

Research Article

Improvements in Drill-Core Headspace Gas Analysis for Samples from Microbially Active Depths

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The IsoJar™ container is widely used in headspace gas analysis for gases adsorbed on cuttings or bore cores from oil and gas fields. However, large variations in the carbon isotopic ratios of CH₄ and CO₂ are often reported, especially for data obtained from depths of <1000 m. The IsoJar™ method leaves air in the headspace that allows microbial oxidation of CH₄ to CO₂, meaning that isotopic fractionation occurs during storage. This study employed the IsoJar™ method to investigate the causes of differences in δ¹³C data reported by previous studies in the Horonobe area of Japan. It was found that after 80 d storage, δ¹³C_{CO2} values decreased by ~2‰, while δ¹³C_{CH4} values increased by >30‰, whereas samples analyzed within a week of collection showed no such fluctuations. The conventional amount of microbial suppressant (~0.5 ml of 10% benzalkonium chloride (BKC) solution) is insufficient to suppress microbial activity if groundwater is used as filling water. The significant variations in carbon isotopic compositions previously reported were caused by microbial methane oxidation after sampling and contamination by groundwater from different depths. To avoid these problems, we recommend the following: (1) if long-term sample storage is necessary, >10 ml of 10% BKC solution should be added or >0.3% BKC concentration is required to suppress microbial activity; (2) analyses should be performed within one week of sampling; and (3) for CO₂ analyses, it is important that samples are not contaminated by groundwater from different depths.

1. Introduction

Investigation of the origin of deep hydrocarbons is an important aspect of resource exploration and may lead to an improved understanding of geological environments. In this investigation, gases adsorbed on rock fragments or bore cores were studied by headspace gas analysis (e.g., [1–5]), which provides information on the generation and migration of light hydrocarbons and gases. The IsoJar™ (Isotech Laboratories and Humble Instruments, USA) container, which is widely used in such analyses (e.g., [6, 7]) comprises a plastic container of ~600 ml volume with an aluminum screw cap on which there is a rubber septum through which headspace gas can be taken by syringe. The analysis procedure (e.g., [8]) normally involves storage of wet cuttings or cores in the jar with water and an air headspace for several days or weeks, the addition of a microbicide such as benzalkonium chloride

(BKC) to minimize bacterial activity, the partitioning of gas into the headspace during storage, and analysis of these gases (e.g., [9–11]). The use of distilled or tap water avoids contamination from dissolved gases. The δ¹³C_{CH4} values of gases from depths of <1000 m, in the biogenic region, are usually in the range of –70‰ to –60‰, with isotopic compositions becoming heavier as depth increases towards the thermogenic region (e.g., [12, 13]). Large variations in carbon isotopic ratios in CH₄ and CO₂ are often reported for depths of <1000 m, with δ¹³C_{CH4} values sometimes reaching –20‰ (e.g., [14–17]). These variations are associated with the effects of microbial activity on methane production or oxidation in underground environments [18]. There are a number of factors that control the rate of methanogenesis [19], including temperature [20], groundwater salinity [21], pH [22], and pore space [23]. Peak microbial activity occurs at 35–45°C, which corresponds to depths of <1000 m [19, 24].

At greater depths, microbial action decreases as thermogenic production increases with the onset of catagenesis (subsequent to diagenesis at shallower depths [8]). More importantly, pore diameters of at least $1\ \mu\text{m}$ are necessary for in situ methanogenesis, as microbes are in the $1\text{--}10\ \mu\text{m}$ size range [25], which suggests that active methane production occurs at depths of $<1500\ \text{m}$ [24]. At shallow depths (less than several meters) below the ocean floor, where the concentration of dissolved gas is relatively low, considerable care was taken to avoid contamination and microbial activity (e.g., [26]). Hachikubo et al. [27] adjusted the concentration of BKC in samples to $\sim 2.5\%$ using 25 ml vials to obtain precise depth profiles of gases relative to hydrates. While the concentration and/or amount of microbicide normally added to IsoJar™ vessels are often omitted in reports, it is considered that the final concentration in IsoJar™ containers should be of the order of 0.01%, which is two orders of magnitude less than that reported by Hachikubo et al. [27]. It is speculated that another possible cause of variations in carbon isotopic composition may be microbial activity in the headspace after sampling, as the amount of microbicide commonly used with samples from microbially active depths might be insufficient to suppress microbial activity.

In a previous study, gas samples from two boreholes (PB-V01 and SAB-1, both $\sim 500\ \text{m}$ deep) in the Horonobe area, Hokkaido, were processed using IsoJar™ containers [14]. In that study, cores were stored in IsoJar™ containers with water and a few drops of BKC solution [14] for up to three months before headspace analysis. Because sampling date of cores and analysis date of gases, which are necessary for the calculation of the storage period, have not been presented in Funaki et al. [14], these unpublished information are summarized in Tables S1a and S1b. Concentration of the BKC solution and amounts of cores and water also have not been reported in Funaki et al. [14]. In the conventional way of using IsoJar™ headspace gas analysis, it is considered that the concentration of BKC solution is lower than 10%, in which case concentrations of BKC in jars are in order of 0.01%. In the construction of the Horonobe Underground Research Laboratory (URL), including two boreholes (PB-V01 and SAB-1), water was collected from groundwater at $\sim 50\ \text{m}$ depth, and this was used by Funaki et al. [14] as filling water for the IsoJar™ containers. Gases dissolved in deep groundwater from the URL were also analyzed, using an evacuated-vial (EV) method [28]. Measured $\delta^{13}\text{C}$ values for CH_4 and CO_2 from both sets of analyses are plotted against each other in Figure 1. Large variations in $\delta^{13}\text{C}_{\text{CH}_4}$ values from the IsoJar™ measurements (Figure 1) were attributed to methane-oxidizing bacterial activity using sulfate ions in the deep underground environment or to isotopic fractionation during gas migration through fractures [14]. However, these possible causes are considered unlikely because (a) geochemical studies in the Horonobe area indicate that reducing conditions are maintained deep underground and sulfate ions are either absent or present at very low concentrations [29–32]; (b) studies of iodine enrichment [33] indicates that any traces of methane oxidation by sulfate in pore waters of sediments would have been erased during upward fluid flow due to compaction during burial; and

