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Variation in Soil Methane Fluxes and Comparison between Two Forests in China

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Abstract: Methane (CH₄) is a vital greenhouse gas with a 28-fold higher global warming potential than carbon dioxide when considering a molar basis for the time horizon of 100 years. Here, we investigated the variation of soil CH₄ fluxes, soil physiochemical properties, and CH₄-related bacteria community composition of two forests in China. We measured CH₄ fluxes using static chambers and analyzed soil bacterial communities using next-generation high-throughput sequencing in a temperate broad-leaved deciduous forest at Baotianman Nature Reserve (TBDF-BTM) and a tropical rainforest at Jianfengling National Natural Reserve (TRF-JFL). Our results showed that the soils from both sites were CH₄ sinks. Significant variation in soil CH₄ fluxes was found at TBDF-BTM exclusively, while no seasonal variation in the CH₄ uptake was observed at TRF-JFL. The CH₄ fluxes at TBDF-BTM were substantially higher than those at TRF-JFL during all seasons. One genus of methanotrophs and three genera of methylotrophs were detected at both sites, though they had no direct relationship with soil CH₄ fluxes. Water-filled pore space and soil total carbon content are the main factors controlling the soil CH₄ fluxes at TBDF-BTM. At TRF-JFL, the soil CH₄ fluxes showed no significant correlations with any of the soil properties. This study improves our understanding of soil CH₄ fluxes and their influencing factors in forests in different climatic zones and provides a reference for future investigation of forest soil CH₄ fluxes, the forest ecosystem carbon cycle, and the forest CH₄ model.

Keywords: CH₄ fluxes; soil physiochemical properties; bacterial communities; temperate deciduous broad-leaved forest; tropical rainforest

1. Introduction

Methane (CH₄) is a major greenhouse gas. Although CH₄ occurs at lower concentrations than carbon dioxide (CO₂) in the atmosphere, the former has a 28-fold higher global warming potential than the latter when compared on a molar basis for the time horizon of 100 years [1]. CH₄ contributes to 20% of the global greenhouse effect [2]. Well-aerated forest soils are known to be significant global carbon sinks for mitigating the atmospheric CH₄ [3,4]. The annual CH₄ uptake of soil in the world is 26–36 Tg·a⁻¹, among which forest soil accounts for 52% [5,6]. China has a large forest area of 2.08 × 10⁸ hm², covering 22% of the total area of the country, which has a huge potential for CH₄

uptake [7,8]. The annual CH₄ uptake of forest soils in China is 0.675 Tg·a⁻¹, with Chinese forest soil methane uptake representing about 3.6–5% of the global CH₄ sink [7,8]. Wang et al. calculated the mean CH₄ flux from forests in China, which is comparable with CH₄ uptake rates by other forests in the world [9–11]. It is generally believed that the dynamic changes in the CH₄ uptake of forest soil are one of the important factors affecting the changes in atmospheric CH₄ concentration [4].

Changes in CH₄ fluxes of forest soil are influenced by multiple factors, such as soil water content [12,13] and soil temperature [14,15]. Soil water content is a key driver of CH₄ fluxes in forest soils, as it limits the diffusive transport of gases in the soil [16]. The effect of water content on CH₄ consumption typically follows a parabolic curve in soils, with reduced rates under the conditions of low or high water content [17–20]. Another important environmental driver of CH₄ flux in soils is soil temperature [21]. However, results about the influence of temperature on CH₄ flux are not consistent [22–27]. In addition to soil moisture and temperature, pH [28], organic matter content [29], nitrogen availability [30,31] also affect CH₄ fluxes.

In addition to the numerous abiotic factors listed above, biological factors also affect CH₄ emission and consumption. CH₄ is produced by methanogens under anaerobic conditions and oxidized by methanotrophs under aerobic conditions [31]. Therefore, CH₄ fluxes have been shown to be closely associated with the community composition [32–34], abundance, and activity [35] of both methanogens and methanotrophs [36]. Methylophages cannot consume CH₄ directly but can make use of oxidized products of CH₄ (e.g., methanol and formaldehyde) and are therefore considered important in CH₄ removal processes in natural environments [36].

Methanotrophs are classified into three types (I, II, and X) on the basis of the type of the unique enzymes of methane monooxygenases (MMOs) they contain [37]. Methanotrophs can also be divided into ‘high-affinity’ or ‘low affinity’ groups based on their activity. The ‘low affinity’ methanotrophs are adapted for growth at high CH₄ concentrations (several thousand ppm in air), such as those arising from wetlands, lakes, and rice paddies [38]. The ‘high affinity’ methanotrophs can make use of the trace amounts of methane (around 1.8 ppm) in the atmosphere (i.e., in forest soils) [39,40]. Type I methanotrophs have been grouped into the ‘low affinity’ group and are prevalent in CH₄-rich and poorly aerated environments, while type II ‘high-affinity’ are predominant in CH₄-poor and well aerated environments such as *Methylosinus* and *Methylocystis* [41]. From the perspective of phylogeny, the type I methanotrophs belong to γ -Proteobacteria, and the type II methanotrophs to α -Proteobacteria. Although the type X species also belong to the γ -Proteobacteria, they are distinct from the group of type I methanotrophs. The type I and type X methanotrophs are both in the family Methylococcaceae. It seems that within the α - and γ -Proteobacteria, they are related to methane oxidation [42]. A species of type II methanotroph *Methylocystis bryophila* exhibited the highest methanol production [37]. The culture-independent studies of methanotrophic communities of soils with high-affinity methane oxidation capacity have showed their presence and a correspondingly frequent superiority of methanotrophs from the novel phylogenetic pmoA gene from α -proteobacteria and γ -proteobacteria named upland soil cluster (USC α) and USC-gamma (USC γ) [43,44]. The USC α bacteria are related to *Methylocapsa acidiphila* [45] and the USC γ bacteria are distantly related to Methylococcaceae [43].

The Baotianman Nature Reserve (BTM) and the Jianfengling National Natural Reserve (JFL) represent the typical temperate and tropical forests, respectively, and provide ideal places to study the ecological function in temperate and tropical forests in China. Previous research at the temperate broad-leaved deciduous forest (TBDF)-BTM has mainly investigated litter decomposition [46,47], biomass carbon storage [48], spatial variability of temperature sensitivity [49], soil respiration [50,51], and nutrient resorption [52]. Research at the tropical rainforest (TRF)-JFL has generally focused on soil respiration [53], biomass, and carbon dynamics [54], in addition to N₂O fluxes [55]. To date, many previous studies related to CH₄ in the temperate and tropical forest ecosystems have mostly concentrated on artificial interference experiment such as fertilizer application, increases of temperatures, indoor control culture experiment, and the comparison of soil CH₄ fluxes under

different forest ages. There are many published reports on CH₄ fluxes and their influencing factors, such as the soil physiochemical properties and microbial communities associated with CH₄ dynamics in different forest types without human disturbances, but there have not been published reports within the TBDF-BTM and TRF-JFL.

