Biogeochemistry (2006) 81:33–44
DOI 10.1007/s10533-006-9028-4

GENERAL PAPER

Hydrogen concentration and stable isotopic composition of methane in bubble gas observed in a natural wetland

Atsuko Sugimoto · Noboru Fujita

Received: 18 March 2005 / Accepted: 20 April 2006 / Published online: 3 June 2006

Abstract Bubble gas samples were collected at three different vegetation sites and two different depths (surface and 40 cm) in a natural wetland, Mizorogaike in Kyoto city, to investigate hydrogen concentration and δD and δ^{13}C values of CH_4. Hydrogen concentration in bubble gas varied from 1 to 205 ppm, and that collected during summer was higher than that during winter. Bubble samples collected at 40 cm at sphagnum site usually showed the lowest H_2 concentration among the samples collected at the three sites and two depths on the same day. The lowest H_2 concentration observed at 40 cm at sphagnum site was similar to that expected for environmental water in which H_2 producer and consumer need to assemble for free energy requirement. Low δ^{13}C and high δD (relatively small hydrogen fractionation; $\Delta\delta D_{water-CH_4} \cong 220‰$) were observed in CH_4 collected at a deeper (40 cm) layer of sphagnum site during winter, when H_2 concentration was low (typically 2–4 ppm). On the other hand, CH_4 in the bubble samples collected during summer showed high δ^{13}C and low δD (relatively large hydrogen fractionation; $\Delta\delta D_{water-CH_4} \cong 300‰$), when H_2 concentration was high. Carbon and hydrogen isotope fractionation during CH_4 production were variable, possibly depending on the H_2 concentration and the production rate. Difference in enzymatic reaction and magnitude of hydrogen isotope exchange among water, CH_4, and H_2 may cause the variation in isotope fractionation during CH_4 production.

Keywords Methane · Hydrogen · Isotopic composition · Wetland · Fractionation factor

A. Sugimoto (✉)
Graduate School of Environmental Earth Science, Hokkaido University, Kita 10 Nishi 5, Kita-ku, Sapporo 060-0810, Japan
e-mail: atsukos@ees.hokudai.ac.jp

N. Fujita
Center for Ecological Research, Kyoto University, 509-3 Hirano-2-chome, Otsu 520-2113, Japan

Introduction

Methane is a final product of anaerobic decomposition of organic matter. Methanogens, strict anaerobic microbes, produce CH_4 from CO_2 and H_2, or acetate, which are the main substrates for CH_4 production in the natural environment (Koyama 1955; Takai 1970). These substrates for CH_4 production are produced by other bacteria in a microbial community which decomposes organic matter, that is, the microbial syntrophic system produces CH_4 in the natural environment (Oremland 1988). Hydrogen is a key material for the community producing CH_4.

It is well known that H_2 partial pressure must be kept low to acquire enough energy for microbes producing acetate, CO_2 and H_2 through fermentation (Zehnder and Stumm 1988). Methane producing bacteria can act as a H_2 scavenger in the community. Therefore, a syntrophic system between the H_2 producer and methanogen (the H_2 consumer) can be established. Actually, syntrophic systems consisting of H_2 producers and methanogens have been found (e.g. Zinder and Koch 1984; Krylova and Conrad 1998), and interspecies transfer of H_2 between them is well known. In such syntrophic system, H_2 production may be a limiting factor of CH_4 production.

It is believed that H_2 partial pressure in the CH_4 producing natural system is usually very low. However, only a few data sets on the H_2 partial pressure in a natural system are available. Lovley and Goodwin (1988) showed that dissolved H_2 concentration in CH_4 producing natural sediments ranged from 5 to 10 nM. Similar range (3–12 nM) of H_2 concentration has been also observed in the CH_4 producing sediment at Cape Lookout Bight (Hoehler et al. 1998).

Sugimoto et al. (1998) tried to probe the microbial community in a hindgut of termites producing methane by observing H_2 and CH_4 emissions from the termites, and pointed out that the location of methane producing bacteria (existing in protozoa or on the hindgut wall) may affect the amount of H_2 emitted from the termite. The idea is that emitted H_2 is leakage from the system and the emission rate of H_2 depends on how H_2 is transferred in the system. How H_2 is transferred may affect the partial pressure of H_2 in the microsite of CH_4 production in aquatic ecosystems as well.

The isotopic composition of CH_4 has been investigated by different approaches. One is a determination of fractionation factors during CH_4 production by culture experiments using isolated microbes (Games et al. 1978; Krzycki et al. 1987; Balabane et al. 1987; Gelwicks et al. 1994; Botz et al. 1996). Anothor one is a determination of the isotopic composition of CH_4 by incubation experiments using natural sediments (Sugimoto and Wada 1993, 1995; Waldron et al. 1998; Avery et al. 1999; Alperin et al. 1992; Blair and Carter, 1992; Conrad et al. 2002). Another is observations of δD and δ^{13}C of CH_4 collected in the natural environment (Whiticar et al. 1986; Hornibrook et al. 1997, 2000; Lansdown et al. 1992; Popp et al. 1999). Although many interesting results have been shown, basic phenomena on carbon and hydrogen isotope fractionations during CH_4 production are not fully understood yet.

Reported values for carbon and hydrogen isotope fractionation factors are variable with considerably wide range. For example, different values (1.045 and 1.061 at 40°C) by Games et al. (1978) and a range from 1.048 to 1.079 by Botz et al. (1996) have been shown for carbon isotope fractionation during CH_4 production from CO_2/H_2. Recently, Valentine et al. (2004) showed that carbon isotope fractionation during CH_4 production from CO_2/H_2 was affected by a partial pressure of H_2, and differential irreversibility hypothesis was proposed. As pointed out by Whiticar et al. (1986), large variability of fractionation values have also been reported in various field data, based on a comparison of δ^{13}C between CH_4 and co-existing CO_2. The difference in δ^{13}C between CO_2 and CH_4 observed in freshwater sediments (around 40‰) are generally smaller than that (55–90‰) observed in marine sediments (Whiticar et al. 1986). Observations at natural wetlands and rice paddies also show large variabilities. A smaller difference in δ^{13}C (about 40–50‰) between CO_2 and CH_4 has been observed for CH_4 produced in soil with labile organic matter (Hornibrook et al. 2000; Popp et al. 1999). Difference in contributions of CO_2 reduction and acetate contribution for the CH_4 production is one of the reasons for the variation. Besides, the effect of H_2 partial pressure on the carbon isotope fractionation may also be responsible, nevertheless it has not yet been tested in the natural system.