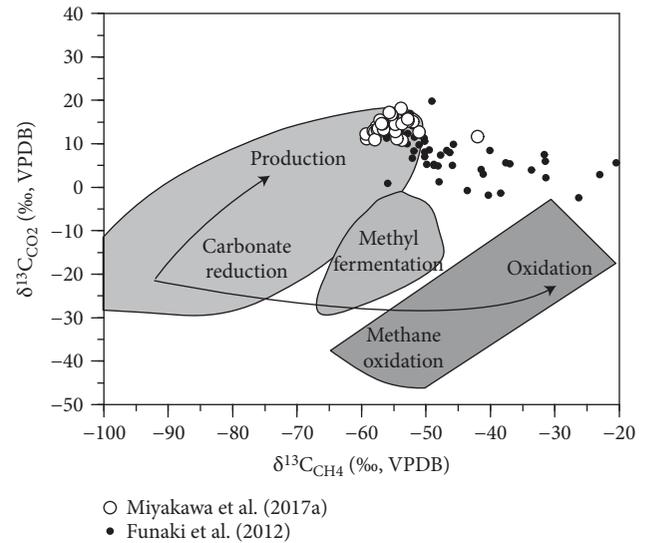


FIGURE 1: Plot of $\delta^{13}\text{C}_{\text{CH}_4}$ vs. $\delta^{13}\text{C}_{\text{CO}_2}$ in coexisting gases, showing fields relating to different gas sources and isotopic shifts resulting from production and oxidation (adapted from [50]). The data are from Funaki et al. [14] and Miyakawa et al. [28].

(c) there is no evidence in the study area of isotopic fractionation in gases during migration [34, 35]. The $\delta^{13}\text{C}_{\text{CH}_4}$ and $\delta^{13}\text{C}_{\text{CO}_2}$ values obtained by the EV method (Figure 1) show little scatter plotting in the carbonate reduction field.

Differences between these data sets could be attributed to factors such as aerobic microbial oxidation of methane in the containers after sampling and/or using groundwater from the different depths of core samples. Possible causes were investigated in the present study to improve the methodology of headspace gas analysis using IsoJar™. Gases from the Wakkanai Formation in the Horonobe area were sampled using the methods of Funaki et al. [14] and Miyakawa et al. [28]. The effects of the sampling method (storage period, water type, and additives) on carbon isotopic ratios in CH_4 and CO_2 were investigated, and improvements in headspace gas analysis techniques are suggested.

2. Geological Setting

The Horonobe area is located in northwestern Hokkaido, in a Neogene–Quaternary sedimentary basin (Figure 2). Since August 2006, the Japan Atomic Energy Agency (JAEA) has been excavating the URL for a research associated with the development of technologies related to the geological disposal of high-level radioactive waste. Geologically, the URL area comprises marine sediments of the Wakkanai Formation (Neogene siliceous mudstone containing opal-CT) and Koetoi Formation (Neogene–Quaternary diatomaceous mudstone containing opal-A). Burial and subsidence of these formations occurred throughout the Neogene and Quaternary, when they underwent early diagenetic thermal alteration at temperatures of $<60^\circ\text{C}$ [36]. The URL and surrounding geology are depicted in Figure 3. The high-pressure boreholes have steel casings, with valves allowing the sampling of groundwater from multiple depths or zones

