International Journal of Agriculture, Environment and Bioresearch

Vol. 2, No. 03; 2017

ISSN: 2456-8643

ABUNDANCE OF METHANOGENS IN FLOODED RICE SOILS AND SURVIVAL OF METHANOGENS IN AIR- DRIED SOILS OF PADDY FIELD OF ZHEJIANG, CHINA

Brahima Traore[1], Min Hang[2], Fasse Samake[1], Chen Meici[2], Amadou Babana[1]

1 Département de biologie de l'Université des Sciences des Techniques et des Technologies de Bamako .USTTB Mali

2 Department of Environmental Engineering, Huajiachi Campus, Zhejiang University Hangzhou, Zhejiang PR China

ABSTRACT

Methane (CH4) emissions from Chinese paddy soil (Zhejiang province) were measured over the rice growing seasons. Emissions of methane were high during two periods (05 days after peak tillering and 07 days after heading flowering stage). The amounts of methanogenic microbes, including hydrolytic fermentative, hydrogen producing acetogenic and methanogenic bacteria, were measured during the different growth stages of rice. The results showed that the amounts of hydrolytic fermentative bacteria increased or decreased with the growth of rice but that of hydrogen-producing acetogenic bacteria and methanogenic increased with the growth of rice. The amounts of methanogens decreased from 1.1×10^6 g^{-1}dry soil after 15 days to 0.4×10^2 g^{-1} dry soil after 1 year in air dried condition. It was obvious that obligatory anaerobic methanogenic bacteria were very sensitive to air (O_2). The obligate anaerobic methanogenic bacteria which were most sensitive to air (O_2) ,could survive in air-dried soil stored for long time. Flooding (or water saturation) and high moisture content appeared to be an important condition for the increased methanogens amounts in soil. No significant differences between the amounts of methanogens in different depths of soil were observed 0-10; 10-15; 15-20.

Keywords: methane emission; field; methanogenic flora; air-dried soil; different depths of rice soil.

Introduction

The rapid increase of the world population over the past 50-100 years has led to increased atmospheric concentrations of Carbon Dioxide (CO_2), Methane (CH_4), and Nitrous Oxide (N_2O). These gases along with additional trace gas species (greenhouse gases) are causing an increase in global temperature. CH_4, following CO_2, is the second important gas contributing to the radiative forcing of the atmosphere (Peter E. Levy, 2012). In terms of the potential of increasing temperature, CH_4 contributes 15% to the greenhouse effect and atmospheric methane increases at a rate of 1% every year (H-Y.Huang et *al*; 2014).

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Agricultural soils are a primary source of anthropogenic trace gas emissions. Rice paddies have been identified as a major source of atmospheric CH_4 and N_2O. The CH_4 emission from global rice fields was estimated to be 25.6 Tg yr^{-1}(Yan et *al*; 2009). Rice, one of the most important crop plants worldwide with a total cultivation area of 155 million hectares, is traditionally grown under flooded or wet conditions during most of the cultivation period (Ma et *al*; 2010). In general, soil becomes an increasing anaerobic environment after being submerged and forms a suitable environment for methanogenic bacteria with the decrease of soil redox potential (Wang 1993; Min 1993). In flood-irrigated rice fields, anaerobic soil conditions lead to CH_4 generation as the final product of organic compost decomposition by methanogenic bacteria. Soils can also act as a significant sink for CH_4, via oxidation by methanotrophic bacteria, and the net efflux is the balance between production and oxidation ((Dalal et *al*. 2008). Rice plants play a decisive role in methane production by providing substrates to methanogens (Tokida et *al;* 2011) and by acting as a conduit to transfer methane from the soil to the atmosphere (Cicerone and Shetter 1981). Report on the application of rice straw in paddy soil proved that decomposing rice straw is not only a substrate of methane production, but in addition stimulates methane production from soil organic and root organic carbon(Yuan et *al* ; 2014). The rhizosphere in soil-plant systems is a potential zone of hotspots of methane production. The rhizosphere is the volume of soil occupied and influenced by plant roots (Philippot et *al*; 2013).

FAO estimated that rice production must increase by 40% the end of 2030 (FAO 2009). This significant increase in rice production can lead to increased methane emission to the atmosphere (Kim et al; 2014; Roy et al; 2014; Haque et al; 2015). CH_4 emission from every rice growing country needs to be measured and assessed from different conditions. In this study, we aimed to measure the amount of methanogens in flooded rice soils, and their survival in air-dried soils.

MATERIALS AND METHODS

General condition for the field test

The test was carried out in pots 30cm in diameter and 45cm in height, the soil in the pots was submerged with tap water. Before transplanting the rice seedlings 1g of urea was applied as basal fertilizers and 1g of urea as dressing fertilizer 15days after transplanting in pot. Three pots were planted with rice seedling named Zhe 852. Three hills of rice seedling (6 rice seedling for each hill) were planted in each pot.