Hydrogen isotope fractionation factor is also still controversial. When CH_4 is produced from CO_2/H_2, it has been believed that all four hydrogen atoms come from water with a certain isotope fractionation. The small values of difference in δD between water and CH_4 ($\Delta\delta D_{water-CH_4}$) has been reported to be 160‰ for natural gas in a marine environment (Nakai et al. 1974), which is similar to the value (180‰) shown by Whiticar et al. (1986) for CH_4 in marine

sediments. Methane with relatively high δD have been believed to be produced from CO_2/H_2. On the other hand, a much larger $\Delta\delta D_{water-CH_4}$ (lower δD of CH_4) is generally observed in freshwater environments; it has been believed that this CH_4 is dominantly produced from acetate. However, the large $\Delta\delta D_{water-CH_4}$ (about 300‰) were also observed for CH_4 produced from CO_2/H_2 in incubation experiments using rice paddy soil (Sugimoto and Wada 1995) and landfill soil (Waldron et al. 1998), and for that observed in a *Carex* dominated fen (Popp et al. 1999). For those systems (rice paddy soil and fen), labile organic matter is expected to be rich. Furthermore, intermediate values between marine sediments (160 and 180‰) and a labile organic rich system (about 300‰) have been reported in several peat bogs (Hornibrook et al. 1997; Lansdown et al. 1992), where less labile organic matter is expected.

It has been pointed out that the reported discrepancies are caused by the difference in H_2 partial pressure during CH_4 production (Burke 1993; Sugimoto and Wada 1995; Hornibrook et al. 1997). In the natural environment, H_2 partial pressure is expected to be high in the system decomposing labile organic matter, because a high decomposition rate could cause a high H_2 production rate.

Hydrogen may be a key factor as described above. However, only a few data set on the H_2 concentration have been available from field observations. In this study, H_2 concentration in bubble was observed with carbon and hydrogen isotope ratios of CH_4 collected in a natural wetland, to investigate the H_2 partial pressure as a controlling factor of a microbial system producing CH_4, and its isotopic composition.

Observations and analysis

Observation site

Observation was carried out on a floating mat of sphagnum peat at Mizorogaike pond, Kyoto, Japan, from June 1995 to July 1996 every month or twice a month. Bubble methane was sampled at three sites with different typical vegetations (reed, marsh trefoil, and sphagnum sites). Reed and mash trefoil sites are covered by single species of *Phragmites australis* (Cav) Trin. ex Steud, and *Menyanthes trifoliata* L., respectively. *Sphagnum palustre* and *S. caspidatum* are dominant species at sphagnum site, and various species of sedge, grass, iris and tree species are found on it.

Each site shows a characteristic hydrologic regime. The reed site is waterlogged throughout the year, while the water table at the marsh trefoil site varies seasonally depending on the seasonal buoyancy of the floating mat. The sphagnum site is a small hummock (a ridge of microtopography). Details of the observational sites were described in Sugimoto and Fujita (1997).

Sampling of bubbles and water

Bubble gas was taken at the surface and the depth of 40 cm with an inverted funnel with a rubber stopper, by agitating the soil with shaking the funnel. Funnels at 40 cm were pre-installed in the peat soil during the observational period, while those at the surface were set at every time of sampling. At the sphagnum site bubble gas was sampled at the depth of 40 cm only, because it was not possible to collect bubble gas above the water table. Collected gas was transferred into a glass vial with butyl rubber septum, and was brought to the laboratory. Sample was taken from a funnel, and single or double samples were taken depending on the volume of the obtained gas.

Water was sampled at each site at the surface, 30, 60 and 90 cm. When the site was waterlogged, surface water was taken as the surface water sample. When the free water table was below the surface, water was sampled by squeezing the surface. For water samples at 30, 60, and 90 cm, a cylinder (about 5 cm in diameter) with tapered end and holes was inserted into the peat soil, and water which entered the cylinder through holes at its end was sampled.

Precipitation was sampled with a funnel fixed on a container to store the rainwater. To avoid evaporation, liquid paraffin was applied to form a cover on the water surface in the container. Sampling was usually done monthly, but rainwater was sampled more frequently depending on

the amount of rainfall when heavy rainfall was observed.

Analysis

Hydrogen and CH_4 concentrations were analyzed with a gas chromatograph with a semi-conductor detector (model GS-15, Sensertech Co. Ltd., Japan) and a gas chromatograph with an TCD (GC8A, Shimadzu, Japan), respectively. Detection limits were lower than 0.6 ppm for H_2 and about 100 ppm for CH_4, and analytical errors were 5% for both.

For the isotopic composition of bubble methane, first CO_2 was cryogenically removed from the bubble sample, then methane in it was combusted in a CuO filled furnace in a vacuum and the CO_2 and H_2O produced from the CH_4 were collected. The produced CO_2 was purified and H_2O were reduced to H_2 with pre-treated Zn shot. The $\delta^{13}C$ and δD values of CH_4 were obtained by analyzing CO_2 and H_2 with isotope ratio mass spectrometers (delta S or MAT252, Thermo Electron, USA).

Isotopic composition of water was obtained for the sample taken in 1996 with an automatic CO_2/H_2/H_2O equilibration system (Thermo Electron, USA). Water samples taken in 1995 were analyzed only for the oxygen isotope ratio by the CO_2/H_2O equilibration method manually.

All analyses were carried out at Center for Ecological research, Kyoto University.

Results

CH_4 and H_2 concentrations in bubbles

Methane concentrations in bubble gas samples collected at surface and 40 cm of reed site were lower than those collected at 40 cm of marsh trefoils site and sphagnum site (t-test, $P < 0.001$), and that of marsh trefoil at surface was intermediate (Fig. 1a). The maximum value (46%) was observed at 40 cm at the marsh trefoil site at the end of August, while minimum value (2.3%) was obtained at the surface of the reed site in December. Methane concentrations observed here were mostly similar to those observed by Lansdown et al. (1992) at a temperate peatland. The lowest value of CH_4 concentration was close to those observed by Tyler et al. (1997) at rice filed in Texas. Uptake of water by dense roots of grass plants may be attributed to the low concentration of CH_4.

