

Dual stable isotope characterization of excess methane in oxic waters of a mesotrophic lake

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Abstract

To determine the source of excess methane in oxic, surface-water columns often found in freshwater environments, we measured the in situ concentration and stable isotopic compositions ($\delta^{13}\text{C}$ and $\delta^2\text{H}$) of methane in Lake Biwa, a mesotrophic lake in Japan. The values from the littoral zone and lake-floor sediments were determined, besides those in the water column of the pelagic zone. Furthermore, we conducted incubation experiments to measure microbial oxidation rates and alterations in the isotopic signatures of methane. We found significant vertical and seasonal variations in both in situ concentrations and stable isotopic compositions of methane measured in the pelagic zone. We concluded that active microbial oxidation was primarily responsible for the variation in $\delta^{13}\text{C}$ and $\delta^2\text{H}$ values of methane in the pelagic water column. As a result, we defined a new indicator $\Delta(2,13)$ to characterize the sources of dissolved methane, in which variations in both $\delta^{13}\text{C}$ and $\delta^2\text{H}$ during methane oxidation had been corrected. The excess methane in oxic, surface-water columns exhibited $\Delta(2,13)$ values similar to those in the littoral zone. We concluded that excess methane at the surface of the pelagic zone originated from the littoral zone via lateral transport. Anoxic near sediments and inflowing rivers were responsible for methane enrichment in water of the littoral zone and in the surface water columns of the pelagic zone.

Atmospheric methane (CH_4) is a known greenhouse gas whose tropospheric concentration continues to increase (IPCC 2014). The hydrosphere is a major source of atmospheric methane (IPCC 2014), and changes in the aquatic environment could result in increases in the concentration of tropospheric methane and contribute to global warming. Generally, methane is produced in anoxic environments and decomposed in oxic environments (Kiene 1991). Nevertheless, the supersaturation of methane in lacustrine water columns, relative to its concentration in equilibrium with the atmosphere, has been frequently observed in oxic lakes (Rudd and Hamilton 1978; Utsumi et al. 1998; Nishimura et al. 1999; Bastviken et al. 2004; Tang et al. 2014, 2016).

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Anoxic sediments in the littoral zone and in the mouths of inflowing rivers (estuaries) have been assumed to be a potential source of methane enrichment in oxic, lacustrine water columns (Schmidt and Conrad 1993; Miyajima et al. 1997; Murase et al. 2003; Bastviken et al. 2004; Fernández et al. 2016; Delsontro et al. 2018; Peeters et al. 2019). However, other in situ sources have also been examined recently. Karl et al. (2008) proposed that methane can be formed as a byproduct when nitrogen-fixing bacteria decompose methylphosphonate (MPn) in a marine environment. Based on an experiment conducted using floating mesocosms in a Canadian temperate shield lake (Lac Cromwell), Bogard et al. (2014) proposed that in situ methanogenesis in oxic waters can be the primary source of lacustrine methane in the atmosphere, and that the same mechanism can also operate in other large, deep lakes and open oceans. During a metagenomic survey of the surface water in Lake Matano (a deep, permanently stratified lake in Indonesia), Yao et al. (2016) found genes related to the C–P lyase pathway, which cleaves C–P bonds in phosphonate compounds. During in vitro laboratory experiments, substantial methane production was detected from the marine alga *Emiliania huxleyi* (Lenhart et al. 2016) and cyanobacteria (Bižić et al. 2020). All these studies proposed that in situ formation of methane in the water column contributes to methane

accumulation in oxygen-saturated marine and lacustrine surface waters. Donis et al. (2017) estimated that approximately 90% of lacustrine methane emissions in the atmosphere are derived from the methane produced within the oxic surface mixed layer, based on the calculated mass balance in a temperate mesotrophic lake (Lake Hallwil) in Switzerland during its stratified period. They also concluded that the production pathways and precursors of in situ methane are different from those of sedimentary methane, based on the difference in the stable carbon isotopic composition ($\delta^{13}\text{C}$) between methane in the surface mixed layer (-62‰ to -60‰) and methane in the sedimentary layer (-65‰ to -75‰). They reported that similar ^{13}C -enriched $\delta^{13}\text{C}$ values in the surface mixed layer, relative to sedimentary methane, can be found in other oxic lakes, such as Lake Stechlin (-50‰ ; Tang et al. 2014), Lake Lugano (-55‰ ; Bleses et al. 2015), Lake Cromwell (-40‰ ; Bogard et al. 2014), and Lake Biwa (-62‰ to -21‰ ; Murase et al. 2003).

The stable isotopic composition of methane has been widely used as a tool for identifying methane sources (Craig 1953; Tsunogai et al. 1998, 2012; Sasakawa et al. 2008). However, to determine the methane source in oxic water columns using $\delta^{13}\text{C}$ as a tracer, we need to correct for the isotopic fractionation from the progression of microbial methane oxidation within the water column, because microbial oxidation can result in ^{13}C -enriched values (Coleman et al. 1981; Nishimura et al. 1999; Tsunogai et al. 2000; Feisthauer et al. 2011). Because microbial methane oxidation is generally rapid in oxic lacustrine waters (Jannasch 1975; Lidstrom and Somers 1984; Whitticar and Faber 1986; Utsumi et al. 1998; Nishimura et al. 1999; Murase et al. 2005), the $\delta^{13}\text{C}$ of methane in the water column can differ from the original value immediately after its production. Because the mass balance calculation conducted by Donis et al. (2017) also admitted of doubts in recent studies (Peeters et al. 2019), further studies are needed to clarify the source of excess methane in oxic, surface-water columns often found in freshwater environments.

During the microbial oxidation of methane, both $\delta^{13}\text{C}$ and $\delta^2\text{H}$ are discriminated (Coleman et al. 1981). The ratio of hydrogen to carbon isotopic discrimination during methane oxidation has been found to be similar, irrespective of the types of methane monooxygenases (Feisthauer et al. 2011), so that the rate of change of $\delta^{13}\text{C}$ and $\delta^2\text{H}$ in residual methane is almost constant in oxic environments (see Experimental section for details). Therefore, by simultaneously measuring both $\delta^{13}\text{C}$ and $\delta^2\text{H}$ values of methane in oxic water columns, and by comparing them with the values in potential sources (such as methane in the sedimentary layer and that in the littoral zone), we can distinguish whether each potential source contributed to methane in the oxic surface waters, irrespective of the progression of microbial oxidation subsequent to the production.

In this study, we determined the concentration, $\delta^{13}\text{C}$, and $\delta^2\text{H}$ value of methane in a pelagic water column of Lake

Biwa—a temperate, mesotrophic freshwater lake in Japan—where the spatial distribution and dynamics of methane have been studied (Miyajima et al. 1997; Murase and Sugimoto 2001, 2002; Murase et al. 2003, 2005). Although the $\delta^{13}\text{C}$ value of methane has been determined in oxic, lacustrine water columns in the world (Nishimura et al. 1999; Murase et al. 2003; Bogard et al. 2014; Tang et al. 2014; Bleses et al. 2015), little has been reported on the simultaneous dynamics of $\delta^{13}\text{C}$ and $\delta^2\text{H}$ in methane. We calculated the vertical distribution and seasonal variations of these values to determine the dual isotopic characteristics of methane oversaturated in the oxic water column. Additionally, from our comparison of the dual isotopic characteristics of methane in the oxic water column with those of methane in potential sources, we discuss the contribution of each possible source to the methane present in the oxic pelagic water column.

Experimental section

Site description

Lake Biwa, located in the central part of the Japanese Islands, is the largest freshwater lake in Japan (Fig. 1). It has a surface area of 670 km² and a total catchment area of 3174 km². More than 120 rivers flow into the lake, but the Seta River, at the southern end of the lake, also known as the Yodo River, is the only natural outflow (Fig. 1b). The main basin of Lake Biwa is mesotrophic. Thermal stratification is present in the lake from May to December, and vertical water convection toward the lake bottom occurs regularly from January to April. Based on seasonal changes in the thermal stratification of the lake water column, we classified each month in our study into one of the following three seasons: stratified (June–September), mixing (November–April), and transitional (May and October).

The average depth and water residence time of the basin are 43 m and 5.5 years, respectively. The depth of the euphotic zone during the stratified season ranges from 10 to 21 m, with an average depth of 15 m (Urabe et al. 1999). Although the emergence of suboxic water in the lake bottom (less than 10% of the saturation level), resulting from insufficient vertical water convection during the preceding winter, has been reported (Kitazawa and Kumagai 2007), the water column was generally oxic during our study.