In the present study, we analyzed the temporal and spatial dynamics of CH₄ flux and the relevant differences between different forest sites and the relationship with important biotic and abiotic controls. Considering the differences in climatic factors, soil physical, and chemical properties, and the possible differences in microbial community at both TBDF-BTM and TRF-JFL, we hypothesized that there would be significant differences in the CH₄ flux between two sites.

2. Materials and Methods

2.1. Site Description

This study was conducted in two forest sites in national natural reserves in China. The temperate broad-leaved deciduous forest at Baotianman Nature Reserve (TBDF-BTM) (33°20′–33°36′ N, 111°47′–112°04′ E) is located in the southwest of Henan Province, east of Qinling Mountains and on the southern slope of the Funiu Mountains (Figure 1). Baotianman Nature Reserve is located between a northern subtropical climate and a warm temperate climate [56], where the deciduous broad-leaved forest ecosystems are considered to be more sensitive to disturbances and susceptible to climate changes, especially global warming [57]. TBDF-BTM has a continental monsoon climate with four distinctive seasons. The mean annual precipitation is 885.6 mm (60% from July–August) and the mean annual air temperature is 15.1 °C [51]. Our study sites were selected in a *Quercus acutidentata* (Maxim. ex Wenz.) Koidz. natural secondary forest (20 m × 20 m) in the vicinity of a flux tower at TBDF-BTM. The dominant tree species are *Quercus aliena* var. *acuteserrata* Maxim., *Q. glandulifera* var. *brevipetiolata* (A.DC.) Nakai, *Q. variabilis* Blume, *Carpinus cordata* Blume, *Cornus controversa* Hemsl., and *Tilia americana* L. [46,50,51]. The main soil type is brunisolic soil for TBDF-BTM [58].

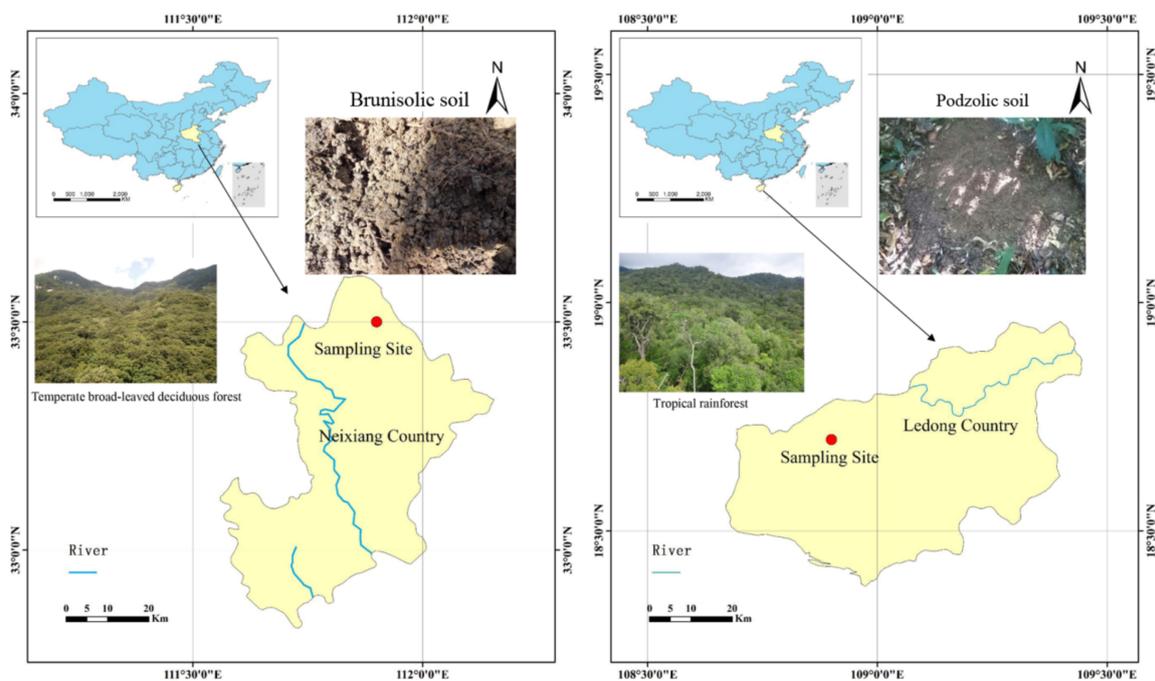


Figure 1. Map of the study areas. Baotianman National Natural Reserve in Neixiang County, Henan Province (**left**) and Jianfengling National Natural Reserve in Ledong County, Hainan Province (**right**), China.

The tropical rainforest at Jianfengling National Natural Reserve (TRF-JFL) (18°23′–18°52′ N, 108°36′–109°05′ E) is situated on Hainan Island, which is located at the northern edge of tropical Asia and has the best-protected tropical mountain rainforests in China [59] (Figure 1). The TRF-JFL tropical mountain rainforest is located between subtropical evergreen broad-leaved forest and tropical rainforest. TRF-JFL has a tropical monsoon climate with distinct rainy (May–October) and dry (November–April) seasons. The mean annual temperature is 19.8 °C and the mean annual precipitation is 2449 mm [54,55]. More than 80% of the annual precipitation falls during the rainy season. Our study site was selected in a primary rainforest (20 m × 20 m) in the vicinity of a flux tower at TRF-JFL. The dominant trees are *Gironniera subaequalis* Planch., *Cryptocarya chinensis* (Hance) Hemsl., *Livistona saribus* (Lour.) Merr. ex A. Chev., and *Mallotus hookerianus* (Seem.) Müll. Arg. The dominant herbs are *Psychotria rubra* (Lour.) Poir. and *Prismatomeris connata* subsp. *hainanensis* Y. Z. Ruan. [54,55]. The main soil type is podzolic soil for TRF-JFL [60].

2.2. Measurement of Soil CH₄ Fluxes

Soil CH₄ fluxes were measured using the static chamber-gas chromatography technique [52]. At each site, five chambers were respectively made of 20 cm internal diameter polyvinylchloride pipes, which consisted of two parts: a base and a chamber. The base was installed approximately 5 cm into the soil. The height of the chamber was 50 cm. There were five replicates of gas samples per month from September 2015 to February 2016 (non-growing season) and twice per month from March to June 2016 (growing season). We collected gas samples with vacuumed 10 mL blood test tubes. Gas samples were collected at intervals of 0, 10, 20, and 30 min after chamber closure. The air temperature inside the chamber at 0 and 30 min was recorded. We did not collect gas samples during rainy or snowy days at TBDF-BTM or during typhoons or rainstorms at TRF-JFL. All the gas samples were sent to the laboratory and analyzed by an Agilent 7890 gas chromatograph (Agilent Technologies, Palo Alto, CA, USA). CH₄ fluxes (J) were calculated accounting for air temperature, time, and atmospheric pressure:

$$J = \frac{dC}{dt} \frac{PM}{RT} H \quad (1)$$

where dC/dt is the rate of CH₄ concentration change, P is the atmospheric pressure of the study site, M is the molar mass of CH₄, T is the absolute temperature at the sampling time, R is the universal gas constant, and H is the chamber height above the soil surface. The units of each measurement were: J , mg·m⁻²·h⁻¹; dC/dt , ppm·h⁻¹; P , kPa; M , g·mol⁻¹; R , J·mol⁻¹·K⁻¹; T , K; H , m.