Methane determination

Collection of methane gas in situ

Glass cylinders with a capacity of 2600 ml were used to cover various representative rice hills

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for 24 hours. Gas samples were taken by syringe from a late pipe fixed on the glass cylinders. First, the air inside the glass cylinders was mixed well by using the syringe, then the sample gas was withdrawn and the needle of the syringe was sealed by a rubber cap to prevent the escape of the sample gas from the syringe.

Methanogenic microflora in soil during growth stages of rice

The population of each group of methanogenic flora was measured by MPN method with triplicate. The formation of H_2 and CH_4 were used as the indexes of amounts for hydrogen – producing acetogenic and methanogenic bacteria, respectively. All the data in this paper are average values of triplicates.

Determination of methane and hydrogen

Methane was measured by 102 GC type of gas chromatograph with a hydrogen flame ionization detector. Under the following conditions: carrier GD-X-102, air speed 700ml/min, H_2 40ml/min, N_2 25ml/min, chromatographic room temperature 40^{o}C, standard time for appearance of methane peaked at 17s.

Hydrogen was measured by 102GC type of gas chromatograph with thermal conductivity detector under the following condition: carrier (h) X 104 N2 40ml/min, chromatographic room temperature 40oC, standard time for appearance of hydrogen peaked at 15 sec.

Composition and preparation of media for determination and isolation of methanogenic flora

Medium composition for determination of hydrolytic fermentative bacteria is as follows (g 1^{-1}); glucose, 10; beef extract, 3; peptone, 5; NaCl, 3; cystein, 0.5; resazurin, 0.002; pH7.2-7.4, distilled water

Medium composition for determination of hydrogen-producing acetogenic bacteria is as follows (g 1^{-1}); CH_3CH_2COONa, 30 mmoles; $CH_3(CH_2)_2COONa$, 30 mmoles; sodium lactate, 30 mmoles; sodium succinate, 30 mmoles; CH_3CH_2OH, 30 mmoles; yeast extract, 2; $MgCl_2$, 0.1; NH_4Cl, 1; K_2HPO_4, 0.4; KH_2PO_4, 0.4; cystein, 0.5; resazurin, 0.002; trace element solution, 10mL; soil extract solution, 300mL; pH, 7.0-7.3. The composition and preparation of trace element solution and soil extract solution are same as that used in medium for determination of methanogens.

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Medium composition for determination of methanogens is as follows (g 1^{-1}): NH_4Cl, 1; $MgCl_2$, 0.1; K_2HPO_4, 0.4; KH_2PO_4, 0.4; KH_2PO_4, 0.4; yeast extract, 1; cystein, 0.5; HCOONa, 5; CH_3COONa, 5; CH_3OH, 5 ml; H_2/CO_2(80/20,v/v); soil extract, 300ml trace element solution 10ml.

Preparation of soil extract is as follows: take several kg paddy soil and add tap water (soil water about 1:1.5), stir it and then let it stand still for 24 hours, filter the supernatant with filter paper, sterilise the filtrate and store it in a refrigerator.

Composition of trace element solution is as follows (g 1^{-1}) ; $N(CH_2COOH)_3$, 4.5 ; $FeCl_2.4H_2O$,0.4; $MnCl_2.H_2O$,0.1 ; $CoCl_2.6H_2O$,0.12 ; $ZnCl_2$, 0.1 ; $AlK(SO_4)_2$, 0.01 ; NaCl,1 ; $CaCl_2$, 0.02 ; Na_2MoO_4, 0.01 ; H_2BO_3, 0.01; and distilled water 11. Store it in a refrigerator.

The media was prepared according to Hungate's anaerobic technique. Before the medium for methanogens was used, 0.1 ml anaerobic sterilised Na_2S (10 g kg^{-1})/$NaHCO_3$(50 g kg^{-1}) mixed solution was added into each tube with 4.5ml medium to decrease further redox of the medium and then 0.1 ml of 160000 units ml^{-1} of penicillin to inhibit eubacteria.

RESULTS AND DISCUSSION

Methane emission

General trend of methane emission

The emission of methane by rice plants at different stages of growth was lower during the early growth stages. It then increased gradually and peaked 5 days after the peak tillering stage and decreased during the end tillering period, it increased again 1 week after the heading flowering stage as shown in **Fig1**.