Sampling

All samples were collected during 17 field campaigns from 2013 to 2018. The pelagic, lacustrine water samples were collected at approximately 5 m intervals through both epilimnion and thermocline, and approximately 20 m intervals through hypolimnion using a 5 L Niskin bottle onboard a research vessel, either the R/V HASU (Center for Ecological Research, Kyoto University) or the R/V Hassaka (University of Shiga Prefecture), at two stations: H1 (35°23'41"N, 136°7'57"E; depth = 85 m; until the May 2016 sampling campaign) or H5

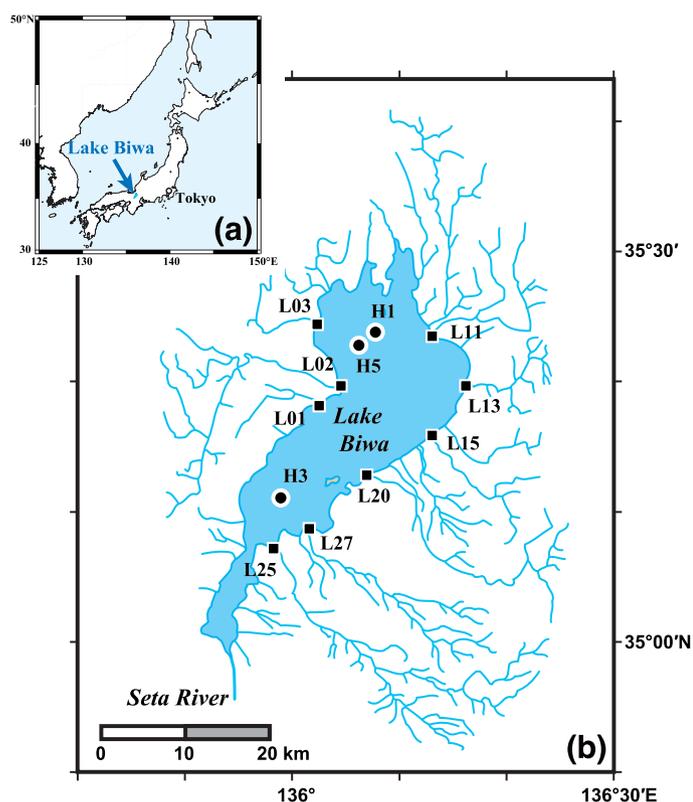


Fig. 1. Map showing the location of Lake Biwa in Japan (a), along with the locations of sampling stations at the pelagic zone (black circles) and the littoral zone (black squares) (modified from Tsunogai et al. 2016) (b).

(35°22'46"N, 136°6'10"E; depth = 95 m; from the July 2016 campaign). Besides the routine stations (H1/H5), pelagic, lacustrine water samples were also collected at Sta. H3 (35°11'39"N, 135°59'39"E; depth = 65 m) during six preliminary sampling campaigns in 2013 and 2014, to check for potential spatial differences in the lake. Vertical profiles of temperature and conductivity were determined using Sea-bird SBE911 plus (Table S1). The locations of the Sta. H1 and H3 corresponded to those of the monthly lake water-monitoring Sta. 17B and 12B, respectively, managed by the Lake Biwa Environmental Research Institute. The details of the field campaigns have already been reported, together with concentration and stable isotopic compositions of nutrients in the lake water samples collected simultaneously in 2013 (Tsunogai et al. 2018).

To determine the concentration and stable compositions of methane in the littoral zone, surface water samples were collected using a bucket at nine stations (L01, L02, L03, L11, L13, L15, L20, L25, and L27; Fig. 1b) along the lake shore, during the six sampling campaigns in 2013 and 2014. Because Lake Biwa's shore was often covered by dense aquatic plants during the summer, it was difficult to take water samples using the research vessels without causing significant disturbances. Instead, all littoral zone water samples were collected from

bridges that crossed over the mouths of major rivers during the base flow periods. Because the mouths of the major rivers flowing into Lake Biwa have been dredged to prevent flooding in the basin area, the apparent flow rates were almost zero, and thus, the water was well-mixed with surficial lake water during the base flow periods. As a result, we regarded lacustrine water from the mouths of major inflowing rivers as representative of the littoral zone. Sampling surface water from the bridges also minimized the sample disturbance.

The subsamples for determining the in situ concentration and stable compositions of methane were slowly transferred to either 125- or 250-mL vials from the Niskin bottle or the bucket. After approximately threefold volume overflow to prevent air contamination, 0.5 mL of HgCl₂ solution (3% by weight) was slowly added as a preservative (Tsunogai et al. 1998, 2012; Nishimura et al. 1999). The HgCl₂ solution was degassed before its addition to minimize the atmospheric contamination. The vials were sealed without headspace with a butyl stopper and an aluminum crimp (Tsunogai et al. 1998, 2000; Nishimura et al. 1999), and stored in the dark at 6°C until being analyzed in our laboratory. Most samples were analyzed within a month, and the longest storage period was 3 months.

Incubation experiments were conducted to evaluate changes in stable isotopic composition ($\delta^{13}\text{C}$ and $\delta^2\text{H}$) due to microbial activity. The subsamples used in the incubation experiments were collected during 8 of the 17 campaigns (Table S3) and treated similarly to other samples, except for the addition of a preservative (Utsumi et al. 1998; Nishimura et al. 1999). The vials for the incubation experiments were cleaned in an acid bath before use. After 36 h of incubation at 6°C, these vials were poisoned with HgCl₂ by injection through each butyl stopper, to stop further microbial methane oxidation activity in the vials. Besides the samples incubated for 36 h, we simultaneously collected additional water samples from each Niskin bottle or bucket for a longer incubation. The maximum incubation period was 240 h (Table S3). A sample collected at 90 m on 19 December 2016 was incubated at 25°C (Table S3). All vials were stored at 6°C in a refrigerator until analysis. All samples were analyzed within a month after poisoning with HgCl₂.

The surface sediment samples were collected using a gravity core sampler (HR-type; Rigo, Japan; inner diameter = 11 cm; 50 cm long) at Sta. H5 from R/V HASU, during two sampling campaigns (June and November 2018) (Table S5). Then, using a porous cup soil-solution sampler (DIK-8390-11; DAIKI, Japan), the pore water samples (~ 5 mL for each) from every 5 cm depth, starting from the surface, were extracted into 20 mL pre-evacuated glass bottles, in which both HgCl₂ (15 mg for each) and phosphoric acid (1 g for each) had been added before the sampling. The headspace of each glass bottle was filled with helium (> 99.99995%) until atmospheric pressure was reached, and the bottles were stored at 6°C in a refrigerator until analysis.

Definition of stable isotopic compositions

The natural stable isotopic composition of methane is represented by its $\delta^{13}\text{C}$ and $\delta^2\text{H}$ values. The delta (δ) values are calculated as $R_{\text{sample}}/R_{\text{standard}} - 1$, where R is the $^{13}\text{C}/^{12}\text{C}$ ratio for $\delta^{13}\text{C}$ (or the $^2\text{H}/^1\text{H}$ ratio for $\delta^2\text{H}$) in both the sample and the respective international standard (VPDB for carbon and VSMOW for hydrogen).

Methane concentration, $\delta^{13}\text{C}$, and $\delta^2\text{H}$ analyses

The concentration and $\delta^{13}\text{C}$ of methane in the samples were determined using a CF-IRMS analytical system, as documented by Hirota et al. (2010). The system comprises an automatic methane and nitrous oxide extraction system (AMEXs; Hirota et al. 2010), a gas chromatograph (HP5890; Agilent Technologies), and a Finnigan MAT 252 isotope ratio mass spectrometer (Thermo Fisher Scientific) with a Combustion III interface (Thermo Fisher Scientific). Helium carrier (>99.99995%) was passed through a column packed with a molecular sieve 5A maintained at liquid nitrogen temperature just before use.

In low-methane samples (<50 nmol kg⁻¹), the sample vial was connected manually to the AMEXs via two stainless-steel needles piercing the butyl rubber stopper. All water in the vial was then transferred to a sparging bottle using helium for the extraction of dissolved gas. In samples with high methane concentration (>50 nmol kg⁻¹), approximately 30% of the water in each vial was replaced by high-purity helium. Then, the vials were vigorously shaken for more than 10 s, followed by more than 30 min at 25°C, to complete the equilibration between aqueous and gaseous phases. Depending on the methane concentration, 0.05–5 mL of gas was extracted from the gas phase in the vial by using a gas-tight syringe (VICI Precision Sampling), and injected into AMEXs via an injection port (Ijiri et al. 2003).

The extracted/injected gas was sent through a purification trap maintained at dry-ice temperature (−79°C) to remove H₂O, and through a chemical trap filled with magnesium perchlorate and Ascarite II (NaOH), to remove residual H₂O and CO₂. The CH₄ was first collected on a Silicosteel tubing trap packed with Porapak-Q immersed in liquid oxygen, and then cryofocused at the head of the PoraPLOT-Q capillary column maintained at liquid oxygen temperature. By removing the liquid oxygen bath from the cryofocusing unit, the trapped components in the unit were injected into the column maintained at room temperature (30°C), separated at a flow rate of 0.5 mL min⁻¹. After elution from the capillary column, CH₄ was quantitatively converted to CO₂ through combustion in a furnace (CuO) maintained at 960°C. They were then introduced into the MS to determine both concentration and $\delta^{13}\text{C}$ values.

The analytical precision of the concentration and the $\delta^{13}\text{C}$ determination were better than 3% and 0.2‰, respectively. Units in the form of mol per kg (such as nmol kg⁻¹ and $\mu\text{mol kg}^{-1}$) were used to present concentrations of the water

samples in this study. The values in mol per kg units were equal to those in the mol per L units (such as nmol L⁻¹ and $\mu\text{mol L}^{-1}$, respectively) in the samples.

$\delta^2\text{H}$ was determined according to Rice et al. (2001) and Yamada et al. (2003), with several modifications described below. Methane, extracted and purified in the same manner as that in the $\delta^{13}\text{C}$ analysis, was introduced into a furnace held at 1350°C via a ceramic tube (35 cm long, 1 mm i.d.) to thermally decompose it into H₂ + C. Our furnace temperature was approximately 100°C lower than that in a previous study (Burgoyne and Hayes 1998). The produced H₂ was separated from residual methane using a MolSieve 5A PLOT column (5 m long, 0.32 mm i.d., DF 30 μm ; Chrompack) held at 35°C, and then introduced into the CF-IRMS system (Thermo Fisher Scientific, Delta V) to determine $\delta^2\text{H}$ through the simultaneous monitoring of H₂ isotopologs at $m/z = 2$ and 3. To achieve a high precision in $\delta^2\text{H}$ determination, we automated the entire analytical system and controlled it with a digital-output electronic sequencer DODES (Komatsu et al. 2011).