The monthly averages were used to estimate the annual gas balances based on chamber flux measurements. To improve the accuracy and avoid the influence of system shifts on the analysis results, before the sample analysis, we used the working standard of the known concentration continuous sample until the analysis results were stable. During the sample analysis, three samples and one work standard were analyzed. We injected 5 mL of gas sample into the gas chromatograph for each sample and a quantitative ring was used to ensure only 1 mL of gas sample flowed to the column.

2.3. Soil Sampling and Analysis

In September and December 2015 and March and June 2016, a total of 20 soil samples (five replicates per season × four seasons) were collected using auger boring at each forest site from 0–10 cm depth below the surface. We did not collect soil samples for the rainy or snowy days at TBDF-BTM or during typhoons or rainstorms at TRF-JFL. The soil samples were transported to the laboratory in an ice box, immediately sieved through a 2 mm mesh to remove stones and coarse roots, and then were divided into three parts through equisection method. One part was stored at –80 °C before DNA extraction. The second part was air dried and stored at room temperature until used for total carbon [53], total nitrogen [54], pH, and water-filled pore space (WFPS) analyses. The third part was stored at 4 °C for microbial biomass carbon (MBC), microbial biomass nitrogen

(MBN), ammonium-nitrogen ($\text{NH}_4^+\text{-N}$), nitrate-nitrogen ($\text{NO}_3^-\text{-N}$), and dissolved organic carbon (DOC) analyses. This portion of the fresh soil sample was oven dried at 105°C for 24 h to measure soil water content (WC).

Soil temperature at 10 cm below the surface was monitored by a geothermometer when collecting soil samples. Fresh soil was mixed with deionized water at a mass ratio of 1:2.5 and the pH was measured with a pH meter (PB-10, Sartorius Instruments Inc., Göttingen, Germany). Volumetric soil water content was measured and converted to WFPS using soil porosity data. The water filled pore space (WFPS) was calculated as follows: $\text{WFPS} = ((\text{gravimetric water content} \times \text{soil bulk density}) / \text{total soil porosity})$, where $\text{soil porosity} = 1 - \text{soil bulk density} / 2.65$ (2.65 being the assumed particle density of the soil). MBC, MBN, and total carbon (TC) were analyzed using the chloroform fumigation-extraction method [53,55] on a TOC analyzer (LIQUIC TOCII, Elementar Analysensysteme GmbH, Hanau, Germany). $\text{NH}_4^+\text{-N}$, $\text{NO}_3^-\text{-N}$, and DOC were extracted with 2 mol L^{-1} KCl and their concentrations were analyzed using a continuous-flow analyzer (San++, Skalar, Breda, The Netherlands). The total nitrogen (TN) was detected by the Kjeldahl method [60] using a Kjeltec 8400 Analyzer (FOSS, Hillerød, Denmark). In order to detect the accuracy of TC, WC, T, pH, $\text{NH}_4^+\text{-N}$, $\text{NO}_3^-\text{-N}$, MBN, and WFPS, we kept one significant figure, whereas for other physical and chemical factor detections, we kept two significant figures.

2.4. DNA Extraction, Amplification, and Illumina HiSeq Sequencing

Total genomic DNA was extracted from 0.25 g of soil sample using the MoBio PowerSoil® DNA Isolation Kit (Carlsbad, CA, USA), according to the manufacturer's instructions [61,62]. DNA concentration and purity were monitored on 1% agarose gels. According to the measured concentration, DNA was diluted to $1 \mu\text{g } \mu\text{L}^{-1}$ using sterile water [62]. The V3 and V4 regions of bacterial 16S rDNA were amplified with the primer pair 515F/806R and the barcode [56]. Polymerase Chain Reactions (PCRs) with five replicates were performed in $30 \mu\text{L}$ reactions with $15 \mu\text{L}$ of Phusion® High-Fidelity PCR Master Mix (New England Biolabs, Ipswich, MA, USA) [63], $0.2 \mu\text{M}$ forward and reverse primers, and approximately 10 ng of template DNA. Thermal cycling conditions consisted of initial denaturation at 98°C for 1 min, followed by 30 cycles of denaturation at 98°C for 10 s, annealing at 50°C for 30 s, and elongation at 72°C for 30 s, and a final extension at 72°C for 5 min. PCR products were purified with a GeneJET Gel Extraction Kit (Thermo Fisher Scientific, Waltham, MA, USA) [64]. Next, 400–450 bp PCR products were selected for analysis of population structure [65]. Library quality was assessed using the Qubit® 2.0 Fluorometer (Thermo Fisher Scientific, Waltham, MA, USA) and Agilent Bioanalyzer 2100 system (Agilent Technologies, Santa Clara, CA, USA). The library was sequenced on an Illumina HiSeq2500 platform (Illumina Inc., San Diego, CA, USA) and 250 bp paired-end reads were generated [66].

2.5. Bioinformatics Analysis

Sequencing reads were assigned to each sample according to the unique barcode tags. Sequences were analyzed using the QIIME (Quantitative Insights into Microbial Ecology) software package and UPARSE pipeline [59]. The reads were first filtered by QIIME quality filters using the default settings for Illumina processing. Then, the sequences were clustered into operational taxonomical units (OTUs) with a 97% sequence similarity cutoff value. For each OTU, a representative sequence was selected and a taxonomic group was assigned using the SILVA database project (<http://www.arb-silva.de>). The species richness of each sample was estimated by rarefaction analysis using mothur (<http://www.mothur.org/>).

Sequence data was deposited at the National Center for Biotechnology Information (NCBI) Sequence Read Archive database (accession number: SRP130793).

2.6. Statistical Analysis

Differences in soil physicochemical properties, CH₄ fluxes, and bacterial community composition in each site were tested using the general linear model univariate analysis of variance with repeated measures analysis and Levene's test for homogeneity of variance. Pearson correlation analysis was used to examine the relationships among CH₄ fluxes, methanotrophs, methylotrophs, and soil physicochemical properties for all data from the four seasons at each site. Differences at $p < 0.05$ level were considered statistically significant. All the analyses were performed using SPSS Statistics version 19.0 (IBM SPSS, Somers, NY, USA). Redundancy analysis (RDA) was used to visualize bacterial communities constrained by environmental factors based on Bray-Curtis distance with 'vegan' package in R [67].