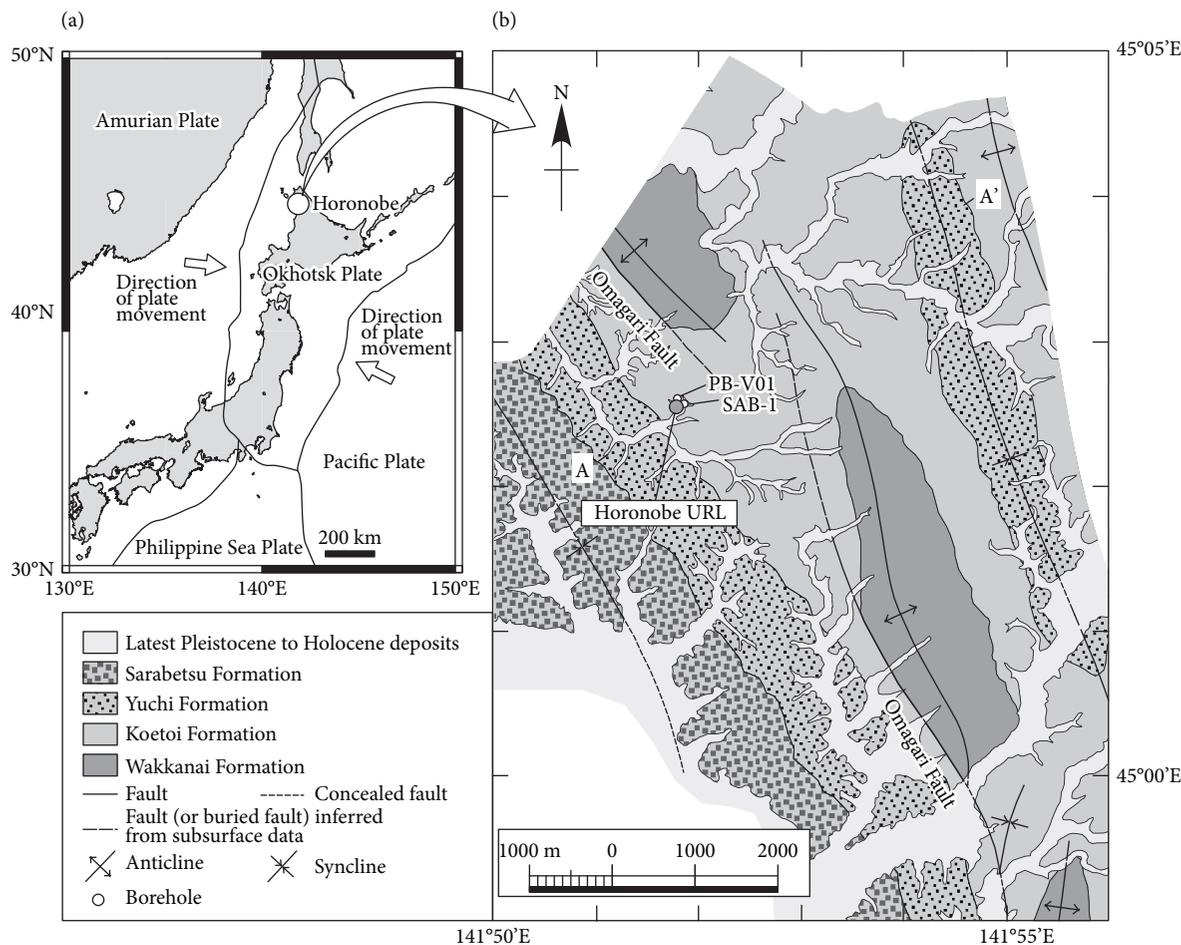


FIGURE 2: Maps showing the location of the Horonobe URL site and the boreholes: (a) location map and (b) geological map (after [51]). The plate boundaries and the direction of plate movement in (a) are from Wei and Seno [52].

[37]. The δD vs. $\delta^{18}O$ plots for groundwater from the Wakkanai and Koetoi Formations indicate that it is a mixture of local meteoric water and altered seawater [38, 39]. Methanogenic and methane-oxidizing microbial communities have played an important role in these formations [40–42], where secondary microbial gas with ^{13}C -enriched isotopic values ($\delta^{13}C_{CH_4}$, -74% to -28% ; $\delta^{13}C_{CO_2}$, -7% to $+31\%$) formed through CO_2 reduction after uplift of the area [28].

3. Sampling and Analytical Methods

3.1. IsoJar™ Samples

3.1.1. Effect of Storage Period. Core samples including in situ pore water (300–400 g), crushed to pieces roughly 30–50 mm in diameter, were placed in IsoJar™ containers with 250–300 g of “filling” water. $HgCl_2$ -saturated or 10% BKC aqueous solution (10 drops (~ 0.5 ml) of either) was added to suppress microbial activity, and the jars were sealed with an air headspace. Final concentrations of both microbicides in the jars were about 0.01%–0.02%. The jars were kept in the dark at room temperature for 5–92 d before analysis. Details of each experiment are summarized in Table 1 and Table S2.

The cores were obtained immediately after drilling of borehole 350-Fz-01 from the bottom of the east shaft (Figure 3). Although distilled or tap water is usually used in the IsoJar™ method, groundwater from depths of 53.5–64.5 m and 350 m in borehole 13-350-C01 (drilled in the 350 m gallery; Figure 3) was used to match the 50 m groundwater used by Funaki et al. [14]. The priority in this study was to evaluate the effects of sampling method on the carbon isotopic ratios of CH_4 and CO_2 , rather than to obtain accurate in situ values. A large portion of CH_4 dissolved in groundwater around the URL had already escaped due to the pressure decrease associated with excavation, so it was expected that only small amounts of gas would remain in cores. Groundwater, rather than tap water, was used to compensate for this (with water from borehole 13-350-C01 being used because water was not available from borehole 350-Fz-01). Core samples IJ1–IJ25 were from depths of 384–416 m (Table S2). Core samples IJ26–IJ28 were from a depth of 470 m (Table S2) and kept in vacuum storage for one month after drilling. At these depths, the C isotopic ratios for CH_4 and CO_2 had similar values to those from 350 m depth [28].

3.1.2. Effect of Additives. The effective amount of additives was investigated as follows. IsoJar™ samples were prepared

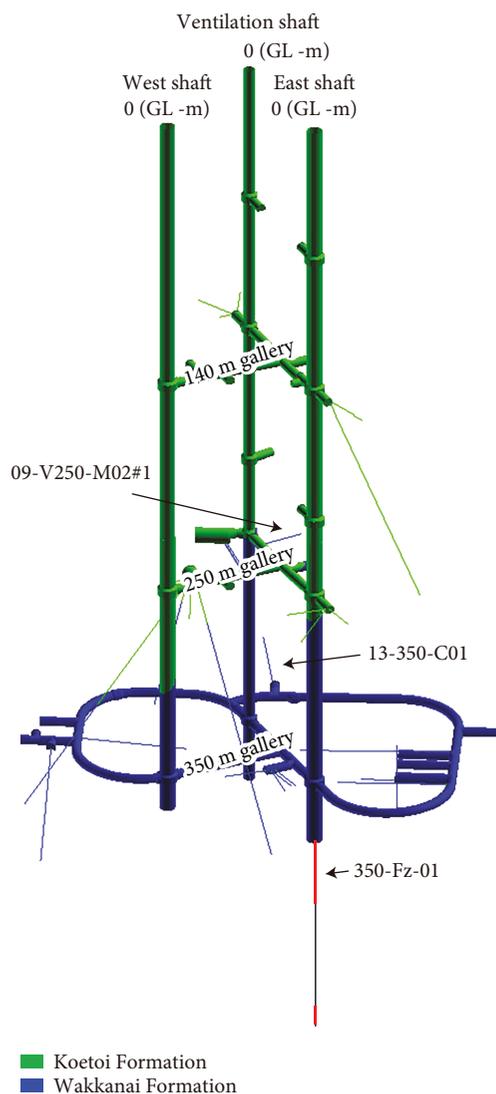


FIGURE 3: Layout of the Horonobe URL, locations of boreholes, and surrounding geology. Red color-labelled “borehole 350-Fz-01” indicates core sampling depths (Table S2).

as described in Section 3.1.1, with up to 20 ml of BKC and HgCl_2 solutions per jar (Table 2 and Table S3). All core samples were from a depth of 480 m in 350-Fz-01. Fresh cores taken immediately after drilling were not available for analysis, and the samples used in this study had been kept in vacuum storage for about six months. Groundwater from borehole 13-350-C01 was used as filling water. Headspace gas compositions were determined after storage in IsoJar™ for one month.