Hydrogen concentration in bubbles varied from atmospheric level (about 1 ppm) to 205 ppm (Fig. 1b). Deviation of the H_2 concentration was large, though the bubble at the surface of the marsh trefoil site showed higher concentration than the other sites and depth ($P < 0.01$). Low concentration of H_2 (< 25 ppm, and typically less than 10 ppm) was observed during winter period from November to April. Average concentration of H_2 during the winter period was lower than that for the other period at all sites and depths, although the difference in H_2 concentration between the winter period and the other season was statistically significant only for the surface and 40 cm of the reed site and 40 cm of the marsh trefoil site ($P < 0.05$).

$\delta^{13}C$ and δD values of bubble methane

Observed $\delta^{13}C$ and δD values of CH_4 in bubble samples were shown in Fig. 2a and b, respectively. Those were within the ranges yet reported for fresh water environment in temperate region (e.g. Hornibrook et al. 1997, 2000; Lansdown et al. 1992; Tyler et al. 1997), however, despite the small area of our observational site, the ranges observed here (–76.7 to –52.8‰ for $\delta^{13}C$ and –371 to –254‰ for δD) were quite large, reflecting vertically and seasonally different conditions of the peat as described later. This is contrasting with a result for the CH_4 produced in an uniform and constant condition, for example the $\delta^{13}C$ of CH_4 (–79 to –71‰) observed in the bottom sediment of north basin of Lake Biwa (Murase and Sugimoto 2001).

The $\delta^{13}C$ of values of bubble methane collected at 40 cm were lower than those at surface for both reed and marshtrefoil site ($P < 0.001$). Surface bubble $\delta^{13}C$ at both sites showed characteristic seasonal variation with a high $\delta^{13}C$ (>–60‰) during summer and relatively low values (< –60‰) during winter, although the difference was statistically significant only for the reed site

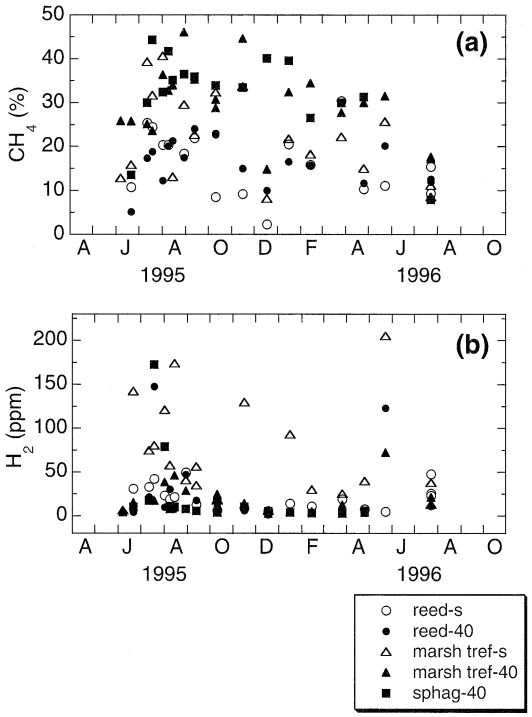

Fig. 1 Seasonal variations of concentration of CH_4 (**a**) and H_2 (**b**) in bubble gas samples collected at the surface and at 40 cm at reed and marsh trefoil sites and those collected at 40 cm at the sphagnum site

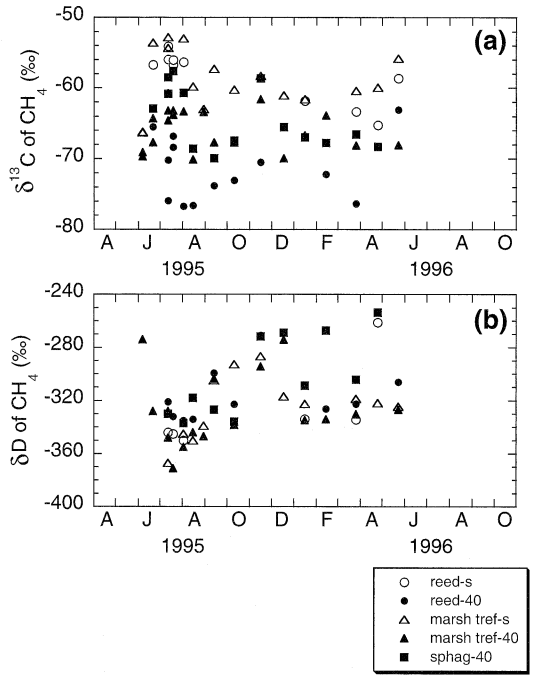

Fig. 2 Seasonal variations of $\delta^{13}C$ (**a**) and δD (**b**) values of CH_4 in bubble samples

($P < 0.01$). At the marsh trefoil site, since large volume of bubble is produced and stored in deeper layer of the soil as its temperature increases in late summer (Sugimoto and Fujita 1997), bubble collected at the surface could be contaminated by the gas produced in deeper layer of the soil and its $\delta^{13}C$ of CH_4 could be also affected. On the other hand, the $\delta^{13}C$ of CH_4 collected at 40 cm at the reed site showed a different seasonal variation: winter maximum was found in contrast to that at surface.

Bubble CH_4 collected at 40 cm at the sphagnum site showed higher $\delta^{13}C$ than that at 40 cm of the reed site ($P < 0.0001$) and the difference between sphagnum site and marsh trefoil site was insignificant.

Observed δD values of bubble CH_4 ranged from –367 to –254‰. During the period from the end of June to the end of August in 1995, all sites showed δD mostly lower than –320‰. Then the δD values of bubble CH_4 at the reed and marsh trefoil sites increased during the period from September to December. The δD value then decreased again and showed a relatively low value, around –330‰ during the period from January to May except for the surface of reed site at the end of April. At the sphagnum site, the bubble CH_4 δD value at 40 cm during winter fluctuated at two different levels of δD values (–270 and –305‰). High δD values (–270 to –260‰) were also found occasionally (e.g. the beginning of June in 1995 at 40 cm of marsh trefoil and the end of April in 1996 at the surface of reed site), which were accompanied by extremely low concentration of H_2 (4.1 and 7.6 ppm for the earlier and latter cases).