During replicate analyses on the same standard gas mixture containing methane (111.3 ppm), the analytical precision (1σ) of $\delta^2\text{H}$ was 2‰ for injections with 10 nmol of methane and 12‰ for injections with 1 nmol of methane into the analytical system (which corresponds to 250 mL of the water sample containing 4 nmol kg⁻¹ of methane). However, when we reduced the amount of methane injected, its average $\delta^2\text{H}$ value changed in accordance with the amount of methane injected, probably due to changes in the magnitude of isotope fractionation during the conversion to H₂, in accordance with the amount of methane injected. Thus, various volumes of a standard methane gas mixture (2020 ppm methane in helium) were injected daily into the analytical system before the sample analyses to determine daily correction factors for the isotope fractionation during conversion to H₂. This correction method allowed us to reduce the methane amount in the analyte to as low as 1 nmol without reducing the accuracy of $\delta^2\text{H}$ analyses.

To calibrate the δ values of methane to the international scale, we measured two reference materials (IAEA natural gas standards NGS-2 and NGS-3) containing methane, at least once per day, in the same manner as that for the measured samples. The data were corrected according to the international scale (VPDB for $\delta^{13}\text{C}$ and VSMOW for $\delta^2\text{H}$) using the following values for the reference materials: $\delta^{13}\text{C} = -44.84\text{‰}$ (vs. VPDB) and $\delta^2\text{H} = -173\text{‰}$ (vs. VSMOW) for NGS-2, and $\delta^{13}\text{C} = -73.27\text{‰}$ (vs. VPDB) and $\delta^2\text{H} = -176\text{‰}$ (vs. VSMOW) for NGS-3.

Specific oxidation rate (k), isotope enrichment factor (ϵ), and lambda (Λ) during methane oxidation

Compared to the initial (= in situ) concentration, we found a significant reduction in methane concentration in many samples without treatment during the incubation experiments. Assuming that methane oxidation in the water

column was a first-order process with respect to the methane concentration (Ward et al. 1987; De Angelis et al. 1993), the specific oxidation rate (i.e., the first-order rate constant) k (day^{-1}) was estimated for each depth or season from changes in methane concentration in each bottle, using the following equation:

$$C_t = C_0 e^{-kt}, \quad (1)$$

where C_0 and C_t are the concentrations of methane at the initial (= in situ) (0) and temporal sampling (t) during oxidation. Only samples with incubation periods of 36 ± 8 h and with incubation temperature of 6°C were used for estimating each specific oxidation rate k .

In accordance with the progression of oxidation in each incubation bottle, the residual methane showed enrichment in both ^{13}C and ^2H compared to the initial (= in situ) $\delta^{13}\text{C}$ and $\delta^2\text{H}$ values due to kinetic fractionation. The change in the isotopic compositions of methane can be approximated by the following Rayleigh equations (Coleman et al. 1981; Elsner et al. 2007; Feisthauer et al. 2011):

$$\delta^{13}\text{C}_t - \delta^{13}\text{C}_0 = \varepsilon_C \ln f, \quad (2)$$

$$\delta^2\text{H}_t - \delta^2\text{H}_0 = \varepsilon_H \ln f, \quad (3)$$

where ε_C and ε_H denote the isotopic enrichment factors (constants) of carbon and hydrogen, respectively; $\delta^{13}\text{C}_0$ and $\delta^{13}\text{C}_t$ are the stable carbon isotopic compositions of methane at the initial (0) and temporal sampling (t) during the oxidation; $\delta^2\text{H}_0$ and $\delta^2\text{H}_t$ are the stable hydrogen isotopic compositions of methane at the initial (0) and temporal sampling (t) during the oxidation; and f is the fraction of methane remaining (equal to C_t/C_0).

As a result, we can estimate ε_C from the slope of linear regression between $\delta^{13}\text{C}_t - \delta^{13}\text{C}_0$ and $\ln f$, as well as ε_H from the slope of the linear regression between $\delta^2\text{H}_t - \delta^2\text{H}_0$ and $\ln f$. Additionally, the approximate ratio of hydrogen vs. carbon discrimination during methane oxidation ($\Lambda = \varepsilon_C/\varepsilon_H$) can be estimated from the relation between $\delta^{13}\text{C}_t - \delta^{13}\text{C}_0$ and $\delta^2\text{H}_t - \delta^2\text{H}_0$ using the following equation (Elsner et al. 2007; Feisthauer et al. 2011):

$$\Lambda = \frac{\delta^2\text{H}_t - \delta^2\text{H}_0}{\delta^{13}\text{C}_t - \delta^{13}\text{C}_0}. \quad (4)$$

Therefore, we can estimate the Λ values from the slope of the linear regression between $\delta^2\text{H}_t - \delta^2\text{H}_0$ ($= \Delta\delta^2\text{H}$) and $\delta^{13}\text{C}_t - \delta^{13}\text{C}_0$ ($= \Delta\delta^{13}\text{C}$) of methane during the methane oxidation, without estimating the fraction of methane remaining (f). Although both ε_C and ε_H during the methane oxidation determined using aerobic methanotrophic strains are variable, ranging from -14.8% to -27.9% for ε_C and from -110.0% to -231.5% for ε_H (Feisthauer et al. 2011), the ratios of

hydrogen isotopic discrimination vs. carbon isotopic discrimination (Λ) during methane oxidation are known to be fairly stable at 12.0 ± 4.6 (Feisthauer et al. 2011). If Λ is stable in the field, we can constrain the relation between the original carbon isotopic composition ($\delta^{13}\text{C}_0$) and the original hydrogen isotopic composition ($\delta^2\text{H}_0$) from the isotopic compositions of residual methane ($\delta^2\text{H}_t$ and $\delta^{13}\text{C}_t$), irrespective of the progression of oxidation. As a result, we verified whether Λ was stable in the water column, based on the changes in $\delta^{13}\text{C}$ and $\delta^2\text{H}$ during the incubation experiments.

Results and discussion

Horizontal heterogeneities of methane in the pelagic zone

When we compared the in situ methane concentrations between Sta. H3 and H1 that were collected at the same depth during the same sampling campaign, the methane concentrations in Sta. H3 were, on average, 30% higher than those in Sta. H1. However, we could not find any significant difference between the stations in their increasing or decreasing trend related to changes in season and depth. Additionally, while the in situ $\delta^{13}\text{C}$ and $\delta^2\text{H}$ values showed a range of variation greater than 50‰ and more than 600‰, respectively, over the span of the entire water column, the differences between the samples collected at the same depth during the same sampling campaign were, on average, less than 0.5‰ and 10‰, respectively, implying that the primary source of excess methane was the same between the stations. As a result, we continued our water sampling only at the deeper and more methane-depleted Sta. H1 (or H5, since July 2016) starting in 2016. In our discussion, we assume the in situ concentration, $\delta^{13}\text{C}$, and $\delta^2\text{H}$ values obtained at Sta. H1 (or H5, since July 2016) to be representative of the lake pelagic zone.

Distribution of the concentration and stable isotopic compositions of methane in pelagic zone water columns

Methane concentrations in the epilimnion and thermocline of the pelagic zone were always higher than those in equilibrium with atmospheric methane ($\sim 3 \text{ nmol kg}^{-1}$; Wiesenburg and Guinasso 1979), irrespective of the year or season of observation (Fig. 2b). As already established previously (Miyajima et al. 1997), this lake has been an emission source of methane to the atmosphere over years, and particularly high concentrations of methane (i.e., more than 150 nmol kg^{-1}) can be found during stratified seasons (May to August), showing maxima at depths of either the epilimnion or upper thermocline (10–20 m depth). In contrast, methane concentrations in the hypolimnion were always lower than those in both the epilimnion and thermocline during stratified seasons, similar to other deep lakes and oceans with the oxic water column throughout the year (Sasakawa et al. 2008; Bles et al. 2015; Donis et al. 2017). However, in accordance with the deepening of the surface mixed layer in wintertime,