3.2. EV Samples. The EV sampling procedure involved the preliminary evacuation of septum-topped 50 ml glass vials containing ~1 ml phosphoric acid (85 wt%). Acidification removed any inorganic carbon as CO_2 , with the CO_2 concentration being measured as total inorganic carbon (TIC). Groundwater (15–30 ml, from borehole 09-V250-M02#1 drilled from the 250 m gallery; Figure 3) was introduced by a syringe through a $0.22\ \mu\text{m}$ membrane filter to remove

microsized carbonate grains and microbes. Samples were stored in the dark at room temperature for 5–98 d, after which ultrapure He was added by a syringe to equalize headspace gas and atmospheric pressures. The sample was left to stand overnight for gas exchange equilibrium to be established between the headspace and dissolved gases. The composition of the headspace gas was determined by GC, with the concentration of dissolved gas being calculated using Henry’s law and the ideal gas equation.

3.3. Analytical Procedure. Gases adsorbed on rock fragments in the IsoJar™ containers were desorbed into the headspace by ultrasonic shaking. Concentrations of O_2 , N_2 , CO_2 , CH_4 , C_2H_6 , and C_3H_8 in headspace gas were determined by gas chromatography (GC) using a GC7890A Valve System (Agilent Technologies, USA). Carbon isotopic values ($\delta^{13}\text{C}_{\text{CH}_4}$ and $\delta^{13}\text{C}_{\text{CO}_2}$) were determined by GC combustion isotope-ratio mass spectrometry (GC-C-IRMS), using an IsoPrime GC-MS system (GV Instruments, UK), and are expressed in the usual VPDB δ notation. The lower limit of determination of carbon isotope ratios ($\delta^{13}\text{C}_{\text{CH}_4}$ and $\delta^{13}\text{C}_{\text{CO}_2}$) requires concentrations of 0.01%. Details of GC and GC-C-IRMS procedures can be found in Waseda and Iwano [43].

4. Results

Headspace concentrations of the gases analyzed and $\delta^{13}\text{C}_{\text{CH}_4}$ and $\delta^{13}\text{C}_{\text{CO}_2}$ values for the IsoJar™ samples prepared as described in Section 3.1.1 are listed in Table 1 and Table S2, and the results for the EV samples are listed in Table 3 and Table S4.

Sample contamination by air does not affect the present discussion of isotopic ratios because concentrations of CH_4 and CO_2 in air are much lower than those in the free gas from groundwater (where $\text{CH}_4 = 74\%–100\%$; $\text{CO}_2 = 1\%–20\%$; [28, 34]).

CO_2 in the free gas undergoes isotopic fractionation during exchange with dissolved inorganic carbon (e.g., $\text{CO}_{2(\text{aq})}$, HCO_3^- , and CO_3^{2-}), with HCO_3^- being the dominant aqueous species at around neutral pH. To compare $\delta^{13}\text{C}_{\text{CO}_2}$ values of the IsoJar™ samples with those of the EV samples, a fractionation correction (intrinsic isotopic fractionation factor) of +7.9‰ between CO_2 gas and HCO_3^- at 25°C [44] was added to $\delta^{13}\text{C}_{\text{CO}_2}$ values measured for the IsoJar™ samples. The range of room temperatures was 20°C–25°C, giving an error of up to –0.6‰.

Variations in $\delta^{13}\text{C}_{\text{CO}_2}$ and $\delta^{13}\text{C}_{\text{CH}_4}$ values with storage time are shown in Figures 4(a) and 4(b), respectively. IsoJar™ samples with groundwater (350 m) stored for less than one week give values similar to the EV samples. Storage periods of ≤ 7 d and ≥ 20 d were considered, as no data are available for the interval of 7–20 d.

The $\delta^{13}\text{C}_{\text{CO}_2}$ and $\delta^{13}\text{C}_{\text{CH}_4}$ ratios for the EV samples are relatively constant, although $\delta^{13}\text{C}_{\text{CH}_4}$ values show a small variation (<4‰) after 98 d (Figure 4(b)), which is consistent with a previous study [28]. It is possible that some microbes are not removed by $0.22\ \mu\text{m}$ groundwater filtration (e.g., [45]), and this might have caused the slight variation in $\delta^{13}\text{C}_{\text{CH}_4}$ values. Differences between carbon isotopic values

TABLE 1: Information on IsoJar™ samples and analytical results of headspace gases.

Sample no.	Amount of core sample (g)	Amount of water (g)	Depth of groundwater	Type of additive	Storage period (days)	CO ₂ (%)	O ₂ (%)	N ₂ (%)	CH ₄ (%)	$\delta^{13}\text{C}_{\text{CH}_4}$ (‰ VPDB)	$\delta^{13}\text{C}_{\text{CO}_2}$ (‰ VPDB)
IJ1	220	350	350 m	None	67	6.5	6.9	83	0.030	-45.4	+13.6
IJ2	369	262	350 m	None	41	8.9	2.8	79	6.7	-35.5	+13.2
IJ3	342	304	350 m	None	79	3.1	11.0	80	2.8	-30.8	+13.2
IJ4	360	269	50 m	None	17	0.63	14.6	80	2.2	-49.3	+5.0
IJ5	239	333	50 m	None	68	2.3	9.4	86	0.014	-51.4	+2.1
IJ6	411	247	50 m	None	42	6.7	0.01	79	12.5	-42.5	+3.5
IJ7	339	277	50 m	None	79	2.5	5.4	80	9.6	-36.8	-1.0
IJ8	338	279	50 m	None	79	0.36	18.1	79	0.45	-22.3	-1.4
IJ9	302	274	350 m	HgCl ₂	19	3.9	7.9	80	5.8	-57.7	+15.2
IJ10	263	328	350 m	HgCl ₂	67	5.0	2.4	86	4.8	-40.7	+14.5
IJ11	380	274	350 m	HgCl ₂	41	4.7	5.1	76	12.3	-48.8	+15.7
IJ12	383	241	350 m	HgCl ₂	92	1.49	14.1	80	0.88	-26.8	+12.9
IJ13	358	262	50 m	HgCl ₂	19	2.6	8.1	75	11.9	-55.3	+6.0
IJ14	225	349	50 m	HgCl ₂	68	0.52	16.8	81	0.059	+4.7	+2.7
IJ15	420	252	50 m	HgCl ₂	42	4.0	3.6	83	7.5	-45.0	+4.7
IJ16	338	284	50 m	HgCl ₂	79	0.57	14.2	80	2.0	-38.2	+2.5
IJ17	341	229	350 m	BKC	19	6.2	6.9	82	1.89	-50.3	+14.5
IJ18	264	281	350 m	BKC	67	8.9	2.2	80	5.2	-44.6	+13.5
IJ19	388	253	350 m	BKC	41	7.4	0.02	76	15.5	-48.6	+14.3
IJ20	333	302	350 m	BKC	79	5.0	4.4	79	7.6	-46.2	+14.4
IJ21	320	290	50 m	BKC	19	4.7	1.82	85	5.5	-46.2	+3.7
IJ22	345	256	50 m	BKC	68	3.7	8.2	84	2.3	-26.7	+2.8
IJ23	392	258	50 m	BKC	42	4.4	3.2	80	10.2	-42.7	+4.3
IJ24	368	291	50 m	BKC	79	2.2	4.1	80	10.2	-41.8	+2.2
IJ25	360	266	50 m	BKC	79	2.6	3.0	79	13.4	-43.5	+3.2
IJ26	388	242	350 m	None	5	2.3	14.2	79	3.6	-55.4	+14.5
IJ27	379	272	350 m	None	5	3.0	11.6	78	6.1	-53.8	+14.8
IJ28	333	316	350 m	None	5	2.1	12.7	80	3.5	-55.5	+15.1