δD values of environmental water and difference in δD between water and CH_4

Daily mean air temperature and precipitation observed at Kyoto, and the δD of precipitation collected at Mizorogaike were shown in Fig. 3. The isotopic composition of precipitation in the observed area depends on the amount of precipitation. Rainfall during the Baiu (monsoon rainy season) period (usually in June and July at Kyoto) showed lower δD values (< –50‰) than in other seasons, while the highest δD value (–23‰)

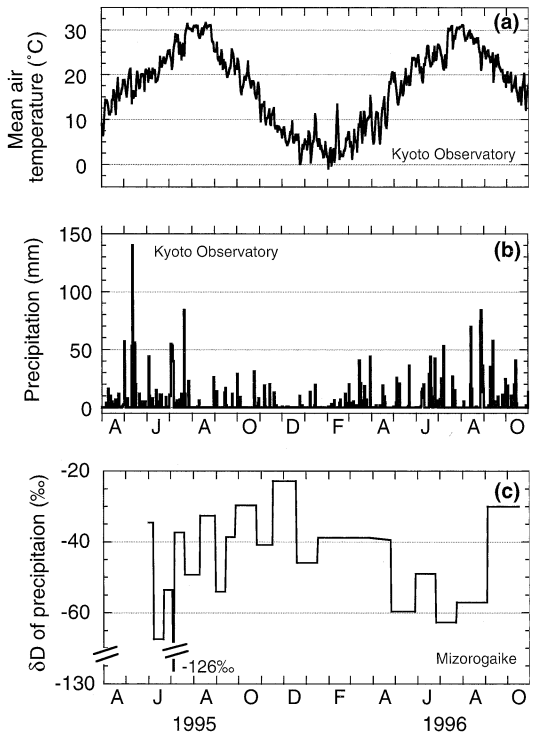

Fig. 3 Daily mean air temperature (**a**) and precipitation (**b**) observed at Kyoto Meteorological observatory, and the δD values of precipitation (**c**) observed at Mizorogaike in Kyoto. The sampling interval for precipitation was usually monthly. However, much more frequent samplings were done depending on the amount of rainfall

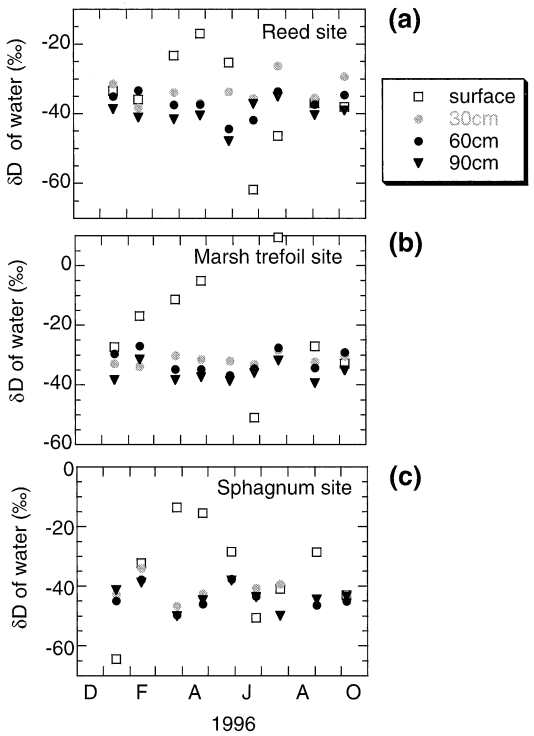

Fig. 4 Surface and soil water δD values at 30, 60, and 90 cm observed at reed site (**a**), marsh trefoil site (**b**) and sphagnum site (**c**). When water lodged on the surface, the lodged water was sampled as surface water. When the water table was below the surface, water was taken from the surface layer by squeezing the surface soil

was observed in November and December in 1995 (Fig. 3c).

Surface water δD values (Fig. 4) showed a large variation reflecting the input of rainwater with low δD value and evaporation of surface water. At the marsh trefoil site, the δD value of the surface water observed in 1996 varied from –51‰ in June to +9‰ in July. On the other hand, soil water at 30, 60 and 90 cm showed relatively constant δD values with slightly lower value at 90 cm than 30 and 60 cm at the reed and marsh trefoil sites. Average δD values of soil water during the observational period in 1996 differed from site to site. Since the difference in the δD values of soil water between 30 and 60 cm was small, average values of them were calculated to be –32, –35, and –43‰ for marsh trefoil, reed, and sphagnum sites, respectively. These differences among sites was caused by the hydrologic difference: at the sphagnum site, summer precipitation with low δD value infiltrates downward because the water table is always below the surface, consequently the δD values of soil water showed relatively lower values than those at the other sites. On the other hand, the free water table at the marsh trefoil site varies seasonally. Surface water evaporates during early summer, then, water with high δD values infiltrates and causes high δD values of soil water at the marsh trefoil site.

The δD values of CH_4 collected at the surface may be influenced by the large fluctuation of δD of surface water, while, the variation in water δD at deeper soil layer was negligible. Difference in δD between water and CH_4, therefore, was calculated for CH_4 collected at 40 cm (Fig. 5), using the values of –32, –35, and –43‰ for water δD at the marsh trefoil, reed, and sphagnum sites, which are the average values for 30 cm and 60 cm during the observational period in 1996.

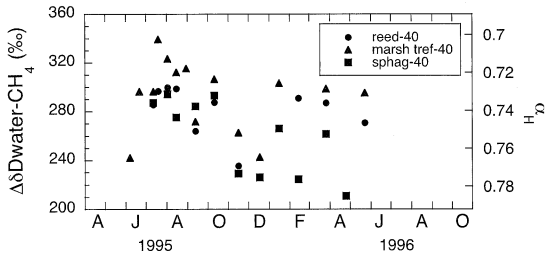

Fig. 5 Difference in δD values between water and CH_4 observed at 40 cm. Corresponding hydrogen fractionation factor defined as $\alpha_H = (D/H)_{CH_4}/(D/H)_{water}$ was also shown in axis at right hand side. For calculation of the difference in δD values, –32, –35, and –43‰ were assumed for the water δD values at reed, marsh trefoil and sphagnum sites, respectively. These are the average values for the δD observed for 30 and 60 cm at each site shown in Fig. 4

Discussion

H_2 partial pressure during CH_4 production and δD and δ^{13}C of bubble CH_4

A large variation in H_2 concentration was observed in the bubble sample (Fig. 1b). This is not exactly equal to the partial pressure in the CH_4 producing microsite nor H_2 concentration in the water. However, it is expected that H_2 observed in the bubble equilibrated with H_2 in the surrounding pore water.