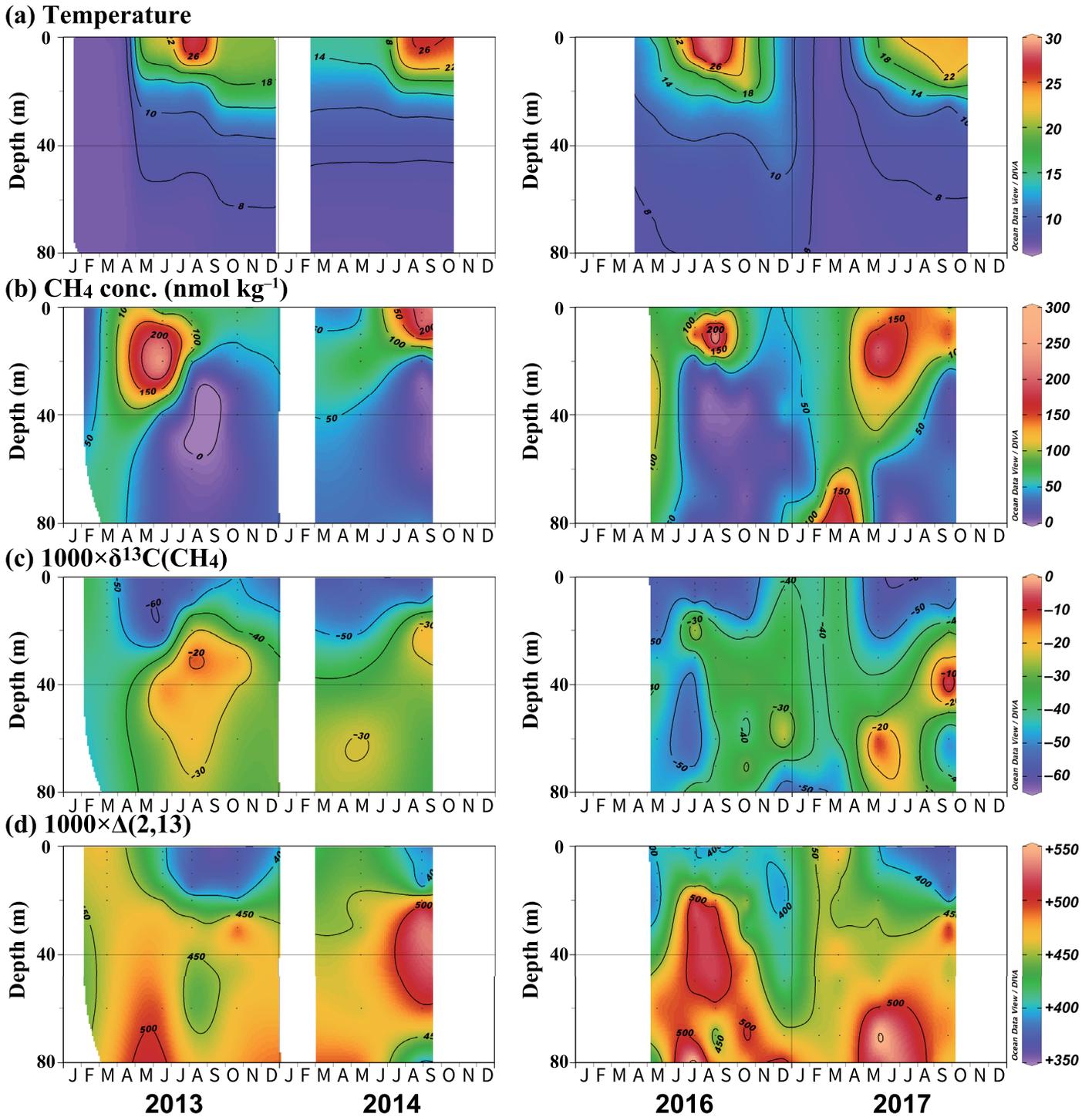


Fig. 2. Temporal variabilities in temperature (a), in situ concentration (b), $\delta^{13}\text{C}$ (c), and $\Delta(2,13)$ (d) of methane, plotted on the time-series contour determined in the pelagic water column (Sta. H1/H5) from January 2013 to December 2014 in the left column and from January 2016 to December 2017 in the right column.

the vertical differences in methane concentrations were small. The minimum concentration during the stratified seasons can be found in the upper hypolimnion (~ 40 m). That is, bottom

waters (> 70 m depth) exhibited methane concentrations higher than those observed in the upper hypolimnion, implying a continuous supply of methane from the lake bottom.

The enriched methane in both the epilimnion and upper thermocline during stratified seasons exhibited low and near-constant $\delta^{13}\text{C}$ values, approximately -50‰ , whereas methane in the hypolimnion showed significant ^{13}C enrichment up to -3.2‰ . It is unlikely that these values in the hypolimnion reflect the actual $\delta^{13}\text{C}$ values of methane at production in and around the lake. Rather, such a ^{13}C -enriched isotopic composition in the hypolimnion can result from the methane-oxidizing bacteria in the lake water column, because the bacteria prefer ^{12}C , leaving unoxidized methane enriched in ^{13}C (Silverman and Oyama 1968; Barker and Fritz 1981; Coleman et al. 1981; Whittar and Faber 1986; Nishimura et al. 1999; Tsunogai et al. 2000).

This hypothesis was also supported by the $\delta^2\text{H}$ values of methane (Fig. 3a). The $\delta^2\text{H}$ values of methane in the lake water column showed significant ^2H -enrichment up to more than $+400\text{‰}$, especially in the hypolimnion, as well as a strong linear correlation with the $\delta^{13}\text{C}$ values (Fig. 3a; $R^2 = 0.92$). Because methane-oxidizing bacteria prefer not only ^{12}C but also ^1H , leaving unoxidized methane enriched in both ^{13}C and ^2H (Coleman et al. 1981; Feisthauer et al. 2011), the linear correlation also implied that bacterial methane oxidation was active in the lake water column. The maximum $\delta^2\text{H}$ value was $+432\text{‰}$ (Fig. 3a), found on 29 May 2017 at a depth of 60 m at Sta. H5 (Table S2). To our knowledge, this value is one of the highest $\delta^2\text{H}$ values of methane found in the natural environment.

Rates and Λ values of methane oxidation in the water column

The specific oxidation rates of methane (k) in the pelagic zone determined through the incubation experiments also supported active methane oxidation in the hypolimnion in the lake (Fig. 4). Additionally, the turnover time of methane in the hypolimnion (a few days) implied a continuous supply of methane from the lake bottom and explains the methane concentration greater than 10 nmol kg^{-1} at a depth of 80 m throughout the year, as also suggested from the vertical distribution of the concentration and stable isotopic compositions of methane in the lake. Compared to the specific oxidation rates of methane in the hypolimnion, the values in the epilimnion exhibited smaller specific oxidation rates. Because the temperature during the incubation experiments (6°C) was lower than that at in situ temperatures in epilimnion (from 7°C to 29°C), the in situ oxidation rates in the epilimnion may be higher than the values determined through the incubation experiments in this study. Still, the specific oxidation rates of methane determined for the epilimnion water in Lake Biwa through incubation under 15°C showed similar small specific oxidation rates of less than $0.1 \text{ (d}^{-1}\text{)}$ (Murase et al. 2005; Murase and Sugimoto 2005). As a result, we concluded that the in situ oxidation rates were negligible (less than 0.1 d^{-1}) throughout the year, probably due to the photo-inhibition of methane oxidation in the epilimnion (Murase et al. 2005; Murase and Sugimoto 2005).

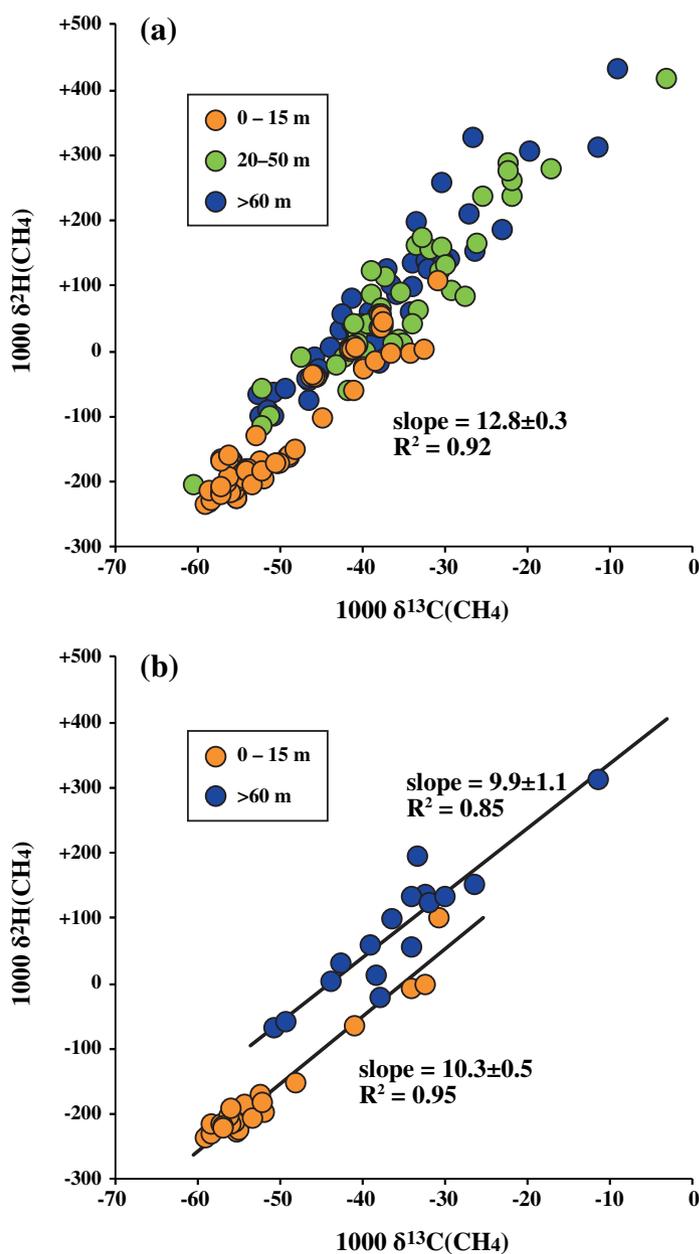


Fig. 3. Relation between the in situ $\delta^{13}\text{C}$ and $\delta^2\text{H}$ values of methane in the pelagic water column at depths ranging from 0 to 15 m (orange), 20 to 50 m (green), and 60 to 90 m (blue) for all water samples collected throughout the years **(a)**, and those during the stratified seasons at depths from 0 to 15 m (orange) and 60 to 90 m (blue) **(b)**. The slope of the regression line (slope) and its correlation coefficient (R^2) determined for the whole dataset are also presented **(a)**, together with those determined for 0 to 15 m data (the lower side) and 60 to 90 m data (the upper side) **(b)**.