of CH₄ and CO₂ ($\delta^{13}\text{C}_{\text{CO}_2} - \delta^{13}\text{C}_{\text{CH}_4}$ = isotopic separation factor; [46]) in the Horonobe area decrease slightly (<1‰) with increasing depth and temperature according to isotopic equilibrium values, with values at depths of 250 m and 350 m being similar to each other within the natural variation of ~2‰ (1 σ) [28]. Therefore, $\delta^{13}\text{C}_{\text{CO}_2}$ and $\delta^{13}\text{C}_{\text{CH}_4}$ values of 250 m groundwater obtained by the EV method (Table 3) were used as reference values for the IsoJar™ samples with 350 m groundwater.

The $\delta^{13}\text{C}_{\text{CO}_2}$ values for IsoJar™ samples show two separate trends for 350 m and 50 m groundwater, with both showing a slight decrease over time, from +15‰ at 5 d to +13‰ at 92 d and from +5‰ at 17 d to -1‰ to +3‰ at 79 d, respectively (Figure 4(a)). The patterns are the same for both BKC and HgCl₂ additives. The two trends suggest that the $\delta^{13}\text{C}_{\text{CO}_2}$ ratios represent dissolved gases from different depths. The concentration of CO₂ in the groundwater, as HCO₃⁻, is relatively high even when degassed at atmospheric pressure (30–50 mmol kg⁻¹; [47]), compared with that of adsorbed CO₂, so carbon isotopic values should be largely dependent on dissolved CO₂. The $\delta^{13}\text{C}_{\text{CO}_2}$ values of samples

with 50 m groundwater (dashed line in Figure 4(a)) are distinct from those of samples with 350 m groundwater, indicating that the former samples are strongly contaminated.

The $\delta^{13}\text{C}_{\text{CH}_4}$ values for IsoJar™ samples (Figure 4(b)) increase markedly with time regardless of additives and fluctuate by more than 30‰ after 80 d; separate trends for different depths are not evident. The concentration of CH₄ remaining in the groundwater at atmospheric pressure after sampling is relatively low (~3 mmol kg⁻¹; [47]), and $\delta^{13}\text{C}_{\text{CH}_4}$ values for the IsoJar™ samples mainly represent gases adsorbed on cores. Therefore, while the relatively large amounts of dissolved CO₂ reduced the effects of isotopic fractionation on $\delta^{13}\text{C}_{\text{CO}_2}$ values, $\delta^{13}\text{C}_{\text{CH}_4}$ values were strongly affected.

The results of the effect of additives as described in Section 3.1.2 are shown and discussed in Section 5.2.

5. Discussion

5.1. Methane Oxidation. During methane oxidation, decreasing CH₄ and increasing CO₂ concentrations are associated

TABLE 2: Information on IsoJar™ samples and analytical results of headspace gases for Section 3.1.2.

Sample no.	Amount of core sample (g)	Amount of water (g)	Type of additive	Amount of additive (ml)	Concentration of additives (wt%)	Storage period (days)	CO ₂ (%)	O ₂ (%)	N ₂ (%)	CH ₄ (%)	$\delta^{13}\text{C}_{\text{CH}_4}$ (‰ VPDB)	$\delta^{13}\text{C}_{\text{CO}_2}$ (‰ VPDB)
IJ29	311	310	None	0	0	27	12.8	1.0	86	0.039	-48.3	+14.2
IJ30	299	282	None	0	0	27	13.7	1.0	85	0.029	-74.6	+15.2
IJ31	284	324	BKC	1	0.03	27	12.5	1.0	86	0.036	-74.2	+14.0
IJ32	323	297	BKC	1	0.03	27	12.4	1.0	87	0.040	-84.1	+13.8
IJ33	332	312	BKC	3	0.10	27	11.6	4.2	84	0.053	-88.5	+13.9
IJ34	352	279	BKC	3	0.11	28	13.1	2.0	85	0.046	-75.7	+13.0
IJ35	368	290	BKC	6	0.20	28	10.2	4.1	86	0.067	-58.1	+12.9
IJ36	382	278	BKC	6	0.21	28	10.2	2.9	87	0.067	-90.4	+12.5
IJ37	309	299	BKC	10	0.32	28	12.8	7.3	80	0.054	-56.2	+14.8
IJ38	310	280	BKC	10	0.34	28	12.8	6.6	80	0.052	-57.9	+14.6
IJ39	340	285	BKC	20	0.66	29	10.9	6.2	83	0.068	-54.5	+13.6
IJ40	336	309	BKC	20	0.61	29	9.0	3.7	87	0.105	-57.9	+13.4
IJ41	272	319	HgCl ₂	1	0.02	29	8.7	10.9	80	0.042	-59.0	+15.0
IJ42	323	296	HgCl ₂	1	0.02	29	10.2	9.9	80	0.041	-63.1	+14.9
IJ43	348	284	HgCl ₂	5	0.13	29	10.8	9.2	80	0.037	-65.4	+15.0
IJ44	319	314	HgCl ₂	5	0.12	29	11.0	9.0	80	0.044	-60.1	+14.8
IJ45	293	322	HgCl ₂	10	0.22	30	12.7	8.4	79	0.043	-62.6	+15.2
IJ46	362	293	HgCl ₂	10	0.24	30	13.3	6.9	80	0.046	-61.9	+15.3
IJ47	285	308	HgCl ₂	20	0.45	30	21	7.0	72	0.033	-62.2	+16.6
IJ48	362	295	HgCl ₂	20	0.47	30	23	6.0	71	0.045	-62.1	+16.1

TABLE 3: $\delta^{13}\text{C}_{\text{CH}_4}$, $\delta^{13}\text{C}_{\text{CO}_2}$, methane, and TIC contents in EV samples.