Comparing the H_2 concentrations observed on a day among the sites and depths, minimum value of H_2 concentration was observed mostly in the bubble collected at 40 cm of sphagnum sites. Since cell wall of sphagnum lacks lignin and instead rich in phenol with sphagnum acid which is genus-specific and forms very stable material with polyphenol (reviewed by Van Breemen 1995). As a result, rate of decomposition of sphagnum tissue is extremely low. Slower decomposition of sphagnum than of other plant material (Sugimoto and Fujita 1997) may cause slower production of H_2 and thus lower concentration of H_2 in the bubble. The H_2 concentration at 40 cm of sphagnum sites was typically lower than 10 ppm (Fig. 1b). Such low H_2 concentration is consistent with the typical concentration of dissolved H_2 observed in a CH_4 producing sedimentary environment. Lovely and Goodwin (1988) showed that dissolved H_2 concentration is typically 7–10 nM in a CH_4 producing sedimentary environment. Concentration of H_2 in gas phase equilibrated with 7–10 nM of dissolved H_2 is 8–11 ppm, assuming 0.02 for H_2 solubility.

Minimum value of H_2 concentration appearing in the day also showed seasonality (Fig. 1b), being slightly higher value (10–17 ppm) during summer than in the winter (1–4 ppm). Increase in H_2 concentration with water temperature has been also observed at Cape Lookout Bight by Hoehler et al. (1998). They showed that dissolved H_2 concentrations in pore water were 11 and 3 nM at 27°C and 14.5°C in August and November, respectively. It has been pointed out that such low H_2 concentrations can be achieved by syntrophic colonization between H_2 producers and consumers fringing organic matter, and that H_2 partial pressure is controlled to obtain a constant free energy required for the syntrophic system (Hoehler et al. 2001). Minimum H_2 concentration on each day seen in Fig. 1b (10–17 ppm in summer and 1–4 ppm in winter) were similar to those values (11 and 3 nM) shown by Hoehler et al. (1998, 2001). It appears, therefore, minimum H_2 partial pressures observed in the bubble samples reflected H_2 concentration of syntrophic system on H_2 producing CH_4.

Not only at the sphagnum site but also the reed and marsh trefoil sites, observed H_2 concentration at 40 cm was lower than that at the surface, probably depending on the difference in the rate of decomposition of organic matter. Adding to the decrease in the decomposition rate of organic matter with depth, larger contribution of sphagnum derived material in the deeper layer of peat may cause the slow-down of decomposition. As a result, H_2 production rate may also be low at deeper layer of the peat soil.

High concentration of H_2 was observed in the surface where a high H_2 production was expected. It is also reasonable that extremely high H_2 concentration was observed in July when temperature increase stimulates decomposition and H_2 production but growth of methanogen lags behind. When the concentration of H_2 is high, it is possible for methanogens to grow apart from H_2 producers (free-living status).

Controlling factor for CH_4 $\delta^{13}C$

Generally, CH_4 $\delta^{13}C$ value depends on its production pathway, substrate $\delta^{13}C$, and fractionation factor. Low $\delta^{13}C$ values of CH_4 and large difference in the $\delta^{13}C$ between CO_2 and CH_4 are a typical result of CH_4 production from CO_2/H_2 (Sugimoto and Wada 1993; Avery et al. 1999; Hornibrook et al. 2000; Conrad et al. 2002). Methane production predominantly from CO_2/H_2 has been observed for natural wetlands, especially for peat bogs (Lansdown et al. 1992; Kelley et al. 1992). Meanwhile, Avery et al. (1999) investigated production pathway and $\delta^{13}C$ of CH_4 at temperate peatland, and showed that CH_4 was predominantly produced from CO_2/H_2 during winter, while acetate was a main substrate of production of CH_4 with high $\delta^{13}C$ during summer. Production pathway was not determined in our observation. However, we expect that main substrate of CH_4 production was CO_2/H_2, especially for the bubbles collected at 40 cm and those collected during winter.

In this study, the $\delta^{13}C$ of CO_2 was not obtained for all samples because of a problem in storage of bubble samples, but several data were available. The $\delta^{13}C$ values of CO_2 in bubbles collected on October 11 in 1995 were –11.5, –6.7, –7.7, –5.6, and –2.9‰ for the surface and 40 cm at the reed site, surface and 40 cm at marsh trefoil site, and 40 cm at sphagnum site, respectively. The $\delta^{13}C$ of CO_2 at the surface was lower than that at 40 cm. The difference in the $\delta^{13}C$ value between CO_2 (described before) and CH_4 (Fig. 2a) observed at 40 cm on October 11, 1995, was 66.3, 62.0, and 65.3‰ at the reed, marsh trefoil and sphagnum sites, respectively, and 64.5‰ in average, whereas that for the surface at the marsh trefoil was 52.5‰, indicating apparent carbon fractionation observed between CO_2 and CH_4 was smaller in the surface than 40 cm.

The $\delta^{13}C$ of CO_2 is generally lower at the surface than 40 cm because of higher rate of CO_2 production at the surface. The $\delta^{13}C$ of CH_4 is, by contrast, higher at the surface than at 40 cm as seen in Fig. 2a, consequently, the difference in the $\delta^{13}C$ value between CO_2 and CH_4 is smaller at the surface than 40 cm. As a result, apparent fractionation between CO_2 and CH_4 at the surface is smaller than that at 40 cm. One of the reasons for observed difference in apparent fractionation is "closed effect" in the deeper soil layer, and another possibility is larger fractionation due to higher partial pressure of H_2 in the surface layer.