Following the progression of oxidation in each incubation bottle, the residual methane showed enrichment in both ^{13}C and ^2H , when compared to the initial $\delta^{13}\text{C}$ and $\delta^2\text{H}$ values. This further confirmed the bacterial preference of both ^{12}C and ^1H , as implied in the distributions of both $\delta^{13}\text{C}$ and $\delta^2\text{H}$

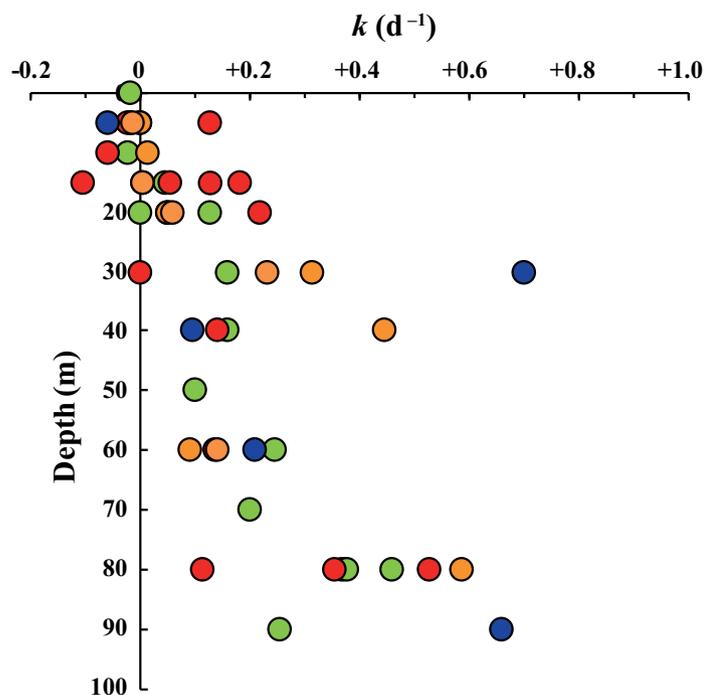


Fig. 4. Vertical distributions of the specific oxidation rates of methane (k) in the pelagic water column in spring (March, April, and May; green), summer (June, July, and August; red), fall (September, October, and November; orange), and winter (December, January, and February; blue).

in the pelagic water columns. The isotopic enrichment factors of carbon (ϵ_C) and hydrogen (ϵ_H) during the oxidation were highly variable, with mean and standard deviations of -31.5‰ and 18.0‰ , respectively, for ϵ_C , and -344‰ and 418‰ , respectively, for ϵ_H . However, changes in $\delta^2\text{H}$ values during incubation ($\Delta\delta^2\text{H}$) showed a strong linear correlation with those in $\delta^{13}\text{C}$ values ($\Delta\delta^{13}\text{C}$), irrespective of the depth or season (Fig. 5; $R^2 = 0.92$). When a subset of the incubation experiments changed the temperature from 6°C to 25°C , we could not find any significant change in the correlation between $\Delta\delta^{13}\text{C}$ and $\Delta\delta^2\text{H}$, and thus, also the Λ values calculated from Eq. 4. Based on the linear correlation between $\Delta\delta^{13}\text{C}$ and $\Delta\delta^2\text{H}$ during the incubation experiments conducted at 6°C , we estimated the average Λ value of the kinetic isotopic fractionation and its standard error in the lake water column to be 10.9 ± 0.2 (Fig. 5).

The estimated average Λ value (10.9 ± 0.2) was significantly smaller than the slope between $\delta^{13}\text{C}$ and $\delta^2\text{H}$ of methane in the water column (12.8 ± 0.3 ; Fig. 3a). If the original $\delta^{13}\text{C}$ and $\delta^2\text{H}$ values of methane emitted into the water column were uniform in the lake, all methane now present would plot in line with the slope of the average Λ value (10.9 ± 0.2). The slope between $\delta^{13}\text{C}$ and $\delta^2\text{H}$ of methane in the water column was larger than the average Λ value determined in bottles, suggesting that the original $\delta^{13}\text{C}$ and $\delta^2\text{H}$ values of methane emitted into the water column were not uniform in the lake.

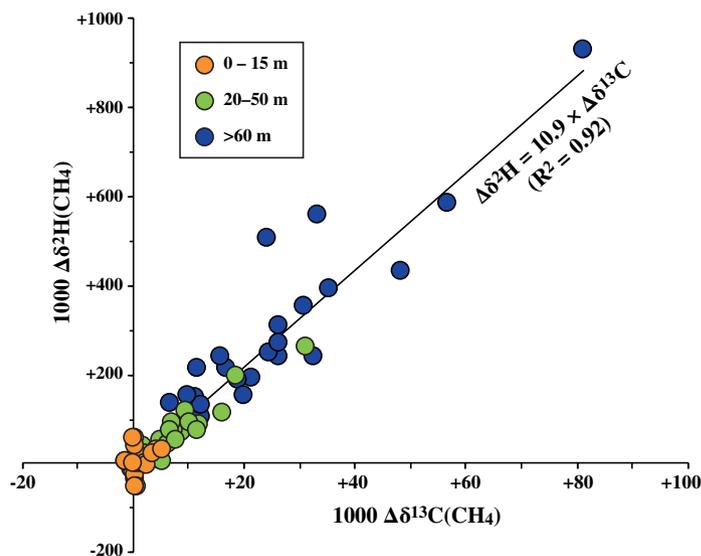


Fig. 5. Relation between the changes in the carbon ($\Delta\delta^{13}\text{C}$) and hydrogen isotopic compositions ($\Delta\delta^2\text{H}$) during the bottle incubation experiments conducted at 6°C for the samples collected during the eight sampling campaigns of different seasons at depths of 0–15 m (orange), 20–50 m (green), and 60–90 m (blue). The solid line represents the least-squares fitting of the whole data.

This was also supported by the relation between $\delta^{13}\text{C}$ and $\delta^2\text{H}$ of methane in the water column. The isotopic compositions of methane in the shallow layers (from the surface to 15 m) had a linear correlation ($R^2 = 0.95$), different from that of the deep layers (from 60 m to the bottom) ($R^2 = 0.86$), for the relation between $\delta^{13}\text{C}$ and $\delta^2\text{H}$ of methane during the stratified season (from June to September), with a significant difference in the values of the intercept ($+365 \pm 26\text{‰}$ for shallow and $+437 \pm 40\text{‰}$ for deep) (Fig. 3b). Additionally, each slope (10.3 ± 0.5 for shallow and 9.9 ± 1.1 for deep) corresponded to an average Λ value (10.9 ± 0.2) within the range of standard errors. These results indicate that the original $\delta^{13}\text{C}$ and $\delta^2\text{H}$ values of methane in the shallow layers were different from those in the deep layers. Additionally, the kinetic isotopic fractionation (Λ value) was the same irrespective of the depth.

Conversely, when we included the isotopic compositions ($\delta^{13}\text{C}$ and $\delta^2\text{H}$) of methane from other seasons (October to May) or depths (20–50 m), the slope of the regression line exceeded the Λ value, as already presented (Fig. 3a). Besides the progression of oxidation, the variation in the isotopic compositions ($\delta^{13}\text{C}$ and $\delta^2\text{H}$) of methane through mixing between those areas with different original isotopic compositions was also likely responsible for the larger slope.

Tracing the sources of excess methane using a new indicator $\Delta(2,13)$

As discussed in previous section, both (1) isotopic fractionation due to the progression of oxidation in the water column

and (2) mixing between these areas with different original isotopic compositions were responsible for variations in the isotopic compositions ($\delta^{13}\text{C}$ and $\delta^2\text{H}$) of methane in the water column. Based on these results, we defined the following $\Delta(2,13)$ as a new indicator for determining whether the original isotopic compositions were the same, irrespective of the progression of oxidation in the water column:

$$\Delta(2,13) = \delta^2\text{H} - \Lambda \times \delta^{13}\text{C}, \quad (5)$$

where Λ denotes the $\varepsilon_{\text{H}}/\varepsilon_{\text{C}}$ ratio during methane oxidation. If the original isotopic compositions are the same for methane in different depths and seasons, their $\Delta(2,13)$ values should also be the same, irrespective of the progression of oxidation in the water column, because the changes in both $\delta^{13}\text{C}$ and $\delta^2\text{H}$ values during methane oxidation in the water column were corrected in $\Delta(2,13)$. We can constrain the source of methane in the water column by comparing the $\Delta(2,13)$ values with those in the possible sources as well. Herein, we used 10.9 for Λ , which was determined through the incubation experiments done in this study (Fig. 5).

Besides temperature, the concentration, and $\delta^{13}\text{C}$ values, the $\Delta(2,13)$ values of methane determined for the pelagic water column were plotted on a time-series contour (Fig. 2d). As presented in the figure, we found a distinct gap in the vertical distribution of $\Delta(2,13)$ values at the thermocline depth (~20 m) during the stratified seasons, implying that the major source of methane was different between the epilimnion and the hypolimnion in the lake water column during the stratified seasons. The sources of methane in the water column can, therefore, be categorized into two major lacustrine sources: methane with lower $\Delta(2,13)$ values (approximately +400‰) that occupied majority of methane in the epilimnion during the stratified seasons and methane with higher $\Delta(2,13)$ values (approximately +500‰) that occupied majority of methane in the hypolimnion during the stratified seasons. Additionally, methane in the other seasons showed intermediate $\Delta(2,13)$ values, irrespective of the depth, and can be explained by mixing between the two major sources due to vertical mixing of the water column. Methane in the thermocline showed intermediate $\Delta(2,13)$ values between the two major sources, which could be explained by the mixing between the two major sources as well.

As already presented, we found particularly high concentrations of methane (greater than 150 nmol kg^{-1}) in the water column during the stratified seasons in the epilimnion or upper thermocline, from 10 to 20 m. The methane in the maxima showed $\Delta(2,13)$ values from +380‰ to +430‰, irrespective of the year (Fig. 2d). These $\Delta(2,13)$ values implied that the major sources of methane in the maxima corresponded to lower $\Delta(2,13)$ values (approximately +400‰) that occupied majority of methane in the epilimnion during the stratified seasons.