Sample no.	Storage period (days)	Sampling depth of groundwater (ground level -m)	$\delta^{13}\text{C}_{\text{CH}_4}$ (‰ VPDB)	$\delta^{13}\text{C}_{\text{CO}_2}$ (‰ VPDB)	Concentration of dissolved CH ₄ (mmol kg ⁻¹)	Concentration of total inorganic carbon (TIC) (mmol kg ⁻¹)
EV1	6	248.5	-56.7	+17.1	—	—
EV2	55	248.5	-54.0	+17.8	7.8	—
EV3	98	248.5	-53.7	+17.3	8.5	—
EV4	6	248.5	-56.8	+16.9	8.7	42
EV5	55	248.5	-54.5	+16.2	6.7	37
EV6	98	248.5	-52.7	+16.7	6.7	35

with carbon isotopic fractionation, resulting in enrichment of ^{13}C in unreacted CH₄ and depletion in CO₂ produced. However, there is no clear relationship between headspace gas concentrations and storage period, possibly because of the variability of adsorbed gas levels in natural samples. An apparent carbon isotopic fractionation factor, α , defined as $\alpha = (\delta^{13}\text{C}_{\text{CO}_2} + 1000)/(\delta^{13}\text{C}_{\text{CH}_4} + 1000)$, was calculated using the $\delta^{13}\text{C}_{\text{CO}_2}$ and $\delta^{13}\text{C}_{\text{CH}_4}$ values in Table 1 (Figure 5). Initial values of α in the Horonobe samples, calculated using the results of the EV method, were around 1.06–1.08, in good agreement with those determined by Miyakawa et al. [28]. The values of α for the IsoJar™ samples decreased from ~1.07 to 1.04 with increasing storage time (Figure 5). An earlier study reported a similar trend, where microbial methane oxidation in marine sediments gave an α value of ~1.08 in the methanogenesis zone, decreasing to ~1.02 in the methane oxidation zone [48].

A bivariate plot of $\delta^{13}\text{C}_{\text{CH}_4}$ vs. $\delta^{13}\text{C}_{\text{CO}_2}$ (Figure 6) indicates two trends for the IsoJar™ samples (Figure 6(b)), as in Figure 4(a). Thus, data plotted in Figures 4–6 indicate that $\delta^{13}\text{C}_{\text{CH}_4}$ values in samples stored for more than one week were affected by CH₄ oxidation to CO₂. $\delta^{13}\text{C}_{\text{CH}_4}$ and $\delta^{13}\text{C}_{\text{CO}_2}$ values reported by Funaki et al. [14] are plotted between the two trends (Figure 6(b)). It seems, therefore, that the variations can be explained by the mixing of the two trends, indicating effects of both methane oxidation and contamination of 50 m groundwater in the IsoJar™ container after sampling.

5.2. Effect of Additives. Significant isotopic fractionation occurred in the IsoJar™ samples, strongly affecting $\delta^{13}\text{C}_{\text{CH}_4}$ values despite the addition of BKC or HgCl₂ (Figure 4), suggesting that the amounts of additives used were insufficient to suppress microbial activity. Results of effect of additives as

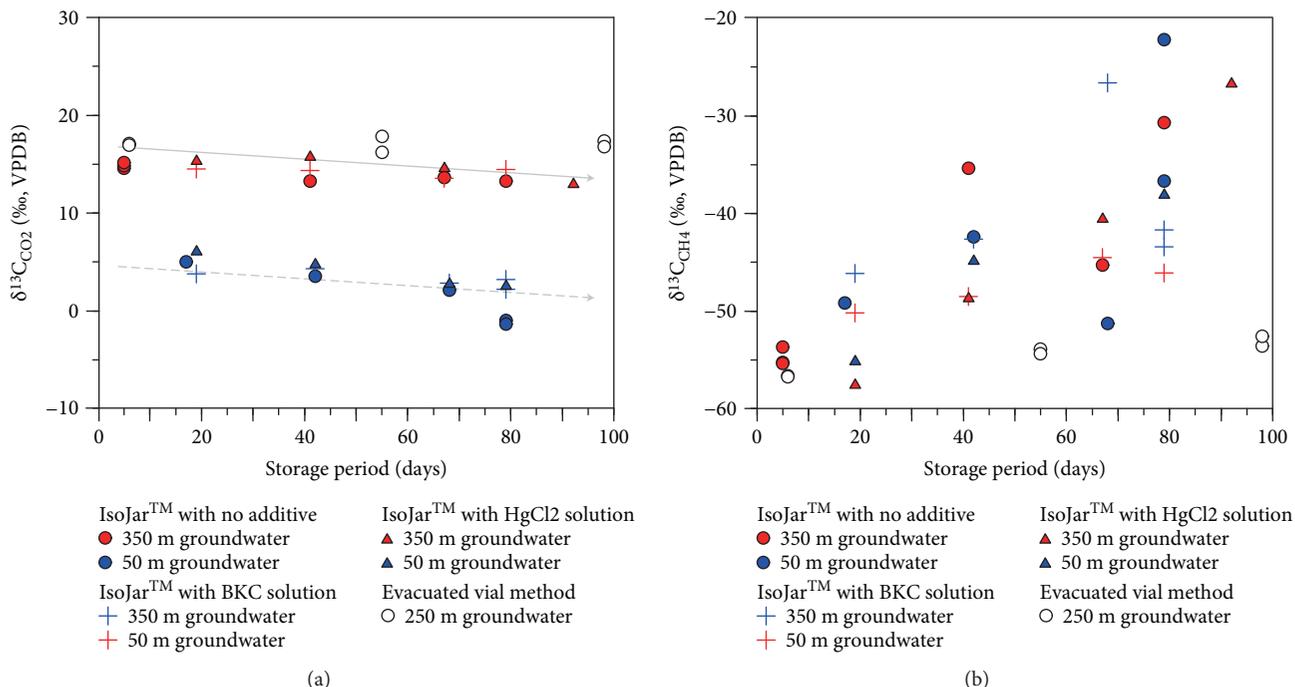


FIGURE 4: Temporal variations in (a) $\delta^{13}\text{C}_{\text{CO}_2}$ and (b) $\delta^{13}\text{C}_{\text{CH}_4}$ after sampling. Solid arrow indicates the trend for samples with 350 m groundwater; dashed arrow indicates the trend for samples with 50 m groundwater.