Figure 6a shows relationship between $\delta^{13}C$ of CH_4 and concentration of H_2. Relationship is not so clear probably due to the close effect as described above, though it can be said that higher concentration of H_2 than 50 ppm was accompanied by high $\delta^{13}C$ of CH_4 observed, while lower $\delta^{13}C$ was observed with low concentration of H_2 in the site of the depth. Recently, Valentine et al. (2004) proposed that carbon isotope fractionation depends on the difference in reversibility of CO_2 molecules which is caused by the difference in H_2 partial pressure in the biochemical reaction steps of CO_2 reduction to CH_4. They suggested that carbon isotope fractionation is large when H_2 partial pressure is low because biochemical reaction is reversible at the fourth step out of 7 steps, while carbon fractionation is small when H_2 partial pressure is high due to irreversibility of the

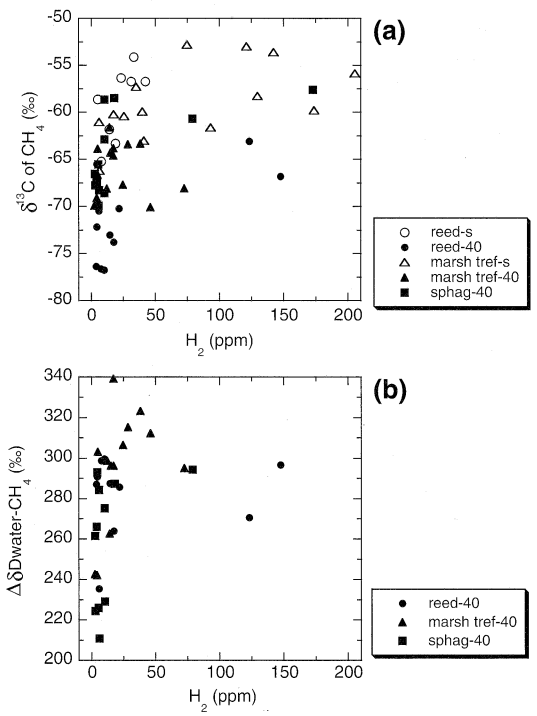

Fig. 6 Relationship between $\delta^{13}C$ of CH_4 and H_2 concentration (**a**) and that between $\Delta\delta D_{water-CH_4}$ and H_2 concentration in bubbles collected at 40 cm (**b**)

reaction at the fourth step. Our observational results are consistent with their hypothesis.

In natural systems, it has been pointed out that decomposition of labile (or fresh) organic matter produces a CH_4 with higher $\delta^{13}C$ values than that of old and less reactive organic matter does (e.g. Jenden and Kaplan 1986; Sugimoto and Wada 1995; Hornibrook et al. 1997). Larger contribution of CH_4 from acetate as shown by Avery et al. (1999) is one of the reasons for the high $\delta^{13}C$ values of CH_4 during summer. In addition, high partial pressure of H_2 in the system decomposing labile organic matter may cause the high $\delta^{13}C$ of CH_4 because of smaller fractionation between CO_2 and CH_4 during CH_4 production as observed at the surface of marsh trefoil site in our observation.

Hydrogen as a controlling factor of CH_4 δD

The δD of CH_4 generally depends on the δD of water and substrates and fractionation during CH_4 production. Since observed δD of water was almost constant at 40 cm, we discuss hydrogen isotope fractionation during CH_4 production at 40 cm here based on the difference in δD between CH_4 and water. Figure 5 shows the difference in δD values between water and CH_4 observed at 40 cm and hydrogen fractionation factor (α_H) defined to be $(D/H)_{CH_4}/(D/H)_{H_2O}$, calculated using the average values for water δD obtained in the section 'δD values of environmental water and difference in δD between water and CH_4'.

Difference in δD value between water and CH_4 ($\Delta\delta D_{water-CH_4}$) at 40 cm ranges from 211 to 339‰ (Fig. 5). The value of $\Delta\delta D_{water-CH_4}$ around 220‰ observed at 40 cm of sphagnum site during winter is similar to that reported by Lansdown et al. (1992), and much larger than those reported for CO_2/H_2 reduction in marine sediments (160–180‰) by Whiticar et al. (1986) and Nakai (1974). The value around 300‰ is similar or slightly smaller than that obtained by Sugimoto and Wada (1995) in an incubation experiment using paddy soil, and much smaller than that (about 400‰) obtained by Balabane et al. (1987) using a pure culture of *Methanobacterium formicicum* in a pressurized atmosphere with 80% of H_2. Obtained value around 300‰ was also similar or slightly smaller than that obtained by Chidthaisong et al. (2002) for soil enrichment incubation experiment.

As seen in Figs. 5 and 6b, $\Delta\delta D_{water-CH_4}$ correlates with H_2 concentration. Smaller value of the $\Delta\delta D_{water-CH_4}$ (about 220‰) was all observed during the period from winter to spring when the H_2 concentration in the bubble was very low (typically 2–4 ppm). Meanwhile, the $\Delta\delta D_{water-CH_4}$ was large (≥300‰) during summer mostly when H_2 partial pressure was high. Although the large $\Delta\delta D_{water-CH_4}$ was also observed during winter at 40 cm of reeds and marsh trefoil sites, despite the low concentration of H_2, it is not surprising that $\Delta\delta D_{water-CH_4}$ is not strictly corresponding to the H_2 concentration, because H_2 partial pressure in the microsite of CH_4 production is expected to be much higher than those in the environmental water and bubble.

Although the mechanism of variation in hydrogen fractionation between water and CH_4 from CO_2/H_2 is not clear at this moment, however, it may vary as a result of isotope exchange among water H_2, and CH_4 as below.

Variation in the hydrogen fractionation means the difference in δD of hydrogen atoms incorporated into CH_4. If all four hydrogen atoms were incorporated into CH_4 from H_2 produced in the water without fractionation during CH_4 production, CH_4 with extremely low δD as low as H_2 δD might be produced, and the CH_4 δD still reflects water δD, because H_2 was produced after isotope exchange with water in the natural system. On the other hand, if CH_4 itself was fully equilibrated with water at the time of its production, the δD of CH_4 would be much higher than that observed. According to the β factor calculated by Richet et al. (1977), $\Delta\delta D_{water-CH_4}$ may be about 720‰ and 80‰ at 20°C for the earlier and the latter cases, respectively. Observed δD value of CH_4 in natural environment is within this range of δD.

One of the possible explanations is variation in the extent of isotope exchange caused by the difference in the rate of CH_4 production. When CH_4 production rate is low under a low H_2 partial pressure, larger extent of isotope exchange between water and CH_4 may cause smaller $\Delta\delta D_{water-CH_4}$, in other words higher δD of CH_4.