To further verify the results, the concentration, $\delta^{13}\text{C}$, and $\Delta(2,13)$ values of methane in the lake water column collected at depths of 0, 10, 20, 40, 60, and 80 m during the stratified seasons are plotted in Fig. 6. The concentration-weighted mean values were calculated and are plotted (diamonds) as well.

The mean pelagic concentrations were highest at 10 m depth. However, when we compared the $\Delta(2,13)$ values at 10 m with those at the surface (0 m), we could not find any significant difference in their $\Delta(2,13)$ values, implying that their major sources were the same and that emission to the atmosphere was likely responsible for the difference in the mean concentrations between them. In contrast, the $\Delta(2,13)$ values at 20 and 40 m were different from those at 0 and 10 m. Additionally, the $\Delta(2,13)$ values showed an increasing trend toward greater depths. Not only the removal of methane through oxidation in the water column but also mixing with methane-depleted water in the hypolimnion was likely responsible for the increasing depth trend in $\Delta(2,13)$.

As a result, we determined the $\Delta(2,13)$ values in the potential sources in and around the lake to compare the $\Delta(2,13)$ values with those in the pelagic water column during stratification. Based on the similarities in the $\Delta(2,13)$ values, we discuss the source of methane enrichment in the epilimnion and upper thermocline of the pelagic water column, showing $\delta^{13}\text{C}$, $\delta^2\text{H}$, and $\Delta(2,13)$ values of $-55 \pm 2\%$, $-197 \pm 25\%$, and $+403 \pm 16\%$, respectively (the concentration-weighted mean values of methane at 10 m depth with 1σ variation ranges during the stratified seasons).

$\Delta(2,13)$ values of methane in the bottom sedimentary layer and in the littoral zone

The concentration, $\delta^{13}\text{C}$, and $\Delta(2,13)$ values are plotted (Fig. 6) for both (1) methane in the water at the littoral zone (L01, L02, L03, L11, L13, L15, L20, L25, and L27) and (2) methane in the sedimentary layer of the pelagic zone at depths of 10, 15, 20, and 25 cm from the surface, irrespective of the season of observation. The concentration-weighted mean values were calculated and are plotted as diamonds.

The sedimentary methane showed almost uniform $\delta^{13}\text{C}$ and $\delta^2\text{H}$ values, and thus, $\Delta(2,13)$ values, of approximately $-73 \pm 1\%$, $-277 \pm 6\%$, and $+517 \pm 11\%$, respectively, regardless of the depth within the sediment or season. The $\delta^{13}\text{C}$ values of methane coincided well with those reported by Murase and Sugimoto (2001) for sedimentary methane in Lake Biwa, ranging from -61% to -80% . The concentration-weighted mean $\Delta(2,13)$ value of sedimentary methane ($+517 \pm 11\%$) agreed with those of pelagic methane, which occupied majority of the hypolimnion during the stratified seasons, showing $\Delta(2,13)$ values of approximately +500‰ (Figs. 2d, 6). Although the $\delta^{13}\text{C}$ and $\delta^2\text{H}$ values of sedimentary methane ($-73 \pm 1\%$ and $-277 \pm 6\%$, respectively) were significantly lower than those in the hypolimnion during the stratified seasons, the deviation can be explained by the progression of microbial methane oxidation within the oxic water

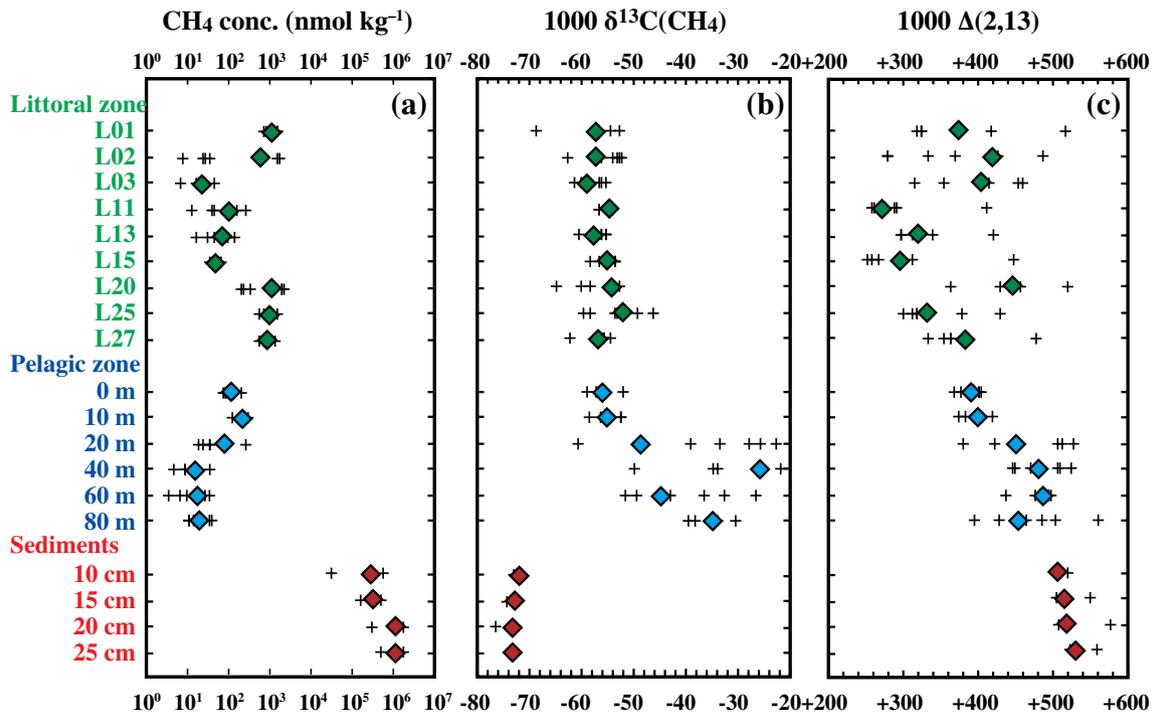


Fig. 6. Compilation of the in situ concentration (a), $\delta^{13}\text{C}$ (b), and $\Delta(2,13)$ values (c) of methane in the water at the littoral zone (Sta. L01–L27), in the lake’s water column of pelagic zone (Sta. H1/H5) collected at depths of 0, 10, 20, 40, 60, and 80 m during stratified seasons, and in the sedimentary layer of the pelagic zone (Sta. H5) at depths of 10, 15, 20, and 25 cm from the surface. While the whole data collected in this study were plotted for the littoral zone and sediments, only the data collected during the stratified seasons were plotted for the pelagic-zone water column. Besides the raw data plotted in plus signs, the annual mean values were calculated and plotted as diamonds (littoral zone: green; pelagic zone water column: blue; sediments: red).

column of the hypolimnion. The progression of microbial oxidation can drive the $\delta^{13}\text{C}$ and $\delta^2\text{H}$ values of residual methane toward ^{13}C - and ^2H -enriched values (Coleman et al. 1981; Nishimura et al. 1999; Tsunogai et al. 2000). We concluded that sedimentary methane was the primary source of methane in the hypolimnion during the stratified seasons (Fig. 7).

In contrast, the littoral zone exhibited generally smaller $\Delta(2,13)$ values with large variations (from +258‰ to +519‰), whereas the $\delta^{13}\text{C}$ values were nearly identical, at approximately -56 ± 2 ‰ (Fig. 6). As a result, large variations in $\delta^2\text{H}$ values of methane (from -121 ‰ to -335 ‰) were primarily responsible for the large variation in the $\Delta(2,13)$ values of methane in the littoral zone. However, when we estimated the concentration-weighted annual mean $\Delta(2,13)$ value for each station, the variation between the stations decreased (from +270‰ to +446‰) (Fig. 6). Furthermore, when we excluded the methane-depleted stations (Sta. L03, L11, L13, and L15) located along the northeastern shore of the lake (Fig. 1b), showing methane concentrations as low as 6 nmol kg^{-1} in winter, the variation between the stations became much smaller, i.e., -256 ‰ to -149 ‰ for $\delta^2\text{H}$ and $+331$ ‰ to $+446$ ‰ for $\Delta(2,13)$.

The methane-depleted samples (methane concentrations of less than 20 nmol kg^{-1}) showed $\delta^2\text{H}$ values significantly

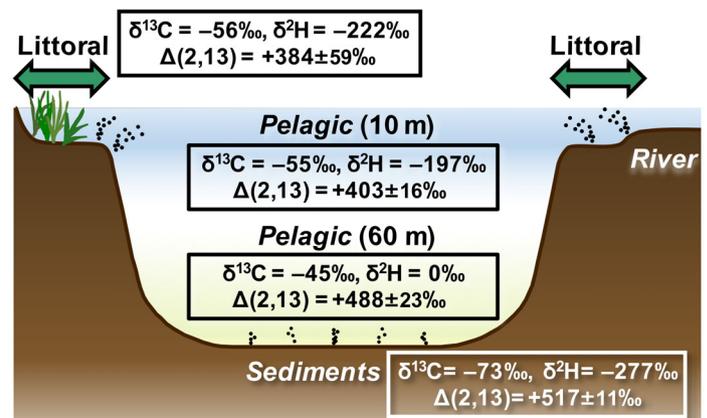


Fig. 7. Compilation of the concentration-weighted mean isotopic compositions of methane ($\delta^{13}\text{C}$, $\delta^2\text{H}$, and $\Delta(2,13)$) in the pelagic zone at depths of 10 and 60 m during the stratified seasons, together with those in the water at the littoral zone and in the sedimentary layer of the pelagic zone.