3. Results

3.1. Soil CH₄ Fluxes

The soils at TBDF-BTM and TRF-JFL oxidized CH₄ throughout the experimental period, with an annual average CH₄ uptake of 13.1 ± 1.8 and 4.9 ± 1.8 kg ha⁻¹ year⁻¹ (0.150 ± 0.02 mg m⁻² h⁻¹ and 0.056 ± 0.02 mg m⁻² h⁻¹), respectively. The annual average value of CH₄ uptake at TBDF-BTM was approximately three times that at TRF-JFL (Figure 2A). The seasonal CH₄ fluxes at TBDF-BTM ranged from -0.23 ± 0.07 to -0.07 ± 0.04 mg m⁻² h⁻¹; the fluxes at TRF-JFL varied between -0.10 ± 0.05 and -0.02 ± 0.03 mg m⁻² h⁻¹. In each season, more CH₄ was taken up by soil at TBDF-BTM than at TRF-JFL. At TBDF-BTM, seasonal CH₄ fluxes markedly decreased from September 2015 to March 2016 and then increased in the coming growing season ($p < 0.01$; Figure 2B).

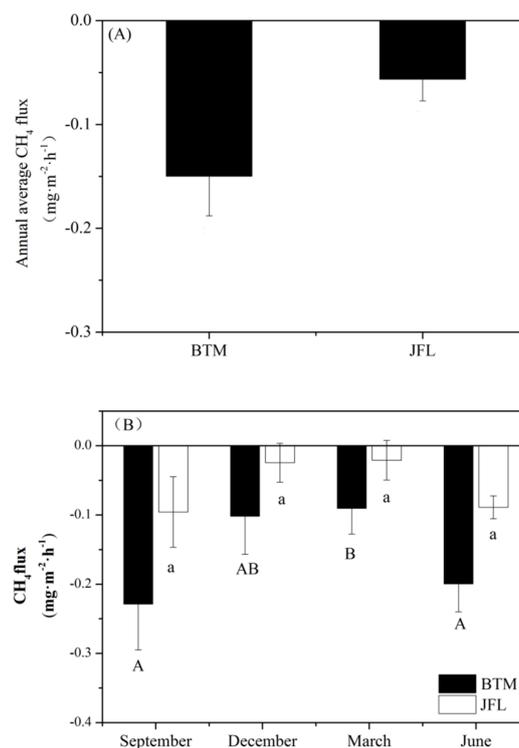


Figure 2. Annual average CH₄ fluxes (A) and their seasonal variation (B) at TBDF-BTM and TRF-JFL. Negative numbers indicate net CH₄ uptake by the soil. Data are annual means \pm standard error. Data for CH₄ fluxes are means \pm standard errors of five replicate plots at the two sites from September 2015 to June 2016. Different capital letters represent significant differences at TBDF-BTM. Different lowercase letters indicated significant differences at TRF-JFL ($p < 0.05$). TBDF-BTM, temperate broad-leaved deciduous forest at Baotianman Nature Reserve; TRD-JFL, tropical rainforest at Jianfengling National Natural Reserve.

3.2. Correlations between CH₄ Fluxes, Soil Properties, and CH₄-Related Bacteria

At TBDF-BTM, the CH₄ flux was positively correlated with WFPS and negatively correlated with MBC and TC ($p < 0.05$, Table 1). At TRF-JFL, the CH₄ flux had no significant correlation with any of the soil properties (Table 1). There was no significant correlation between CH₄ fluxes and methanotroph or methylotroph abundances at the two sites (Table 2). Of the 11 soil physicochemical factors measured at both sites, six factors (WC, DOC, TN, T, pH, and NO₃⁻-N) had the most significant effect on the abundance of CH₄-related bacteria ($p < 0.01$, Figure 3). The first axis (RDA1) explained 32.3% of the variation in the relative abundances of methanotrophs and methylotrophs variability. The second axis (RDA2) explained 12.5% of relative abundances of methanotrophs and methylotrophs, for a combined explanation of 44.8%. At TBDF-BTM, the relative abundance of methanotrophs and methylotrophs showed significant correlations with WC, DOC, TN ($p < 0.01$, Figure 3). At TRF-JFL, T, pH, and NO₃⁻-N showed significantly correlated correlations with the relative abundances of methanotrophs and methylotrophs ($p < 0.01$, Figure 3).

Table 1. Correlations (Pearson's correlation coefficients) among CH₄ fluxes and soil properties at sites.

Variable	TBDF-BTM CH ₄ Flux	TRF-JFL CH ₄ Flux
WC	0.335	0.077
T	-0.407	-0.147
pH	-0.257	0.085
WFPS	0.459 *	-0.06
TC	-0.448 *	-0.181
TN	-0.292	0.034
NH ₄ ⁺ -N	-0.027	0.214
NO ₃ ⁻ -N	0.162	-0.232
MBC	0.522 *	0.35
DOC	-0.175	-0.032
MBN	0.025	0.026

Asterisks indicate significant differences between two sites in the same month (*, $p < 0.05$). WC, water content; T, temperature; WFPS, water-filled pore space; TC, total carbon; TN, total nitrogen; NH₄⁺-N, ammonium nitrogen; NO₃⁻-N, nitrate nitrogen; MBC, microbial biomass carbon; DOC, dissolved organic carbon; MBN, microbial biomass nitrogen.

Table 2. Correlations (Pearson's correlation coefficients) among CH₄ fluxes, methanotrophs, methylotrophs abundances at two sites.

Bacterial Abundance Associated with CH ₄	TBDF-BTM CH ₄ Flux	TRF-JFL CH ₄ Flux
<i>Candidatus</i> Methyloacidiphilum	-0.126	-0.236
<i>Methylotenera</i>	-0.124	-0.183
<i>Methylobacterium</i>	-0.206	-0.027
<i>Methylovirgula</i>	-0.188	-0.194