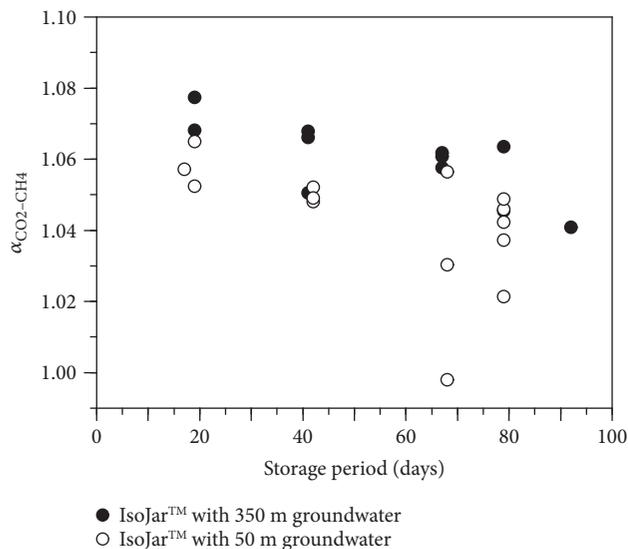


FIGURE 5: Temporal variations in the apparent carbon isotopic fractionation factor, α , between CO_2 and CH_4 .

described in Section 3.1.2 are listed in Table 2 and shown in Figure 7. With <10 ml BKC solution or $<0.3\%$ BKC concentration, $\delta^{13}\text{C}_{\text{CH}_4}$ values fluctuated significantly, and isotopic compositions became lighter. This is opposite to the effect of microbial carbonate reduction and may be due to low CH_4 concentrations (Table 2) resulting from storage of the cores for about six months in a vacuum container (to avoid oxidation by the air and drying), possibly with significant removal of adsorbed gases. An instrumental mass bias of carbon isotope ratio was reported in a very low concentration of

hydrocarbons with respect to mass spectrometry [49]. In this study, all the data of CH_4 concentrations were above the lower limit of determination of 0.01% (Table 2) indicating that large fluctuations of $\delta^{13}\text{C}_{\text{CH}_4}$ values (Figure 7) are not due to instrumental mass bias. The mechanism of any reaction opposing fractionation is not clear. In a low CH_4 concentration, the carbon isotope ratio may be easily disturbed by complex microbial metabolism (e.g., methane oxidation and carbonate reduction). With >10 ml BKC solution or $>0.3\%$ BKC concentration, the $\delta^{13}\text{C}_{\text{CH}_4}$ values were relatively constant at about -56% (Figure 7), which is in good agreement with the values for EV samples.

Although the $\delta^{13}\text{C}_{\text{CH}_4}$ values for IsoJar™ samples with HgCl_2 solution are relatively constant (Figure 7), they are consistently 5‰–6‰ lower than those with BKC solution. Concentrations of CO_2 in the headspace of samples IJ47 and IJ48, to which 20 ml HgCl_2 solution was added, are considerably higher than those in the other samples (Table 2). A possible cause of the decrease in $\delta^{13}\text{C}_{\text{CH}_4}$ values may be isotopic fractionation associated with mercuric reactions with methane, which generate HCl and lead to CO_2 outgassing. Although this mechanism is not clear, considerable care should be required using HgCl_2 as a microbicide with respect to carbon isotope fractionation.

6. Conclusions

This study investigated the possible causes of carbon isotopic variations (in $\delta^{13}\text{C}_{\text{CH}_4}$ and $\delta^{13}\text{C}_{\text{CO}_2}$ values) in borehole drillings to 500 m depth, as reported by Funaki et al. [14]. The results have led to improvements in the IsoJar™ method for the determination of carbon isotopic ratios in CH_4 and

