Short Communication

Sample Preparation for Methanogenic Activities of Heterogeneous Flooded Rice Soils

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Methane in the atmosphere is an important greenhouse gas, and the global increase in methane emissions from wetland rice fields has been thought to be a major cause of the increase in atmospheric methane^{1,13}. When rice fields are flooded, anoxic and reduced conditions develop in the soil and methane is actively produced by methanogens¹⁰. It has been shown that application of rice straw to rice fields causes an increase in methane emission from the paddies^{3,7,8,11,12,14}, and rice roots provide various substrates that enhance microbial activities in the soil⁶. Investigations on microbes in paddy soil are important to understand methane emission from rice fields.

Bulk soil samples collected from paddies are heterogeneous and usually contain various materials such as rice roots, plant residue and small pebbles. Furthermore, two distinct layers, an oxidized surface layer and a reduced layer, develop in the plow layer of flooded paddy soil¹⁰. The heterogeneity of paddy soil makes it very difficult to ensure reproducibility and reliability in microbial investigations of the soil. In view of the heterogeneity, in our previous studies on

the population densities of microbes in paddy soil^{4,5}, we fractionated the bulk soil sample into three parts; soil fractions, living rice roots and plant residue. The heterogeneity of the soil should also be taken into consideration in sample preparations for the determination of microbial activities of flooded rice soil. Thus, in this study, we examined methods of preparing samples for the determination of methanogenic activity after fractionation of paddy soil, as was used for microbial counting in previous studies^{4,5}.

Soil samples for measuring methanogenic activity were obtained from an experimental plot (RS plot) of rice fields of the Shonai Branch, Agricultural Experimental Station of Yamagata Prefecture (Fujishimamachi, Yamagata, Japan). This plot was annually applied with rice straw ($0.5 \text{ t } 10 \text{ a}^{-1}$) and inorganic fertilizers. Rice straw was applied to the soil surface in the autumn after harvesting the rice, and plowed into the soil before flooding the next cropping season. Water was collected from the irrigation canal flowing into the rice field. The water was bubbled with O_2 -free N_2 gas after addition of 0.5 g l^{-1} L-cysteine-HCl $\cdot \text{H}_2\text{O}$ as a reducing agent, then anaerobically autoclaved. This solution was used as the anaerobic diluent for washing and diluting samples in the determination of methanogenic activity.

Bulk core soil samples were collected from the plow

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layer from three different sites in RS plot (depth 0–10 cm) using a core sampler (volume 200 ml, height 10 cm, diameter 5 cm). Pieces of large plant residue (approx. 4–5 mm and greater) and rice roots present in the bulk soil were removed carefully with tweezers by the method described previously^{4,5}. Then, the soil samples from three sites were mixed in a plastic bag to avoid air exposure and used as a soil fraction for the measurement of methanogenic activity. For determination of vertical distributions of methanogenic activity in soil fractions, soil samples obtained by core sampling from the plow layer were divided into four parts depending on the depth (0–1, 3–5, 8–10 and 13–15 cm). For this experiment, soil samples were collected from four sites in RS plot, and soil fractions from different depths were prepared by mixing the samples from the four sites together.

Rice roots were obtained by digging up soil around growing root systems of hills of rice plants using a trowel. Soil adhering to the roots was washed several times with the sterilized anaerobic diluent. Unless otherwise stated, the roots were cut into pieces, washed twice and then used as samples for the measurement of methanogenic activity. Plant residue (mainly rice straw applied to the paddy soil) was collected from the plow layer of the soil. After soil adhering to the plant residue was washed off several times with anaerobic diluent, the plant residue was cut into 1–2 cm lengths, and if necessary, washed several times before use.

Unless otherwise stated, samples of soil fractions were suspended in anaerobic diluent equivalent to the wet weight of the sample (dilution at 1 : 1) and the soil slurries were distributed among glass tubes (18 mm × 180 mm) under O₂-free N₂ gas flow. Rice roots and plant residue samples were also suspended in anaerobic diluent at a 1 : 1 dilution in test tubes. To investigate the effect of homogenization on methanogenic activity, the samples suspended in the diluent at 1 : 1 were homogenized with a Waring blender (10,000 rpm, 10 min) and then distributed into tubes.

Each tube containing the sample (10 ml) suspended in diluent was sealed with a double-butyl rubber stopper under O₂-free N₂ gas flow and immediately incubated at 30°C for 6 h. The methane con-

centration accumulated in the headspace of each tube was measured every two hours by gas chromatography (Hitachi 263 Gaschromatograph: carrier gas: N₂, detector: FID, column: Molecular Sieve 13X-S, column temperature: 60°C, injection temperature: 150°C). The methanogenic activity per dry weight of each sample (mg CH₄ day⁻¹ kg⁻¹ [dry weight]) was calculated from the initial rate of increase in the concentration of methane in the tube and the dry weight

Fig. 1. Effects of suspension and homogenization of samples on methane production. Symbols: ○, with suspension; ●, without suspension; ▲, with homogenization. Error bars represent the standard deviations of replicate experiments.

of the sample. Measurements were carried out in 3–5 replicate tubes.

Increases in methane concentration in the tubes prepared as described above were compared with those of the samples without suspension (Fig. 1). For plant residue samples, methane concentrations increased almost linearly irrespective of whether the samples were suspended. For soil fraction and rice root samples, methane concentrations also increased almost linearly when these samples were suspended in diluent. Without suspension, although methane concentrations rapidly increased during the first two hours of incubation, the increase almost stopped after that. This might indicate that methane was carried over from the samples. This finding is contrary to that reported by Schütz et al.⁹⁾, which indicated linear increases in methane concentration during the incubation of paddy soil without suspension.