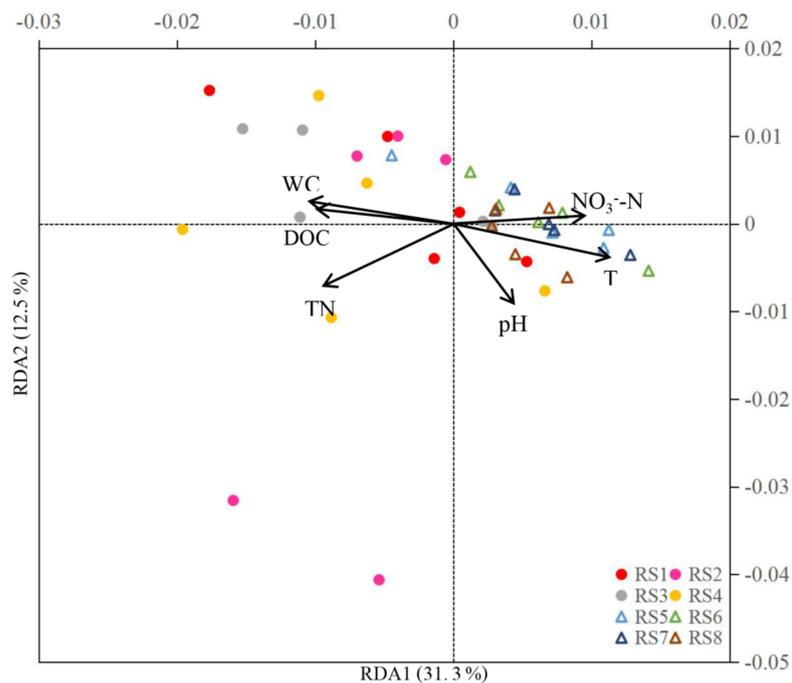


Figure 3. Redundancy analysis (RDA) was used to correlate bacterial communities with environmental factors at the two sites. RS1, RS2, RS3, and RS4 represent September and December 2015 and March and June 2016 at TBDF-BTM, respectively. RS5, RS6, RS7, and RS8 represent September and December 2015 and March and June 2016 at TRF-JFL, respectively.

3.3. Soil Properties

The soil properties at the two forest sites are shown in Table 3. At TBDF-BTM, soil water content increased slightly but not significantly from September to December 2015 and then markedly decreased by a total of 47% over the following two seasons. In contrast, soil temperature and pH first decreased from September to December 2015 and then increased from March to June 2016, both of which were the lowest in December 2015 and highest in June 2016. WFPS also reached its highest level in June 2016, which was 62% higher than that in September 2015. Both TC and TN increased from September to December 2015 by 139% and 68%, respectively, and then leveled off in the following two seasons. NH_4^+ -N and NO_3^- -N showed a decreasing trend, while MBC and DOC did not differ significantly throughout the experimental period. MBN markedly increased by 3.9-fold from September to December 2015 and remained at high levels in March 2016, followed by a 56% decrease thereafter.

At TRF-JFL, soil water content remained stable and generally lower than that at TBDF-BTM, while soil temperature fluctuated with season and was much higher than that at TBDF-BTM. The pH first decreased and then increased across the different seasons within a similar range as that at TBDF-BTM. WFPS and DOC significantly decreased from September 2015 to March 2016 and then increased slightly; both parameters were lower than those at TBDF-BTM, except for WFPS in September 2015. TC showed an increasing trend and was generally higher than that at TBDF-BTM, except for December 2015. TN was lower than that at TBDF-BTM, while NH_4^+ -N and NO_3^- -N were generally higher. MBC and MBN were the largest in December 2015 and March 2016, respectively, compared with the other seasons, and showed similar concentrations between the two sites.

Table 3. Soil physicochemical properties at TBDF-BTM and TRF-JFL during different seasons.

Soil Property	Site	September (2015)	December (2015)	March (2016)	June (2016)
Water content (%)	BTM	33.7 ± 2.5ab	41.5 ± 2.8a	28.6 ± 1.5b	21.9 ± 1.9c
	JFL	26.4 ± 1.6a	22.4 ± 0.6b	22.2 ± 2.0ab	21.2 ± 2.9b
Temperature (°C)	BTM	12.6 ± 0.6b	−0.6 ± 2.2d	6.4 ± 1.2c	17.0 ± 0.5a
	JFL	22.5 ± 0.1b	17.7 ± 0.2c	17.2 ± 0.3c	24.7 ± 0.1a
pH	BTM	4.7 ± 0.1b	4.1 ± 0.2c	5.1 ± 0.1a	5.2 ± 0.1a
	JFL	4.9 ± 0.0b	4.2 ± 0.1c	5.1 ± 0.1ab	5.3 ± 0.1a
WFPS (%)	BTM	37.2 ± 3.6b	51.9 ± 3.8ab	50.3 ± 4.3b	60.4 ± 2.9a
	JFL	52.5 ± 1.7a	43.6 ± 3.0b	34.1 ± 1.2c	41.9 ± 1.9b
TC (g/kg)	BTM	32.3 ± 2.9c	77.4 ± 8.0a	61.3 ± 2.5b	62.0 ± 1.6b
	JFL	40.0 ± 1.6c	43.6 ± 3.5c	62.2 ± 3.2b	121.5 ± 5.7a
TN (g/kg)	BTM	2.29 ± 0.15c	3.84 ± 0.33b	4.21 ± 0.22ab	4.74 ± 0.30a
	JFL	2.45 ± 0.15a	2.29 ± 0.07a	2.24 ± 0.03a	2.34 ± 0.06a
NH ₄ ⁺ -N (mg/kg)	BTM	13.5 ± 0.7a	11.3 ± 2.9ab	8.1 ± 0.4b	7.3 ± 0.8b
	JFL	20.0 ± 4.7a	8.9 ± 0.5a	12.1 ± 5.2a	14.1 ± 3.6a
NO ₃ [−] -N (mg/kg)	BTM	2.6 ± 0.2a	2.1 ± 0.2ab	1.6 ± 0.2b	1.0 ± 0.1b
	JFL	5.6 ± 1.0a	5.9 ± 1.2a	1.1 ± 0.3b	5.7 ± 0.8a
MBC (g/kg)	BTM	0.54 ± 0.08a	0.54 ± 0.15a	0.35 ± 0.08a	0.36 ± 0.09a
	JFL	0.40 ± 0.04b	0.71 ± 0.08a	0.51 ± 0.05ab	0.31 ± 0.09b
DOC (g/kg)	BTM	1.62 ± 0.17a	1.84 ± 0.14a	1.61 ± 0.12a	1.62 ± 0.05a
	JFL	1.50 ± 0.31a	1.36 ± 0.05a	0.34 ± 0.03b	0.53 ± 0.04b
MBN (mg/kg)	BTM	43.5 ± 6.8b	171.0 ± 52.4a	190.7 ± 36.95a	83.8 ± 35.6ab
	JFL	126.4 ± 40.3b	85.4 ± 21.3b	219.4 ± 16.8a	89.4 ± 13.5b

Different letters indicate significant difference between different months at the same site ($p < 0.05$). WC, water content; T, temperature; WFPS, water-filled pore space; TC, total carbon; TN, total nitrogen; NO₃[−]-N, nitrate nitrogen; MBC, microbial biomass carbon; DOC, dissolved organic carbon; MBN, microbial biomass nitrogen.