Valentine et al. (2004) also suggested that hydrogen fractionation may vary with the rate of CH_4 production in pure culture experiment. This may be understood by longer time for CH_4 production and by lower possibility for direct incorporation of H_2 into CH_4.

The second possible explanation is a different hydrogenase functioning under a different H_2 partial pressure. There are various hydrogenases found in vivo (Thauer et al. 1993): hydrogenase functioning in methanogenesis may vary from species to species, and also may vary depending on the H_2 partial pressure. It has been also recognized that some of hydrogenases localize on the cytoplasmic membrane, and are probably functioning as an association with the membrane (Sprott and Beveridge 1993). Those hydrogenases may catalyze hydrogen isotope exchange outside. It is, therefore, expected that each hydrogenase shows specific way of hydrogen uptake and different fractionation among water, H_2, and CH_4, because some of them may take up hydrogen from water after some extent of isotope exchange, and the other may just catch hydrogen in the cell. There may be also a large difference in affinity with H_2, probably causing a variation in the fractionation. Diffusion of H_2 to the site of enzymatic reaction may also affect the δD of hydrogen atoms incorporated into the CH_4 molecule.

Two isofunctional genes have been found for three reactions among seven and for the enzyme to take up H_2, and two types of hydrogenase have been also found, in biochemical reactions during CH_4 production from CO_2/H_2. Luo et al. (2002) showed that expressions of these genes were regulated by H_2 partial pressure in the system, from the comparison between two different conditions on H_2, namely pure culture in the pressurized H_2 atmosphere and co-culture with fatty acid oxidizing bacteria in which H_2 partial pressure was kept low because of interspecies transfer of H_2. It has been observed that availability of H_2 also regulates the growth yield of bacteria (Morgan et al. 1997). Such regulations of the gene expression and bacterial growth with H_2 concentration may be an adaptation to the low H_2 concentration in the natural condition, and hydrogen fractionation may vary with the combination of these enzymes functioning in vivo.

The third possible explanation is the difference in δD between H_2 in the environmental water and that transferred directly from H_2 producer to methanogen. When H_2 partial pressure is high, free-living methanogens (living without tight relationship with another microorganisms) can grow, using H_2 released from H_2 producer, of which δD is expected to be extremely low due to the fractionation between water and H_2. On the other hand, when H_2 partial pressure is very low, methanogen only in a syntrophic system can survive. Since H_2 transferred from H_2 producer to methanogen is not released outside of the system, it is possible that the extent of isotope exchange between water and CH_4 ($\Delta\delta D_{water-CH_4}$) differs between syntrophic system and free-living methanogen.

Concluding remarks

Although the correlation between H_2 concentration and isotopic composition of CH_4 in the bubble samples was not strict, low $\delta^{13}C$ and high δD values of CH_4 (thus large carbon isotope fractionation between CO_2 and CH_4 and small hydrogen fractionation between water and CH_4) tended to be found with low H_2 concentration in the bubble collected at deeper soil layer (40 cm) during winter. On the other hand, high $\delta^{13}C$ and low δD values of CH_4 were observed during summer when H_2 partial pressure was high.

Low value of $\Delta\delta D_{water-CH_4}$ observed for 40 cm at the sphagnum site was around 220‰ during winter when H_2 partial pressure was very low, typically 2–4 ppm, corresponding to 2–4 nM of dissolved H_2 concentration assuming equilibrium with the bubble. Such a low level of H_2 has never been performed in culture experiments. Fractionation between water and CH_4 observed at this condition (220‰) is, however, still larger than that observed in marine sediments (160–180‰). To understand the mechanism of variations in carbon and hydrogen isotope fractionations during CH_4 production, further investigations are required with taking account of way of hydrogen incorporation into the CH_4 molecule under such low concentration of H_2 as observed here in a natural wetland.

Acknowledgements This work was partly supported by Grant-in-aid No. 14301 from Ministry of Education, Sports, and Culture, Japan. The authors thank to Mr. Kojima (CER Kyoto University) for his assistance in field observations and samplings. We are grateful to Prof. Wada and other people working for CER Kyoto University for their help and useful discussions. We also thank to anonymous reviewer for the helpful comments.

References

Alperin MJ, Blair NE, Albert DB, Hoehler TM, Martens CS (1992) Factors that control the stable isotopic composition of methane produced in an anoxic marine sediment. Global Biogeochem Cycles 6:271–291

Avery GB Jr, Shannon RD, White JR, Martens CS, Alperin AJ (1999) Effect of seasonal changes in the pathways of methanogenesis on the $\delta^{13}C$ values of pore water methane in a Michigan peatland. Global Biogeochem Cycles 13:475–484

Balabane M, Galimov E, Hermann M, Letolle R (1987) Hydrogen and carbon isotope fractionation during experimental production of bacterial methane. Org Geochem 11:115–119

Blair NE, Carter WDJ (1992) The carbon isotope geochemistry of acetate from a methanogenic marine sediment. Geochim Cosmochim Acta 56:1247–1258

Botz R, Pokojski H-D, Schmitt M, Thomm M (1996) Carbon isotope fractionation during bacterial methanogenesis by CO_2 reduction. Org Geochem 25:255–262

Burke RAJ (1993) Possible influence of hydrogen concentration on microbial methane stable hydrogen isotopic composition. Chemosphere 26:55–67

Chidthaisong A, Chin K-J, Valentine DL, Tyler SC (2002) A comparison of isotope fractionation of carbon and hydrogen from paddy field rice roots and soil bacterial enrichments during CO_2/H_2 methanogenesis. Geochim Cosmochim Acta 66:983–995

Conrad R, Klose M, Clus P (2002) Pathway of CH_4 formation in anoxic rice field soil and rice roots determined by 13C-stable isotope fractionation. Chemosphere 47:797–806

Games LM, Hayes JM, Gunsalus RP (1978) Methane-producing bacteria: natural fractionations of the stable carbon isotopes. Geochim Cosmochim Acta 42:1295–1297

Gelwicks JT, Risatti JB, Hayes JM (1994) Carbon isotope effects associated with autotrophic acetogenesis. Appl Environ Microbiol 60:467–472

Hoehler TM, Alperin MJ, Albert DB, Martens CS (1998) Thermodynamic control on hydrogen concentration in anoxic sediments. Geochim Cosmochim Acta 62:1745–1756