higher than those showing methane concentrations more than 20 nmol kg^{-1} (Fig. S6; $p < 0.05$). These results imply that the contribution of atmospheric methane (having $\delta^{13}\text{C}$ and $\delta^2\text{H}$ values of -47 ‰ and -90 ‰, respectively; Quay et al. 1999)

to littoral zone water due to gas exchange between the surface water and lower atmosphere in the littoral zone was partly responsible for the large variation found in the $\delta^2\text{H}$ values of methane in the littoral zone water samples, particularly for those in the methane-depleted stations (Sta. L03, L11, L13, and L15). Therefore, while the $\delta^2\text{H}$ values of methane in the methane-enriched samples (approximately -220‰) represented those produced in and around the littoral zone, the contribution of atmospheric methane became significant, and thus, the $\delta^2\text{H}$ values of methane increased in some of the methane-depleted samples. While the contributions of atmospheric methane had little impact on the $\delta^{13}\text{C}$ values, owing to the small differences in $\delta^{13}\text{C}$ values between atmospheric methane (approximately -47‰ ; Quay et al. 1999) and the methane produced in and around the littoral zone (approximately -56‰), the contribution of atmospheric methane had a significant impact on $\delta^2\text{H}$ values, because of the large differences between atmospheric methane (approximately -90‰ ; Quay et al. 1999) and the methane produced in and around the littoral zone (approximately -220‰). Besides the influence of atmospheric methane, the large variation found in the $\delta^2\text{H}$ and $\Delta(2,13)$ values of methane, even in the methane-enriched stations, implies that seasonal and regional changes in the production processes of methane in the littoral zone were also responsible for the variation found in $\delta^2\text{H}$ values of methane in the littoral-zone water samples.

The concentration-weighted mean values of methane throughout the littoral zone stations were estimated to be $-56 \pm 2\text{‰}$, $-222 \pm 57\text{‰}$, and $+384 \pm 59\text{‰}$ for $\delta^{13}\text{C}$, $\delta^2\text{H}$, and $\Delta(2,13)$, respectively. Although data of the methane-depleted littoral zone water samples, in which the contribution of atmospheric methane could be significant, were included in estimating the mean values, the concentration-weighted mean values essentially represented those of the methane-enriched samples so that the influence of atmospheric methane was least in the mean values. Hence, we used the mean values as those produced in and around the littoral zone, and thus, enriched in the littoral zone water.

Based on the distribution of the concentration and $\delta^{13}\text{C}$ of methane in Lake Biwa, including the littoral zone, Murase and Sugimoto (2005) proposed that river water and littoral sediment are potential sources of increased methane in the pelagic zone seen during the stratified seasons. Adding $\delta^2\text{H}$ data, our results also supported these findings (Fig. 7) because the concentration-weighted mean values for methane in the littoral zone ($-56 \pm 2\text{‰}$, $-222 \pm 57\text{‰}$, and $+384 \pm 59\text{‰}$ for $\delta^{13}\text{C}$, $\delta^2\text{H}$, and $\Delta(2,13)$, respectively) coincide well with those of methane in the epilimnion and upper thermocline of the pelagic water column (Fig. 7). We concluded that majority of methane enrichment in the epilimnion and upper thermocline of the pelagic water column during the stratified seasons was derived from the littoral zone, probably through lateral transport via a layer of similar density (Schmidt and Conrad 1993; Murase et al. 2003; Fernández et al. 2016; Delsontro

et al. 2018; Peeters et al. 2019). Although the maximum concentration within the mean concentrations of the pelagic zone water column was found at a depth of 10 m and not at the surface (Fig. 6), the methane concentrations at the surface may have been reduced through emissions to the atmosphere during lateral transport.

Source of methane in the littoral zone

Methane in the littoral zone showed mean values of $-56 \pm 2\text{‰}$, $-222 \pm 57\text{‰}$, and $+384 \pm 59\text{‰}$ for $\delta^{13}\text{C}$, $\delta^2\text{H}$, and $\Delta(2,13)$, respectively (Fig. 7). By using these values as tracers, we discuss the possible sources of methane in the littoral zone, and thus, the methane enrichment in the epilimnion and upper thermocline of the pelagic water column together with the sedimentary methane in the pelagic zone, which showed $\delta^{13}\text{C}$, $\delta^2\text{H}$, and $\Delta(2,13)$ values of approximately $-73 \pm 1\text{‰}$, $-277 \pm 6\text{‰}$, and $+517 \pm 11\text{‰}$, respectively (Fig. 7).

Two primary metabolic pathways are generally recognized for typical biological methanogenesis: acetate fermentation and CO_2 reduction (Whiticar et al. 1986; Sugimoto and Wada 1993). Based on $\delta^{13}\text{C}$ values of methane, together with the relation between the $\delta^{13}\text{C}$ values of sedimentary CO_2 and those of sedimentary methane, Murase and Sugimoto (2001) concluded that the sedimentary methane in Lake Biwa is predominantly produced via the CO_2 reduction pathway in anoxic sediments. The $\delta^2\text{H}$ values determined in this study ($-222 \pm 57\text{‰}$ for the littoral zone and $-277 \pm 6\text{‰}$ for the pelagic sediments) were enriched in deuterium, compared to methane in typical freshwater environments, such as wetlands and rice paddies (Whiticar et al. 1986; Sugimoto and Wada 1993; Nakagawa et al., 2002*a,b*; Chanton et al. 2005). Based on the empirical relation between $\delta^2\text{H}$ of water and $\delta^2\text{H}$ of methane, which was obtained experimentally through incubating freshwater soil (Sugimoto and Wada 1993), we can estimate the $\delta^2\text{H}$ value of methane produced via CO_2 reduction as $-355 \pm 20\text{‰}$ and the $\delta^2\text{H}$ value via acetate fermentation as $-326 \pm 15\text{‰}$, with water (H_2O) having a $\delta^2\text{H}$ value of -55‰ (the $\delta^2\text{H}$ value of lake water; Tsunogai et al. 2018). Still, we concluded that methane production in anoxic sediments via the CO_2 reduction pathway was a reasonable source for these values, as explained below.

In case of sedimentary methane, the $\delta^{13}\text{C}$ values ($-73 \pm 1\text{‰}$) indicated that they were produced through CO_2 reduction in anoxic sediments (Whiticar et al. 1986; Chanton et al. 2005). The observed ^2H -enriched $\delta^2\text{H}$ values of sedimentary methane ($-277 \pm 6\text{‰}$) can be explained by a lower concentration in the methane precursor (H_2) during CO_2 reduction, compared to those in the usual freshwater environments (Burke Jr. 1993; Sugimoto and Wada 1993; Chanton et al. 2005). During a kinetic reaction under a closed system, having significant kinetic fractionation in hydrogen isotopes between the reactant (H_2) and the product (CH_4), the product presents ^2H -depleted $\delta^2\text{H}$ values at the initial stage of the reaction, when the reactant (H_2) is abundant. In accordance with

the progress of the reaction, however, the product will present ^2H -enrichment. At the final stage of the reaction, when the reactant (H_2) is exhausted, the $\delta^2\text{H}$ values of the product (CH_4) would be the highest, showing $\delta^2\text{H}$ values similar to those of the reactant in the initial stage.

In contrast, the ^{13}C -enriched $\delta^{13}\text{C}$ values at approximately $-56 \pm 2\text{‰}$ of methane in the water column of the littoral zone were unusual for those produced through CO_2 reduction in anoxic sediments (Whiticar et al. 1986; Chanton et al. 2005), even considering that the carbon isotopic fractionation factor during CO_2 reduction can be variable (Conrad 2005). Additionally, the $\delta^{13}\text{C}$ values of $-56 \pm 2\text{‰}$ were significantly higher than those of methane determined in the anoxic sediments in the littoral zone of the lake, which showed $\delta^{13}\text{C}$ values from -68.9‰ to -60.1‰ (Murase and Sugimoto 2002). This ^{13}C -enrichment, however, can be explained by the progression of partial oxidation at the sediment surface in the littoral zone (Reeburgh 1983; Sansone et al. 1999; Whiticar 1999; Tsunogai et al. 2002; Riedinger et al. 2010). Because the water column was oxic in Lake Biwa, including the littoral zone, the methane produced in anoxic sediments should pass through the oxic–anoxic interface in the sediments, where aerobic/anaerobic methane oxidation is active (Reeburgh 1983; Sansone et al. 1999; Whiticar 1999; Tsunogai et al. 2002; Riedinger et al. 2010). The progression of microbial oxidation can enrich the $\delta^{13}\text{C}$ and $\delta^2\text{H}$ values of the residual methane (Coleman et al. 1981; Nishimura et al. 1999; Tsunogai et al. 2000), and therefore, can explain the ^{13}C -enrichment of methane in the littoral zone (approximately -56‰) while proposing its production in anoxic sediments.