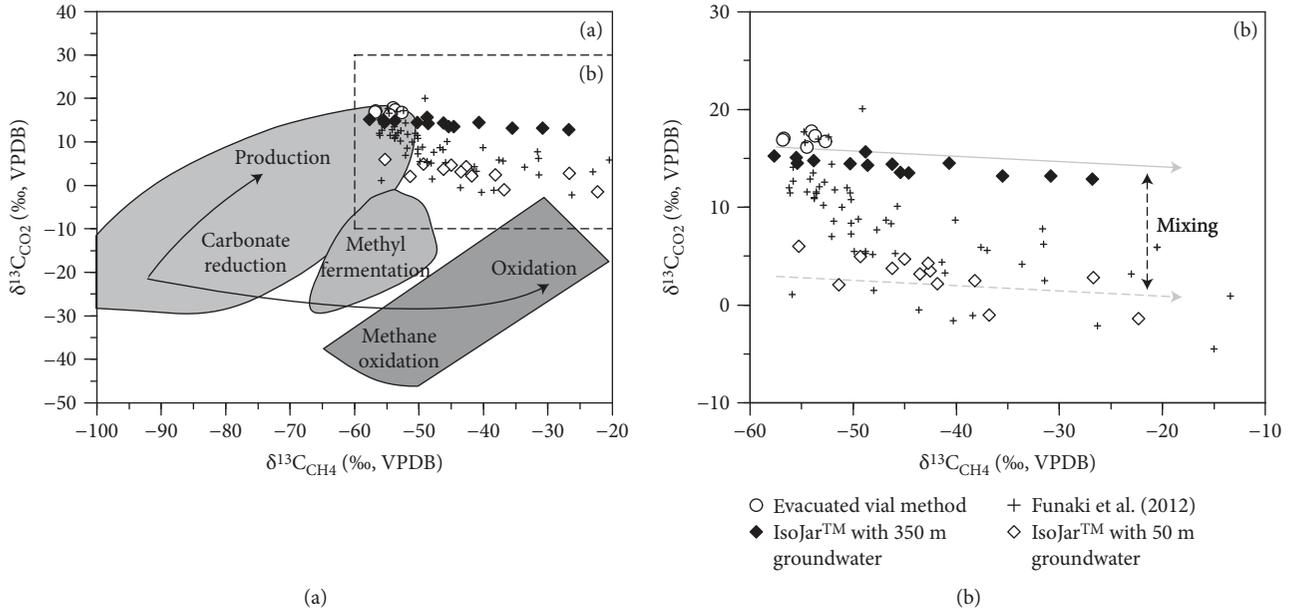


FIGURE 6: Plot of $\delta^{13}\text{C}_{\text{CH}_4}$ vs. $\delta^{13}\text{C}_{\text{CO}_2}$ in coexisting gases. (a) Grey areas show fields relating to different gas sources and isotopic shifts resulting from production and oxidation (adapted from [50]). The area indicated by the dashed rectangle is enlarged in (b). (b) Solid arrow indicates a methane oxidation trend for samples with 350 m groundwater; dashed arrow indicates the trend for samples with 50 m groundwater.

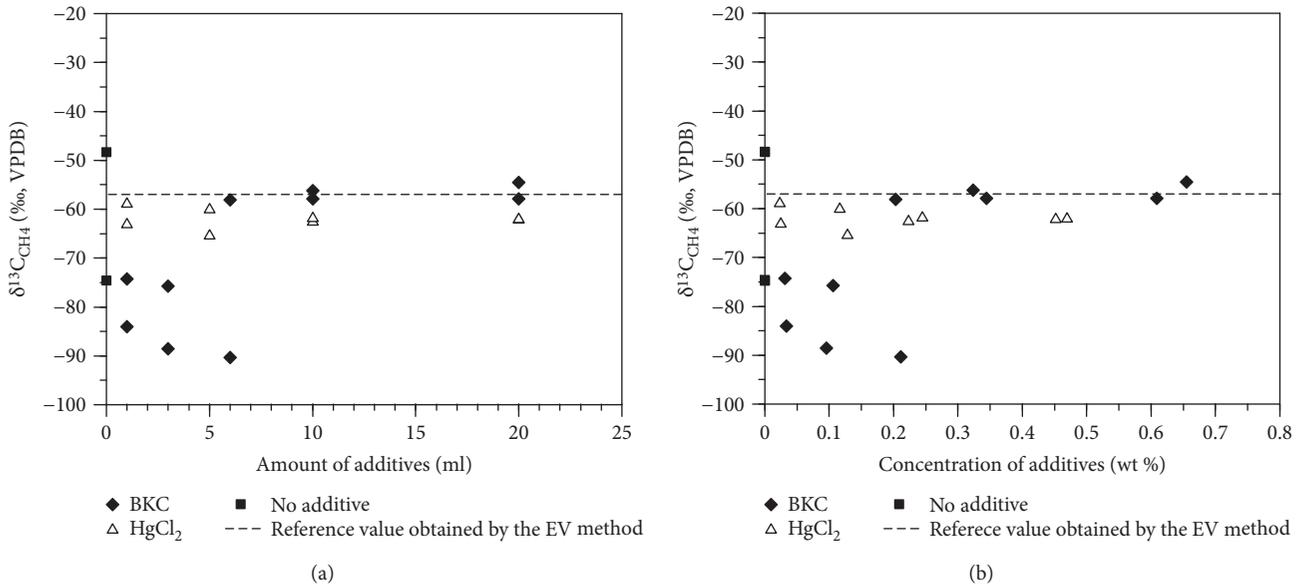


FIGURE 7: Plot of $\delta^{13}\text{C}_{\text{CH}_4}$ values vs. (a) amount of microbial suppressant used and (b) concentration of additives.

CO_2 adsorbed on bore cores. It was found that with air in the IsoJar™ headspace, microbes oxidize CH_4 to CO_2 during storage, accompanied by isotopic fractionation, especially for samples from depths of <1000 m where microbes are more active. Isotopic fractionation resulted in $\delta^{13}\text{C}_{\text{CH}_4}$ and $\delta^{13}\text{C}_{\text{CO}_2}$ values reaching >30‰ and >2‰ after 80 d storage, respectively, while samples analyzed within a week of sampling showed no such effect. The significant isotopic fractionation in CH_4 was due to its low concentration in the sampling container, while the weaker fractionation in CO_2 was due to

its relatively high concentration. The conventional amount of BKC additive (~0.5 ml of 10% solution) was insufficient to suppress microbial activity at least when using in situ groundwater as filling water. The large variations in isotopic compositions reported by Funaki et al. [14] thus appear to have been caused by microbial methane oxidation in the IsoJar™ containers after sampling and contamination with groundwater from different depths. Important technique improvements are summarized as follows: (1) if long-term sample storage is necessary, >10 ml of 10% BKC solution

should be used or >0.3% BKC concentration is required; (2) analysis within a week of sampling is strongly recommended; and (3) for CO₂ analysis, groundwater from different depths should not be used.

Data Availability

The all data used to support the findings of this study are included within the article and Supplementary information file.

Conflicts of Interest

The authors declare that there is no conflict of interest regarding the publication of this paper.

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Supplementary Materials

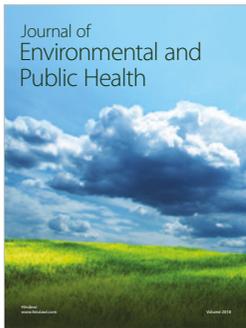
Sampling date, analysis date, sampling depth of cores, and carbon isotope ratios of CH₄ and CO₂ of headspace gases in IsoJarTM samples of Funaki et al. [14] (Tables S1a and S1b). Sampling date, analysis date, sampling depth of cores, and chemical compositions of headspace gases in IsoJarTM samples and EV samples of this study (Tables S2, S3, and S4). (*Supplementary Materials*)

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