanism may have increased N_2O flux after application of fertilizer. Nitrification and denitrification processes are taken place concurrently (Suratno et al., 1998).

Urea was shown to increase the abundance of aquatic earthworms in this study. Mineral N has been shown to stimulate the aquatic earthworm population (Simpson *et al*., 1993). Also, higher N availability increases the growth of photosynthetic aquatic organisms, including microorganisms that are food sources for aquatic earthworms.

Aquatic earthworms

Aquatic earthworms are well known to maintain soil quality (Simpson *et al*., 1993). More particularly, aquatic earthworms in paddy soils have the potential to improve soil health and increase soil fertility and plant production. But very little is known about the role of this invertebrate group on CH_4 emission from paddy field. During our field study in Kamakura, it was observed that CH_4 flux on July was reduced around 40% when aquatic earthworm density reached to 11,000/m^2. In laboratory studies also, aquatic earthworms were seen to reduce CH_4 emission from paddy soil through increasing biomass of methanotrophs. Methanotrophs intercept and then oxidize the CH_4 produced in paddy soil as it escapes into the atmosphere via aerobic interfaces (Dubey, 2005). Aquatic earthworms are 'conveyor belt'-type feeders (Rhoads, 1974). For example, the head of aquatic earthworms is buried in the soil for feeding while its tail remains on the surface for respiration. Through this bioturbation activity, aquatic earthworms are able to introduce O_2-rich water into the lower soil layer, and it also produces a layer of faecal pellets on the soil surface. This soil habitat modification led to an increase in the biomass of methanotrophs, which accelerated the oxidation of CH_4 produced by methanogens. However, contrasting results were reported for the tropical earthworm *Pheretima* sp. (John *et al*. 2015). This species increased CH_4 emissions from flooded soil by bioturbation where methanotrophs were not able to consume CH_4. Since the earthworm used in this study is an aquatic species, we assume that aquatic earthworms are more likely to decrease CH_4 emissions from flooded paddy soil.

Table 29: A comparative evaluation of factors influencing production and emission of CH_4 from paddy field

Factors	Impact on CH_4 production and emission	Mitigation/ emission (%)	Risk
1. Water regime			
Intermittent drainage	Production is reduced due to oxidation and emission is reduced	15-80%	N_2O emission is increased and GWP of N_2O is 265.
Mid-season drainage	Production and emission is reduced	43%	
Prolonged mid-season drainage	Production and emission is reduced	90%	
2. Fertilizer type			
Urea	Enhance CH_4 production after hydrolyzation and emission is increased	95% compared to ammonium sulfate	
Ammonium sulfate	Sulfate decreases CH_4 production by activating sulfate reducers in soil which compete for the same substrates	58%	Root rot of rice plants
3. Tillage practices			
No tillage	Emission of CH_4 is reduced due to increased compactness of soil	93%	Long-term practice increases labile SOC and it is an important methanogenic substrate in irrigated paddy

4. Earthworms		
Aquatic earthworm	Emission of CH_4 is reduced by bioturbation of aquatic earthworms	40-90%
Tropical earthworm (*Pheretima sp.*)	Emission of CH_4 is increased by bioturbation as methanotrophs are not able to consume CH_4.	60%

Conclusions

No tillage farming practice which is being increased in South Asian countries is known as beneficial against soil disturbances, to aggregate stability and organic carbon flux rates. This farming practice is also favorable for enhancing density of invertebrate fauna like aquatic earthworms in paddy field. Water management practices like mid-season drainage, intermittent irrigation suppress CH_4 emission very well but these practices loss density of aquatic earthworms. Nitrogenous fertilizer such as urea, ammonium sulfate which are fundamental for proper growth of rice plants affect CH_4 and N_2O emission where urea emits CH_4 whereas ammonium sulfate controls but nitrogenous fertilizers have risk of N_2O emission. Mineral fertilizers are also able to enhance aquatic earthworm population in paddy field. The most important finding if this research work was mitigation of CH_4 emission by aquatic earthworms. Aquatic earthworms improved biomass of mehanotrophs on upper soil surface in flooded paddy field and showed a potential effect on CH_4 emission mitigation.

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