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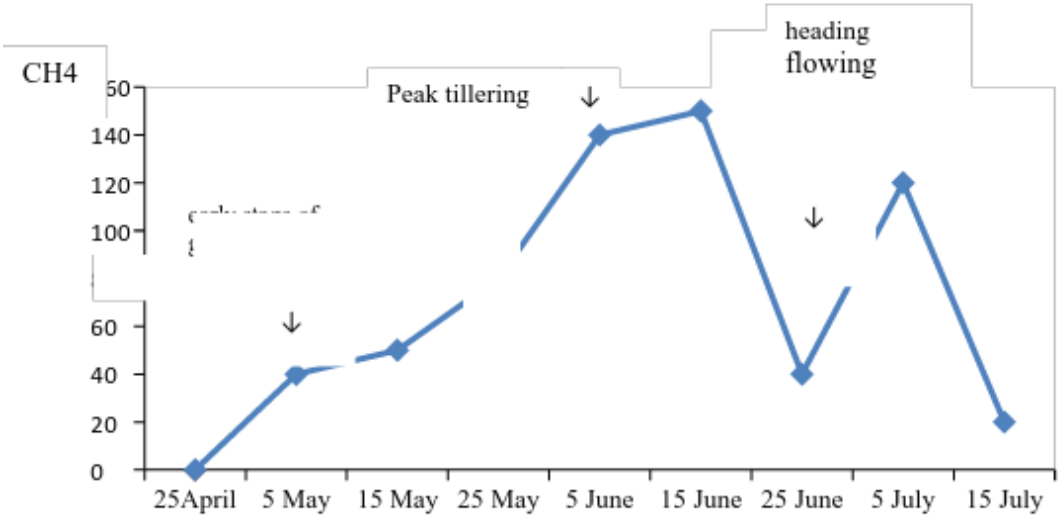

Figure 1 Methane emission during growth stage of rice plan(Methane emitted 10^4mol/(pot.d)

Methanogenic microflora

The amounts of methanogenic microbes, including hydrolytic fermentative, hydrogen producing acetogenic and methanogenic bacteria, were measured during the different growth stages of rice, as presented in Table1. The results showed that the amounts of hydrolytic fermentative bacteria increased or decreased with the growth of rice but that of hydrogen-producing acetogenic bacteria and methanogenic increased with the growth of rice. The amount of hydrolytic fermentative bacteria was higher (10^5--10^8/g dry soil) during the growth stage of rice than the amounts of hydrogen-producing acetogenic bacteria (10^3 --10^6/g dry soil), and than the amounts of methanogenic bacteria (10^2—10^6g dry soil).

Table 1. The amounts of methanogenic bacteria in soil during growth stages of rice (Cells/g dry soil)

Microbial group	**Growth stage of rice**
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	Early stage of growth	Peak tillering stage	Heading lowering stage
HFB	1.60×10^6	1.19×10^5	2.00×10^8
HPAB	3.40×10^3	1.50×10^6	2.70×10^6
MB	7.54×10^2	1.19×10^6	1.30×10^6

Notes: HFB. Hydrolytic fermentative bacteria; HPAB. Hydrogen-producing acetogenic bacteria; MB. Methanogenic bacteria

Amounts of methanogens in different depths of rice soil

The amounts of methanogens grown on H2/CO2 in 0-10, 10-15 and 15-20 cm depths of rice soil were measured during the peak tillering period and the heading flowering period and the results is shown in Table 2. The results showed that no significant differences between the amounts of methanogens in different depths of soil were observed, but amounts in soil of 10-15 cm depths were higher than the 2 others depths.

Table 2. Amounts of methanogens in different depths of rice soil

Sampling date	Substrate	Depth of soil(cm)		
		0-10	10-15	15-20
5 June	H_2/CO_2	4.3×10^7	8.2×10^7	0.4×10^7

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25 June x 10^6	H_2/CO_2	2.6 x 10^6	6.3 x 10^6	2.1

Survival of methanogens in air- dried rice soils

Before the harvest period soil from pots with about 50% of water content was mixed well and air dried. During this time, the amounts of methanogens were measured every 2 months. The results showed that the amounts of methanogens were 1.1 x 10^6 g^{-1}dry soil after 15 days and 0.4 x10^2 g^{-1} dry soil after 1 year (Fig 2). The results also confirmed that obligatory anaerobic methanogenic bacteria were very sensitive to air (O_2) . Fig2