If we assume that (1) Λ during aerobic/anaerobic methane oxidation at the oxic and anoxic interface in the sediments is the same as that in the water column ($\Lambda = 10.9$) and (2) the original $\delta^{13}\text{C}$ value of methane in anoxic sediments in the littoral zone is the lowest $\delta^{13}\text{C}$ of sedimentary methane in the pelagic region (-68.1‰ ; Murase and Sugimoto), the original $\delta^2\text{H}$ value becomes -358‰ . These values are comparable to the $\delta^2\text{H}$ value of methane produced via CO_2 reduction ($-355 \pm 20\text{‰}$), estimated based on the empirical relation between the $\delta^2\text{H}$ values of water and methane obtained experimentally through incubating freshwater soil (Sugimoto and Wada 1993). The ^2H -depleted original $\delta^2\text{H}$ value of methane can be explained by the hydrogen gas concentration during CO_2 reduction, which is higher than that in pelagic sediments, probably due to the rapid degradation rates of organic matter (Burke Jr. 1993; Sugimoto and Wada 1993; Chanton et al. 2005). In conclusion, methane produced in anoxic sediments at the mouths of inflowing rivers (Jones and Mulholland 1998; Smith et al. 2000; Murase et al. 2003), or at the littoral zone (Schmidt and Conrad 1993; Miyajima et al. 1997; Murase et al. 2003; Bastviken et al. 2004; Fernández et al. 2016; Delsontro et al. 2018; Peeters et al. 2019) can be a potential source of dissolved methane in the littoral zone, showing mean $\delta^{13}\text{C}$, $\delta^2\text{H}$, and $\Delta(2,13)$ values of $-56 \pm 2\text{‰}$, $-222 \pm$

57‰ , and $+384 \pm 59\text{‰}$, respectively, and thus, a potential source of increased methane in the pelagic zone during the stratified season, showing mean $\delta^{13}\text{C}$, $\delta^2\text{H}$, and $\Delta(2,13)$ values of $-55 \pm 2\text{‰}$, $-197 \pm 25\text{‰}$, and $+403 \pm 16\text{‰}$, based on similarities in the stable isotopic compositions of methane, particularly in the $\Delta(2,13)$ values.

Possible contributions of methane other than sediments

The methane produced in anoxic sediments at the mouths of inflowing rivers or the littoral zone can be a reasonable source of dissolved methane enriched in the epilimnion and upper thermocline based on the $\delta^{13}\text{C}$, $\delta^2\text{H}$, and $\Delta(2,13)$ values of methane, as presented in previous sections. Other sources, however, could potentially contribute, if they show stable isotopic compositions similar to that of enriched methane in the epilimnion and upper thermocline of the pelagic water column. Thus, we compare our values with those reported for the other possible sources, especially those pertaining to in situ production in the oxic water column (Table 1).

To the best of our knowledge, the isotopic compositions of methane aerobically produced from phytoplankton have not yet been determined. Vigano et al. (2009), however, determined the stable isotopic compositions of methane aerobically produced from plant material under UV irradiation. The median values of the stable isotopic compositions of methane emitted from C3 plants were $-67.0 \pm 4.5\text{‰}$ and $-360 \pm 32\text{‰}$ for $\delta^{13}\text{C}$ and $\delta^2\text{H}$, and thus, yielded values of $+370 \pm 81\text{‰}$ for $\Delta(2,13)$; those emitted from C4 and CAM plants were $-52.3 \pm 5.5\text{‰}$, $-279 \pm 32\text{‰}$, and thus, $+291 \pm 92\text{‰}$ for $\delta^{13}\text{C}$, $\delta^2\text{H}$, and $\Delta(2,13)$, respectively (Table 1). Although the $\delta^{13}\text{C}$ values of methane emitted from C4 and CAM plants coincided well with those of the methane enriched in the epilimnion and upper thermocline of the pelagic water column, showing mean $\delta^{13}\text{C}$ values of $-55 \pm 2\text{‰}$, it is unlikely that phytoplankton or periphyton conducted C4 photosynthesis in the lake. In contrast, while the $\delta^{13}\text{C}$ and $\delta^2\text{H}$ values of methane emitted from C3 plants showed significant differences from those enriched in the epilimnion and upper thermocline, the $\Delta(2,13)$ values of methane emitted from C3 plants ($+370 \pm 81\text{‰}$) corresponded to those of the methane enriched in the epilimnion and upper thermocline of the pelagic water column within the possible range of variation ($\Delta(2,13)$ values of $+403 \pm 16\text{‰}$) (Table 1). As a result, aerobic methane produced from phytoplankton (or phytoplankton-derived organic material) could be a major source if (1) the stable isotopic compositions of methane emitted from C3 plants represented those of aerobic methane production from phytoplankton and (2) methane oxidation was active in the pelagic water column. However, because the oxidation rates were almost zero in the epilimnion and upper thermocline during the stratified seasons (Fig. 4), it is difficult to explain the differences in $\delta^{13}\text{C}$ and $\delta^2\text{H}$ values via oxidation in the water column. We concluded that methane aerobically produced in situ in the pelagic water columns from phytoplankton (or phytoplankton-derived organic material) is likely a minor

Table 1. The mean stable isotopic compositions methane in pelagic zone water (10 m), together with those in the possible sources.

	1000 $\delta^{13}\text{C}$	1000 $\delta^2\text{H}$	1000 $\Delta(2,13)$	References
Pelagic zone (10 m)*	-55 ± 2	-197 ± 25	$+403 \pm 16$	This study
Littoral zone	-56 ± 2	-222 ± 57	$+384 \pm 59$	This study
Sediments [†]	-73 ± 1	-277 ± 6	$+517 \pm 11$	This study
C3 plants	-67.0 ± 4.5	-360 ± 32	$+370 \pm 81$	[1]
C4 and CAM plants	-52.3 ± 5.5	-279 ± 32	$+291 \pm 92$	[1]
Methoxyl groups in C3 plants	-50.4 ± 9.6	-220 ± 26	$+329 \pm 131$	[1]
Sinking particles	-36.7 to $+5.9$	No data	No data	[2]

References: [1] Vigano et al. (2009); [2] Sasakawa et al. (2008).

*The concentration-weighted mean values of methane at 10 m depth during the stratified seasons.

[†]The concentration-weighted mean values of methane dissolved in sediment pore water at depths of 10–25 cm from the surface in the pelagic zone.

source of methane enriched in the epilimnion and upper thermocline of the pelagic water column.

Vigano et al. (2009) also determined the stable isotopic compositions of methoxyl groups in C3 plants as $-50.4 \pm 9.6\text{‰}$, $-220 \pm 26\text{‰}$, and thus, $+329 \pm 131\text{‰}$ for $\delta^{13}\text{C}$, $\delta^2\text{H}$, and $\Delta(2,13)$, respectively (Table 1). Because the stable isotopic compositions of methoxyl groups included those determined for methane enriched in the epilimnion and upper thermocline of the pelagic water column ($-55 \pm 2\text{‰}$, $-197 \pm 25\text{‰}$, and thus, $+403 \pm 16\text{‰}$ for $\delta^{13}\text{C}$, $\delta^2\text{H}$, and $\Delta(2,13)$, respectively) within the possible range of variation (Table 1), the methoxyl groups in C3 plants could be a primary source of methane enrichment in the epilimnion and upper thermocline if the stable isotopic fractionation during conversion from the methoxyl groups to methane was minimal. At present, however, we do not have any knowledge on processes that convert methoxyl groups in organic material to methane (which can proceed at room temperature) accompanying such small isotopic fractionation. As a result, we concluded that the methoxyl groups in C3 plants were likely a minor source of methane enrichment in the epilimnion and upper thermocline of the pelagic water column.

Sasakawa et al. (2008) determined the stable carbon isotopic compositions of methane emitted from sinking particles in the western North Pacific to range from $-36.7 \pm 1.2\text{‰}$ at 40 m depth to $+5.9 \pm 7.5\text{‰}$ at 100 m depth (Table 1), concluding that methane production in sinking particles can be a major source of excess methane in the surface-water column. They also found that the end-member $\delta^{13}\text{C}$ values of excess methane increased with the water depth, probably due to the increasing oxidation/production ratio resulting from a lower CH_4 production rate in the sinking particles at greater depths (Sasakawa et al. 2008). Still, the $\delta^{13}\text{C}$ values of excess methane emitted from sinking particles are greater than -40‰ , on average, at 10 m depth (Sasakawa et al. 2008), while that for methane in the pelagic lake water column in Lake Biwa is $-55 \pm 2\text{‰}$. Furthermore, although the supersaturation ratios in methane concentration were less than 10% in the western North Pacific, where methane emission from sinking particles

was largely responsible for the supersaturation (Sasakawa et al. 2008), they were greater than 3000% at the surface in Lake Biwa, implying that we should assume much more important methane sources in Lake Biwa other than the sinking particles. Thus, we concluded that methane production in sinking particles is not a major source of methane enrichment in the epilimnion and upper thermocline of the pelagic water column either.

Concluding remarks

In this study, the vertical distributions of the concentration, $\delta^{13}\text{C}$, and $\delta^2\text{H}$ values of methane were determined in the water column of a mesotrophic, freshwater lake, together with their seasonal variation, to clarify the dual isotopic characteristics of oversaturated methane in the oxic surface of the pelagic zone during the stratified seasons. Additionally, we used the dual isotopic characteristics of methane in the oxic surface-water column of the pelagic zone and those in potential sources (e.g., sedimentary methane and methane in littoral zone water) to discuss possible sources of the existing methane. In conclusion, methane produced in anoxic sediments in the mouths of rivers flowing into the lake, or in the littoral zone, was the most probable source of methane observed in the littoral zone, and thus, the most probable source of enriched methane in oxic surface water during the stratified seasons. Further determination of the $\delta^{13}\text{C}$ and $\delta^2\text{H}$ values of methane in sediments of inflowing rivers and in the littoral zone will support this conclusion.

Because the stable isotopic compositions of methoxyl groups in C3 plants determined previously overlap with those determined for enriched methane in the oxic surface-water column, the methoxyl groups in C3 plants could be a potential source of enriched methane if the stable isotopic fractionation during conversion of the methoxyl groups to methane was minimal. Thus, further studies are needed to clarify the factors controlling the carbon and hydrogen isotope fractionation associated with the conversion from organic materials (including methoxyl groups) to methane, to verify the present

results. The isotopic compositions of methane aerobically produced from phytoplankton should be determined as well.

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Conflict of interest

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