Hoehler TM, Aplerin MJ, Albert DB, Martens CS (2001) Apparent minimum free energy requirements for methanogenic archaea and sulfate-reducing bacteria in an anoxic marine sediment. FEMS Microbiol Ecol 38:33–41

Hornibrook ERC, Longstaffe FJ, Fyfe WS (1997) Spacial distribution of microbial methane production pathways in temperate zone wetland soil: stable carbon and hydrogen isotope evidence. Geochim Cosmochim Acta 61:745–753

Hornibrook ERC, Longstaffe FJ, Fyfe WS (2000) Evolution of stable isotope composition for methane and carbon dioxide in freshwater wetlands and other anaerobic environment. Geochim Cosmochim Acta 64:1013–1027

Jenden PD, Kaplan IR (1986) Comparison of microbial gases from the Middle American Trench and Scripps Submarine Canyon: implication for the origin of natural gas. Appl Geochem 1:631–646

Kelley CA, Dise NB, Martens CS (1992) Temporal variations in the stable carbon isotopic composition of methane emitted from Minnesota peatlands. Global Biogeochem Cycles 6:263–269

Koyama T (1955) Gaseous metabolism in lake muds and paddy soils. J Earth Sci Nagoya Univ 3:65–76

Krylova N, Conrad R (1998) Thermodynamics of propionate degradation in methanogenic paddy soil. FEMS Microbiol Ecol 26:281–288

Krzycki JA, Kenealy WR, DeNiro MJ, Zeikus JG (1987) Stable carbon isotope fractionation by *Methanosarcina barkeri* during methanogenesis from acetate, methanol, or carbon dioxide-hydrogen. Appl Environ Microbiol 53:2597–2599

Lansdown JM, Quay PD, King SL (1992) CH_4 production via CO_2 reduction in a temperate bog: a source of ^{13}C-depleted CH_4. Geochim Cosmochim Acta 56:3493–3503

Lovley DR, Goodwin S (1988) Hydrogen concentration as an indicator of the predominant terminal electron-accepting reactions in aquatic sediments. Geochim Cosmochim Acta 52:2993–3001

Luo H-W, Zhang H, Suzuki T, Hattori S, Kamagata Y (2002) Differential expression of methanogenesis genes of Methanothermobactor thermoautotrophicus (formerly Methanobacterium thermoautotrophicum) in pure culture and in cocultures with fatty acid-oxidizing syntrophs. Appl Environ Microbiol 68:1173–1179

Murase J, Sugimoto A (2001) Methane in sediments of Lake Biwa: spatial distribution of abundance and stable isotopic composition. Geochem J 35:257–263

Morgan RM, Phil TD, Nolling J, Reeve JN (1997) Hydrogen regulation of growth, growth yields, and methane gene transcription in Methanobacterium thermoautotrophicum DH. J Bacteriol 179:889–898

Nakai N, Yoshida Y, Ando N (1974) Isotopic studies on oil and natural gas fields in Japan. Chikyu Kagaku 7/8:87–98

Oremland RS (1988) Biogeochemistry of methanogenic bacteria. In: Zehnder AJB (ed) Biology of anaerobic microorganisms. A John Wiley & Sons, Inc., New York, pp 641–705

Popp TJ, Chanton JP, Whiting GJ, Grant N (1999) Methane stable isotope distribution at a Carex dominant fen in north central Alberta. Global Biogeochem Cycles 13:1063–1077

Richet P, Bottinga Y, Javoy M (1977) A review of hydrogen, carbon, nitrogen, oxygen, sulphur, and chlorine stable isotope fractionation among gaseous molecules. Ann Rev Earth Planet Sci 5:65–110

Sprott GD, Beveridge TJ (1993) Microscopy. In: Ferry JG (ed) Methanogenesis. Ecology, physiology, biochemistry & genetics. Chapman & Hall, New York, pp 81–127

Sugimoto A, Fujita N (1997) Characteristics of methane emission from different vegetations on a wetland. Tellus 49B:382–392

Sugimoto A, Wada E (1993) Carbon isotopic composition of bacterial methane in a soil incubation experiment: contributions of acetate and CO_2/H_2. Geochim Cosmochim Acta 57:4015–4027

Sugimoto A, Wada E (1995) Hydrogen isotopic composition of bacterial methane: CO_2/H_2 reduction and acetate fermentation. Geochim Cosmochim Acta 59:1329–1337

Sugimoto A, Inoue T, Tayasu I, Miller L, Takeichi S, Abe T (1998) Methane and hydrogen production in a termite-symbiont system. Ecol Res 13:241–257

Takai Y (1970) The mechanism of methane fermentation in flooded paddy soil. Soil Sci Plant Nutr 6:238–244

Thauer RK, Hedderich R, Fischer R (1993) Reactions and enzymes involved in methanogenesis from CO_2 and H_2. In: Ferry JG (ed) Methanogenesis. Ecology, physiology, biochemistry & genetics. Chapman & Hall, New York, pp 209–252

Tyler SC, Bilek RS, Sass RL, Fisher FM (1997) Methane oxidation and pathways of production in a Texas paddy field deduced from measurements of flux, $\delta^{13}C$ and δD of CH_4. Global Biogeochem Cycles 11:323–348

Valentine DL, Chidthaisong A, Rice A, Reeburgh WS, Tyler SC (2004) Carbon and hydrogen isotope fractionation by moderately thermophilic methanogens. Geochim Cosmochim Acta 68:1571–1590

Van Breemen N (1995) How sphagnum bogs down other plants. Trends Ecol Evol 10:270–275

Waldron S, Watson-Craik IA, Hall AJ, Fallick AE (1998) The carbon and hydrogen stable isotopes composition of bacteriogenic methane: a laboratory study using a landfill inoculum. Geomicrobiology 15:157–169

Whiticar MJ, Faber E, Schoell M (1986) Biogenic methane formation in marine and freshwater environments: CO_2 reduction vs. acetate fermentation – isotope evidence. Geochim Cosmochim Acta 50:693–709

Zehnder AJB, Stumm W (1988) Geochemistry and biogeochemistry of anaerobic habitat. In: Zehnder AJB (ed) Biology of anaerobic microorganisms. John Wiley & Sons, Inc., New York, pp 1–38

Zinder SH, Koch M (1984) Non-aceticlastic methanogenesis from acetate: acetate oxidation by a thermophilic coculture. Arch Microbiol 138:263–272