3.4. Composition of Bacterial Communities

A total of 2,375,395 paired-end reads were obtained from 40 soil samples. Next, 51,137 OTUs clustered at 97% sequence identity were mapped to 44 phyla and 556 known genera. The rarefaction curve level led off towards the asymptote, thus suggesting a sufficient sequencing depth and a good coverage of taxonomic diversity for bacterial communities (Figure S1). The good coverage varied throughout the year, but there was no significant difference at each site (Figure 4). Within the bacterial domain, Alphaproteobacteria was the most abundant group (range of relative abundance, 15.1–20.6%) at the class level, followed by Betaproteobacteria (7.3–22.4%) and Gammaproteobacteria (6.1–8.9%). At TBDF-BTM, Proteobacteria (Alpha-, Beta-, Gamma-) accounted for 31.7% to 38.5% of total bacteria; at TRF-JFL, the relative abundance of Proteobacteria was slightly higher, accounting for 32.9% to 51.3% of total bacteria (Figure 5).

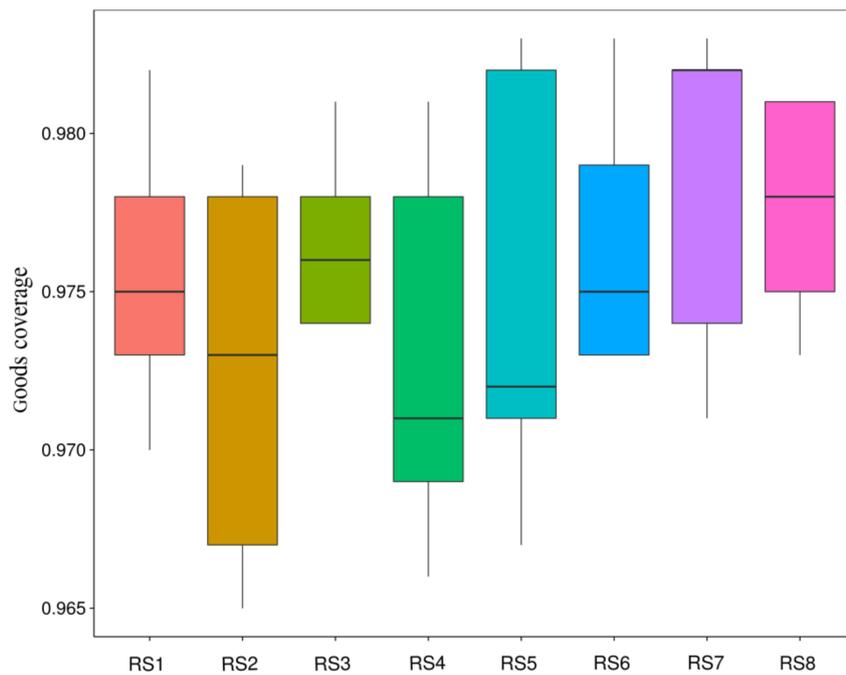


Figure 4. Good coverage analysis at TBDF-BTM and TRF-JFL. RS1, RS2, RS3, and RS4 represent September and December 2015 and March and June 2016 at TBDF-BTM, respectively. RS5, RS6, RS7, and RS8 represent September and December 2015 and March and June 2016 at TRF-JFL, respectively.

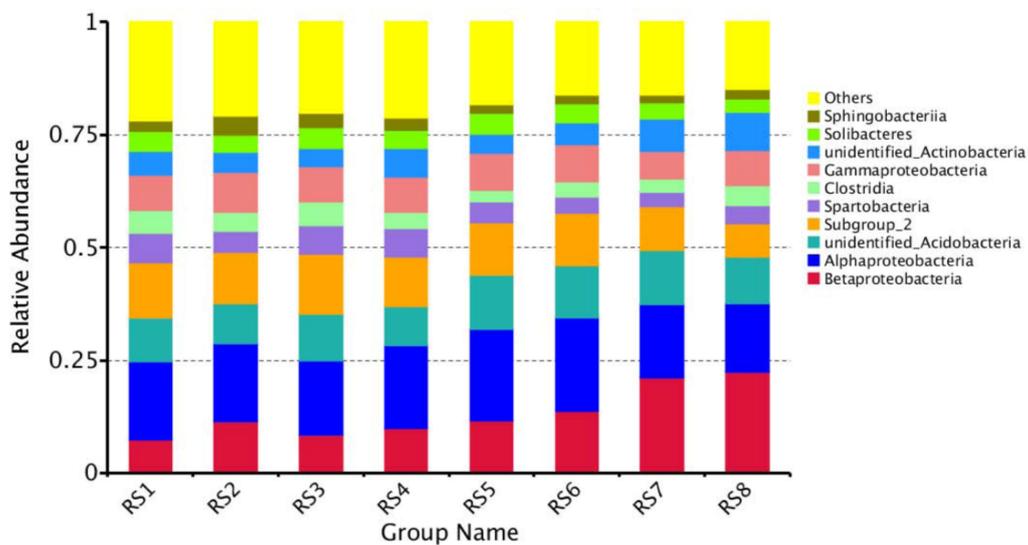


Figure 5. Bacterial community composition at the class level at the two sites (TBDF-BTM, TRF-JFL). The class Proteobacteria is represented by Alpha-, Beta-, Gamma-. RS1, RS2, RS3, and RS4 represent September and December 2015 and March and June 2016 at TBDF-BTM, respectively. RS5, RS6, RS7, and RS8 represent September and December 2015 and March and June 2016 at TRF-JFL, respectively.

At the genus level, we detected one genus of methanotrophs (*Candidatus Methyloacidiphilum*) and three genera of methylotrophs (*Methylobacterium*, *Methylotenera*, and *Methylovirgula*). For the relative abundance of methanotrophs and methylotrophs, there were no significant differences at TBDF-BTM and TRF-JFL in the four seasons ($p > 0.05$; Figure 6A,B). The total relative abundance of the methanotrophs and methylotrophs in all seasons at TBDF-BTM was higher than TRF-JFL.