When rice roots suspended in the diluent were incubated longer, the roots often turned gray within about 12 hrs (data not shown). This suggested that the condition of rice roots in the tubes changed rapidly during the incubation.

When samples were homogenized in diluent (1 : 1), the methane production was strongly or almost completely suppressed during incubation in all samples (Fig. 1). This may have been caused by the physical

destruction of methanogens, and/or of the methanogenic association which supplies substrates for methanogens adhering to rice roots and plant residue, and also in the soil. We previously enumerated various microbes on rice roots and plant residue after homogenizing the samples in the same way as described above, and detected rather high population densities of methanogens by the MPN method⁴⁾. These two findings suggest that a large number of methanogens can survive the mechanical homogenization of these samples but they can not produce methane in the diluent immediately after the treatment. Dannenberg et al.²⁾ found that the rate of methane production of non-agitated paddy soil slurry was faster than that of vigorously stirred slurry. They thought that this was due to the destruction of associations of acetate-utilizing methanogens, syntrophic microorganisms and other latent microorganisms, which were susceptible to physical force.

Methane production in the soil fraction in replicate tubes proceeded almost at the same rate during incubation and, as shown in Table 1, the deviations among calculated activities per dry weight for replicate experiments were low. Table 1 shows that methanogenic activity in both rice roots and plant residue samples was much higher than that of soil fraction on a dry weight basis.

Table 1. Deviations in methanogenic activities of soil fraction, rice roots and plant residue samples among replicate experiments.

Exp.	Tube No.	Methanogenic activity (mg CH ₄ /kg d.w./day)		
		Soil fraction	Rice Roots	Plant Residue
1	1	1.31	17.5	21.1
	2	1.40	20.6	25.2
	3	1.69	21.5	28.3
	4	N.D.	31.8	28.4
	5	N.D.	42.0	30.5
	Mean ± S.D.	1.47 ± 0.16	26.7 ± 9.0	26.3 ± 3.2
2	1	0.47	9.2	21.6
	2	0.59	9.9	50.4
	3	0.70	13.9	54.6
	4	N.D.	14.2	76.3
	5	N.D.	15.5	128.7
	Mean ± S.D.	0.59 ± 0.10	12.5 ± 2.5	66.2 ± 35.7

N.D., Not determined.

There was no significant difference in the increase in the methane concentration among rice roots of different length (Fig. 2). Cutting the roots was thought to increase the amount of exudate from the sections and affect methane production during incubation, but these findings indicate that the number of sections of rice roots does not significantly affect the increase in methane concentration during this incubation period.

When soil fractions were further diluted at 1 : 3 (4-fold) or 1 : 5 (6-fold) with anaerobic diluent, the increased dilution rates resulted in a considerable reduction in methanogenic activity on a dry weight basis of each sample (Table 2). The methanogenic activity in an ecosystem may be affected by the amounts of substrates available such as acetate or H_2 - CO_2 , as well as the population density and the activity of each methanogen. Thus, this finding may indicate that the

supply of substrates for methanogens is limited by the increased dilution rate, or that the microbial associations in the soil are also destroyed by dilution as seen in the homogenized samples.

Figure 3 shows the vertical distribution of methanogenic activity in the plow layer of paddy soil determined four times during the cropping season of rice. The result obtained on June 25 indicates that the distribution of methanogenic activity in the plow layer is highly heterogeneous under some conditions.

Based on these findings, we designed the procedure for the preparation of samples to determine methanogenic activities in various samples from paddy soils. As rice roots and plant residue which are usually mixed in the bulk soil samples, often have much higher activities than those of soil fractions, it is essential to remove large pieces of these material from the bulk soil samples to obtain reliable values of methanogenic activity from the soil itself. Furthermore, a soil fraction obtained from a plow layer must be mixed well to get representative values of methanogenic activity for the soil in the plow layer, and core soil samples must be divided according to depth for more detailed determinations.

It was also shown that samples should be suspended in anaerobic diluent to obtain linear increases in methane concentration during incubation. As for soil fractions, since the dilution to less than 1 : 1 is practically difficult, and the increased dilution rate results in a decrease in the calculated activities per dry weight, it is practical to suspend the samples in anaerobic diluent at 1 : 1. Rice roots and plant residue samples should also be suspended in diluent after being cut to appropriate lengths. They should be in-

Fig. 2. Effects of the length of rice roots on methane production. Error bars represent the standard deviations of replicate experiments. Symbols: ○, 0–1 cm; ●, 4–6 cm; △, 8–10 cm.

Table 2. Effects of dilution rate of soil fraction on methanogenic activity calculated from the dry weight of the sample.

Dilution rate (fold)	Methanogenic activity (mg CH_4 /kg d.w./day)		
	2	4	6
Exp. 1	15.2 (1)	11.4 (0.75)	5.1 (0.33)
Exp. 2	19.1 (1)	11.9 (0.62)	6.2 (0.32)

Values in parentheses indicate relative activities.

Fig. 3. Vertical profiles of methanogenic activity from soil fractions in the plow layer.

cubated in N₂ gas, and the potential of methanogenic activity per dry weight of each sample should be determined from the initial increasing rate in methane concentration in the tubes and the dry weight of each sample. Vigorous agitation during incubation should be avoided as reported²⁾.

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