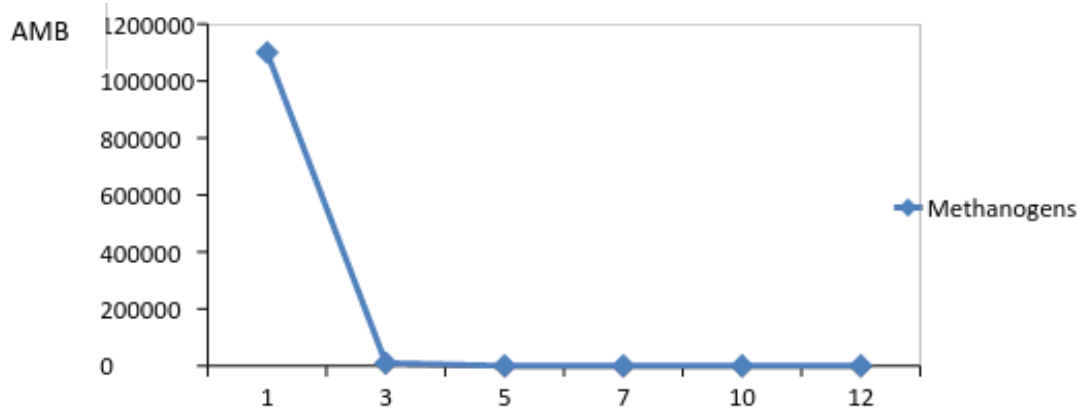

Fig2: Amounts of methanogens in air dried soil. AMB. Amounts of methanogens (cells g^{-1}dry soil) . M. Month

After air dried period soil was broken, sieved by sieve with 60 mesh, stored in sample vials in laboratory.20g of air dried soil with about 1% of water content was put into each serum vial (3serum vial were used.), added with 15 ml tap water and incubated at 35^0C. The amounts of methanogens were measured and results of a MPN determination in triplicate for the methanogens were presented in **Fig 3.** The population of methanogens was present in air dried soil but at low value 0.4 x10^2 g^{-1} dry soil and their amounts increased to 6.4x10^6 g^{-1} dry soil after addition of water and incubation for 14 days. The result showed that obligatory anaerobic methanogenic bacteria which were most sensitive to air (O_2) ,could survive in air-dried soil stored for long time. Flooding (or water saturation) and high moisture content appeared to be an

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important condition for the increased methanogens amounts in soil. Once the air-dried soil got certain content of water, methanogens survived in the air- dried soil might produce methane (Min et *al*; 1993).

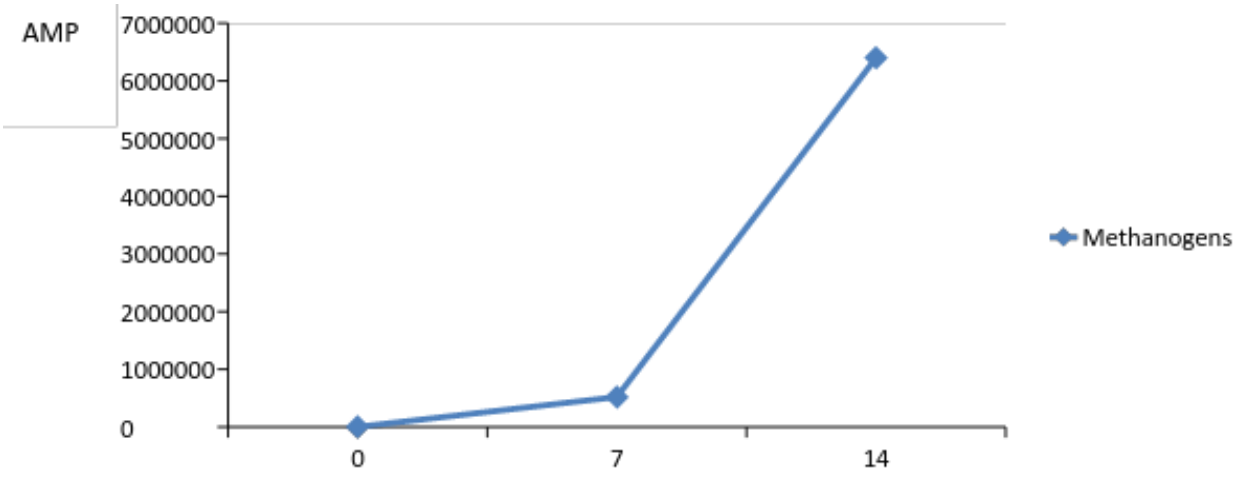

Fig 3: **Amounts of methanogens in 20g air dried soil added water after incubation. AMB. Amounts of methanogens (cells g^{-1}dry soil)**

CONCLUSION

The amounts of methanogenic microbes, including hydrolytic fermentative, hydrogen producing acetogenic and methanogenic bacteria, were measured during the different growth stages of rice. The amounts of hydrolytic fermentative bacteria increased or decreased with the growth of rice but that of hydrogen-producing acetogenic bacteria and methanogenic increased with the growth of rice. The obligatory anaerobic methanogenic bacteria were very sensitive to air (O2) but could survive in air-dried soil stored for long time and high moisture content appeared to be an important condition for the increased methanogens amounts in soil. No significant differences between the amounts of methanogens in different depths of soil were observed.

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