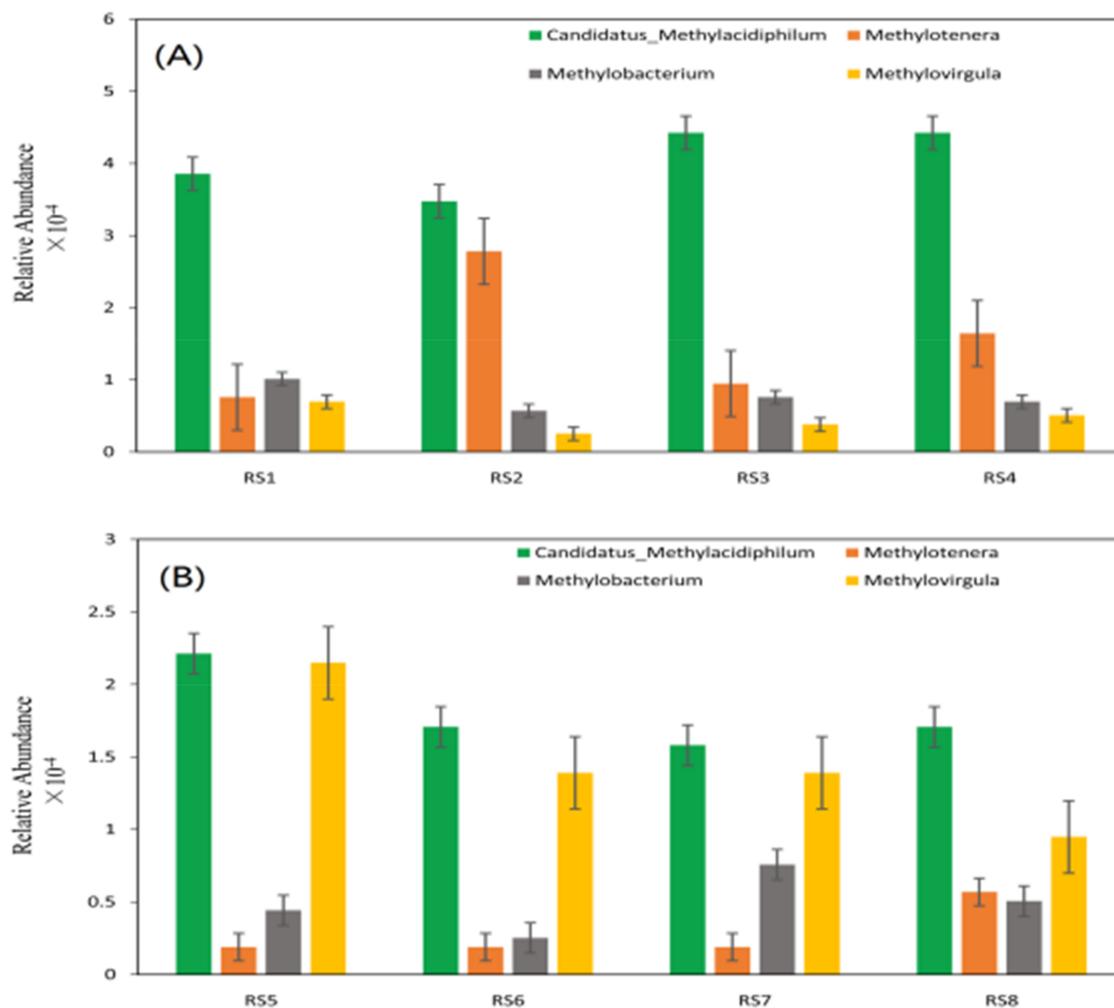


Figure 6. Relative abundances of *Candidatus Methylacidiphilum*, *Methylobacterium*, *Methylovirgula*, and *Methylothera* at TBDF-BTM and TRF-JFL. (A) RS1, RS2, RS3, and RS4 represent September and December 2015 and March and June 2016 at TBDF-BTM, respectively. (B) RS5, RS6, RS7, and RS8 represent September and December 2015 and March and June 2016 at TRF-JFL, respectively. Different letters mean significant difference at $p < 0.05$.

4. Discussion

4.1. Variations in Soil CH₄ Fluxes

During the study period, the TBDF-BTM and TRF-JFL soils were all sinks for CH₄ and the TRF-JFL soils were a weaker CH₄ sink than the TBDF-BTM soils. At TBDF-BTM, we found an obvious decreasing trend in CH₄ uptake from September to the following March. With the decrease of temperature from September 2015 to the following March (Table 3), plants began to enter the non-growing season, soil physicochemical properties changed, and CH₄ uptake decreased. From March to August 2016, CH₄ uptake increased markedly, because during this period, plants began to grow rapidly with the temperature increase and the forest soil reached a higher net CH₄ uptake in the peak growing season. Several studies in temperate forests have shown that the rate of CH₄ uptake increases during summer period compared with winter [68–70]. We found similar results during our study. The oxidation of CH₄ in summer and autumn is higher than that in winter and spring at TBDF-BTM. Soil CH₄ fluxes also showed seasonal variations in the boreal and temperate forests, for example, a higher uptake in summer and emission in winter, respectively [71]. The CH₄ uptake rates of the temperate forest soils in European and North American are ~25.6 kg CH₄ ha⁻¹ year⁻¹ [72], which is nearly twice the

rate observed at TBDF-BTM. However, at TRF-JFL, we found no seasonal variation in the CH₄ uptake from September 2015 to the following June (Figure 2B). Because the TRF-JFL is located in a tropical region, the soil temperature was relatively high and remained stable throughout the study period. Therefore, we did not find any significant change in CH₄ uptake among the different seasons at this site. In addition, the CH₄ fluxes also had no direct relationship with any physical and chemical factors, surprisingly. The subtropical forest soil acted as a net soil CH₄ sink [71]. The rates of CH₄ uptake in tropical forest soils of Queensland, Australia, range from 0.8 to 4.73 kg CH₄-C ha⁻¹ year⁻¹ [68,73]. The annual CH₄ uptake rates in an old-growth tropical forest site of southern China range from 2.3 to 3.4 kg CH₄-C ha⁻¹ year⁻¹ [74]. All these results are lower than the CH₄ uptake rates observed at TRF-JFL. The difference in CH₄ uptake at the two sites is probably related to the different climatic conditions in the two distinct regions. Despite the inconsistency in CH₄ uptake rates, we noted that temperate forest soils show greater CH₄ oxidation capacity than tropical forest soils in the current study.

4.2. Relationships between Soil CH₄ Fluxes and Environmental Factors

A low WFPS indicates that more pore space and larger variations in the WFPS lead to greater variability in CH₄ uptake [75]. Changes in WFPS explained more than 65% of the temporal variation in CH₄ uptake at a primary seasonal tropical rainforest and a rubber plantation site in Southwest China [76]. Soil CH₄ consumption was negatively correlated with WFPS in a tropical forest [74], which is in agreement with previous findings in aerated temperate soils and different forest soils [68,74,76,77]. DOC represents the primary carbon source for CH₄ production, which explained the strong positive correlation found between the seasonal pattern of soil DOC concentrations and CH₄ emissions in a flooded paddy soil [78]. A previous study reported a positive correlation between soil DOC concentration and the CH₄ oxidation rate ($r = 0.76, p < 0.01$), indicating that DOC is an important regulator of CH₄ oxidation in arid soils [79]. In addition, DOC input from forest organic layers can change the inhibition effect of nitrogen deposition on the soil atmospheric CH₄ uptake, which depends on the types of deposited nitrogen [48]. DOC concentrations at TBDF-BTM were all higher in four seasons than that at TRF-JFL (Table 3). In other words, DOC was not one of the main factors affecting CH₄ flux at the two sites. DOC can stimulate methane oxidation, although we did not observe a statistically significant result. This may be a paradox of biological significance and statistical results.

After statistical analysis, we found CH₄ fluxes were significantly associated with WFPS, TC, and MBC, but not WC and other physical and chemical factors at TBDF-BTM. Therefore, the seasonal variation of CH₄ flux is the result of the interaction of environmental factors in the region.

4.3. Relationships between CH₄ Fluxes and CH₄-Related Bacteria

There are numerous studies that describe seasonal changes in microbial communities over time, across a wide range of ecosystems [66,69,70,80]. We found that the relative abundance of methanotrophs was significantly higher in winter than in summer. This implies that the lower temperatures in winter combined with other prevailing conditions [81]. Often in microbial communities, the change in relative abundance between winter and summer is due to the most abundant organisms “dying back” during periods of lower resource availability. In these cases, it may appear that the abundance of a rare species is increasing, but instead the abundance stays the same but appears to be enhanced due to the reduction in abundance of other species. Soil methanotrophs are bacterial consumers of atmospheric CH₄. Consumption of atmospheric CH₄ by methanotrophs has been well documented in forest soils [81]. However, at our two sites, there were no obvious seasonal change in the abundance of methanotrophs and methylotrophs, which was in accordance with Shrestha’s results [82]. The relative abundance of methanotrophs and methylotrophs in soil was higher at TBDF-BTM than that at TRF-JFL (Figure 6A,B), and the amount of methane oxidation at TBDF-BTM ($0.150 \pm 0.02 \text{ mg m}^{-2} \text{ h}^{-1}$) was larger than that at TRF-JFL ($0.056 \pm 0.02 \text{ mg m}^{-2} \text{ h}^{-1}$) (Figure 2A). However, it was found that the abundance of methanotrophs and methylotrophs had no statistically significant relationship with the CH₄ flux in our study ($p > 0.05$; Table 2). The possible reason is that both the relative abundance

of methanotrophs and methylotrophs and CH₄ flux vary greatly between each replicate, leading to statistically insignificant results. In addition, there may be a mismatch in the relationship between the abundance and function of methanotrophs and methylotrophs. Other factors may combine to influence the oxidation of methane in the forest soils of our two sites.

In this study, we detected the presence of CH₄-oxidizing *Candidatus* Methylacidiphilum, a distinct phylogenetic lineage within the phylum of Verrucomicrobia [83] at both sites. Moreover, three methylotrophs (*Methylotenera*, *Methylobacterium*, and *Methylovirgula*) [39] were found in the soil samples. *Methylotenera*, members of Methylophilaceae, are non-CH₄-utilizing methylotrophs that can obtain CH₄-derived carbon [37]. *Methylobacterium* are facultative methylotrophs that are capable of growing on one-carbon compounds, such as methanol, methylamine, formaldehyde, and formate, and members of this genus are ubiquitous in nature [84]. *Methylovirgula*, the genus of facultative methylotrophs, assimilate methanol-derived carbon via the RuBP pathway [85].

At TBDF-BTM, the total relative abundance of the observed methanotrophs and methylotrophs genera had no distinct seasonal variation. However, we observed a seasonal variation in CH₄ flux. This may be due to the fact that the activity of these CH₄-related bacteria in December and March (cold, non-growing season) was lower, despite their higher abundance. Although these CH₄-related bacteria had a rich, active community and the relative abundance of these CH₄-related bacteria increased in winter, they evidently have lower overall cold tolerance than that in the dry site [86]. At TRF-JFL, soil temperature was relatively stable and the activity of these CH₄-related bacteria may remain the same activity during the rainy and dry seasons, and therefore we did not observe a statistically significant seasonal variation in CH₄ fluxes.

WFPS showed a statistically significant positive relationship with CH₄ fluxes and a statistically significant negative relationship with the relative abundance of methanotrophs [87]. Not only did the relative abundances of type I and type II methanotrophs change with temperature, but the composition of the type I methanotrophic community was influenced by temperature [88]. We found that CH₄ flux had a correlation with WFPS, TC, and MBC, whereas no relationship with methanotrophs/methylotrophs was observed at TBDF-BTM. CH₄ flux had no relationship with any physical and chemical factor and methanotrophs/methylotrophs at TRF-JFL. This may be caused by the relative abundance of methane-oxidizing bacteria and CH₄ fluxes varying greatly between each repetition, leading to insignificant statistical results. In addition, there may be a discrepancy between the relationship between the abundance of methane-oxidizing bacteria and their functionality (i.e., CH₄ oxidation). The combination of biotic and abiotic factors certainly affects the oxidation of soil CH₄ fluxes in both sites.

Many studies have detected methane-oxidizing bacteria using the functional gene *pmoA* [89]. Our study analyzed the bacterial diversity using 16S rDNA analysis, which may underestimate the abundance of methane-oxidizing bacteria. With only one site for each forest type, our study is still limited and cannot reach a general conclusion regarding the intrinsic difference in CH₄ fluxes between forest types in the transitional climatic regions and the underlying abiotic and biotic controls. Future work including more study sites from different forest types needs to be conducted targeting the functional genes of methane-oxidizing bacteria.

5. Conclusions

We found that the two sampling sites soils take up CH₄, with higher CH₄ uptakes observed in the TBDF-BTM temperate forest soils and lower CH₄ uptakes in the TRF-JFL tropical rainforest soils. Our results suggest that WFPS and TC have a remarkable effect on CH₄ fluxes at TBDF-BTM and the CH₄ fluxes had no significant correlations with any soil properties at TRF-JFL. The total relative abundance of the methanotrophs and methylotrophs in all seasons at TBDF-BTM was higher than TRF-JFL. Soil water content (WC), dissolved organic carbon (DOC), total nitrogen (TN), temperature (T), pH, and nitrate-nitrogen (NO₃⁻-N) were the main factors controlling the abundance of methanotrophs and three genera of methylotrophs of forest soil at the two sites. Methanotrophs and three genera of

methyloprophs had no correlation with soil CH₄ fluxes at both sites. Water-filled pore space and soil total carbon content were the main factors controlling the soil CH₄ fluxes at TBDF-BTM. At TRF-JFL, the soil CH₄ flux had no significant correlations with any of the soil properties. The methanotrophs in our two forest soils also need to be identified by culture-based approaches to verify their relationship with CH₄ fluxes. Our observations in this study provide a scientific basis for the relationships among soil CH₄ fluxes, methanotrophic and methyloprophic communities, and soil properties in two distinct forest ecosystems. These results advance our understanding about the conditions under which commonly known methanotrophs and methyloprophs occur in forest soils, recognize the undefined microbial populations responsible for oxidation of atmospheric CH₄, and provide a future reference for modeling forest CH₄ fluxes.

Supplementary Materials: The following are available online at <http://www.mdpi.com/1999-4907/9/4/204/s1>, Figure S1: Rarefaction curves obtained for the HiSeq sequencing reads of operational taxonomic units (OTUs) from the V3–V4 region of bacterial 16S rDNA genes in soil samples collected at the two different forest sites.

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Conflicts of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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