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# Freeze–Thaw Cycles Have More of an Effect on Greenhouse Gas Fluxes than Soil Water Content on the Eastern Edge of the Qinghai–Tibet Plateau

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**Abstract:** The Qinghai-Tibetan Plateau (QTP) is sensitive to global climate change. This is because it is characterized by irregular rainfall and freeze–thaw cycles resulting from its high elevation and low temperature. Greenhouse gases (GHGs) mainly contribute to the warming of the QTP, but few studies have investigated and compared the effects of irregular rainfall and freeze–thaw cycles on GHGs. In this study, we conducted a laboratory experiment under four types of freeze–thaw treatments with three soil water content levels to simulate the irregular freeze–thaw and rainfall conditions. The results showed that both the soil water content and freeze–thaw treatment influenced the soil properties, soil enzyme activities, and the microbial biomass; however, the freeze–thaw treatment had significantly higher influences on GHG fluxes than soil water content. In order to explore other biotic and abiotic factors in an attempt to establish the main factor in determining GHG fluxes, a variation partition analysis was conducted. The results revealed that freeze–thaw treatments were the strongest individual factors in predicting the variance in $N_2O$ and $CO_2$ fluxes, and the pH, which was only significantly affected by freeze–thaw treatment, was the strongest individual factor in predicting $CH_4$ flux. Across the water content levels, all the freeze–thaw treatments increased the $N_2O$ flux and reduced the $CH_4$ flux as compared to the CK treatment. In addition, long-term freezing reduced the $CO_2$ flux, but the treatment of slowly freezing and quickly thawing increased the $CO_2$ flux. In summary, these results suggest that the freeze–thaw treatments had quite different effects on $N_2O$, $CH_4$, and $CO_2$ fluxes, and their effects on GHG fluxes are more significant than those of soil water content on the eastern edge of the QTP.

**Keywords:** microcosm; gas flux; soil properties; soil enzymes; microbial biomass

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## 1. Introduction

The Qinghai-Tibetan Plateau (QTP) is known as the "roof of the world" as it is the highest plateau on earth. It is also known as the "Asian water tower", as it is the origin of many major Asian rivers [1]. In addition, the QTP is China's "ecological security barrier" due to its function in soil and water conservation, as a carbon sink, and in biodiversity protection [2]. However, as a result of the high elevation and harsh environment, the QTP has been confirmed as the most sensitive region to global climate changes [3–5], especially global warming. The mean annual air temperature growth rate on the QTP is 0.04 °C, which is twice that of the global average [1], and hard evidence indicates that this warming is mainly contributed by greenhouse gases (GHGs) [6], including carbon dioxide ($CO_2$), methane ($CH_4$), and nitrous oxide ($N_2O$) [7]. For this reason, more investigations should be conducted to clarify the potential factors influencing the GHGs on the QTP.

Precipitation is one of the most important environmental factors that determines the biodiversity and functions of the QTP ecosystem [8]. A significant mechanism that regulates the effect of precipitation on soil biological function may be soil water content, as it can influence the soil oxidation reduction potential, microbial activities, soil aeration, and soil aggregates [9–12]. The effects of soil water content on soil abiotic and biotic factors can also indirectly impact GHG fluxes [13]; for example, Yang et al. [14] found that the soil temperature was the most correlated factor in influencing the GHG fluxes rather than soil water content under a simulated rainfall experiment on the QTP. Previous studies found that the soil water content could also directly increase the GHG fluxes [15,16]. Darenova et al. [15] found that soil water content determined the $CO_2$ flux in an oak coppice forest. Combining these results demonstrates that water content might, directly and indirectly, influence soil GHG fluxes on the QTP. However, there are no studies that have detailed the distinction between the direct and/or indirect effects of soil water content on GHG fluxes on this area.

Soil freeze–thaw fluctuations are common on the QTP due to the low temperature at such a high elevation [17]. This is another important factor for QTP ecosystem stability besides the water content, as it can enhance nutrient release, which promotes plant growth [18,19]. Risk et al. [20] and Xu [21] reviewed that freeze–thaw cycles can also impact the GHG fluxes in various ways. On the QTP, the soil incubation experiment from Wu et al. [22] demonstrated that the GHG fluxes were higher during soil thawing. The results from Chen et al. [23] revealed that a lower thawing temperature had a significantly higher $N_2O$ flux in a QTP alpine meadow, and this study suggested that different freeze–thaw cycle types with, for example, different thawing temperatures, might impact the GHG fluxes, but no recent studies have focused on that on the QTP. Therefore, a study of the effect of freeze–thaw cycle types on GHGs is necessary for the QTP.

In this study, in order to test the effects of water content and freeze–thaw cycles on GHG fluxes on the QTP, we conducted a two-factor microcosm interaction experiment with three soil water content levels and four different freeze–thaw cycles. Furthermore, we also measured another 17 variables related to soil properties, soil enzyme activities, and soil microbes in order to explore the direct and/or indirect effects of the aforementioned two factors via various biotic and/or abiotic factors using variance partition analysis.

## 2. Materials and Methods

### *2.1. Site Description and Soil Collection*

The experimental soil samples were collected from Hongyuan County (31°51′–33°33′ N, 101°51′–103°22′ E) on the eastern edge of the Qinghai–Tibet Plateau, China (Figure 1a). The mean temperature in this area is 1.0 °C, the mean annual precipitation is 735 mm, and the soil type is mainly alpine meadow soil. The natural vegetation is dominated by alpine meadow vegetation types, including *Elymus nutans*, *Koeleria litwinowii*, and *Care xenervis*, and the artificially cultivated vegetation includes conventional forage grasses such as *Lotus corniculatus*, *Phalaris arundinacea*, and *E. sibiricus*. Hongyuan County is located in the watershed of the Yangtze River and Yellow River systems, which play a great role in water conservation and biodiversity maintenance of the QTP and basins of the Yangtze River and Yellow River [24].

Soil samples were collected from four different land-use types to represent the local condition, including the artificial forage grassland of *L. corniculatus* and *P. arundinacea* and pasture land grazed by sika deer and yak. The forage grasslands were established by Sichuan Academy of Grassland Sciences and have been governed by them for 7 years. Each forage grassland area was nearly 100 × 100 $m^2$. The pastures belong to the local pastoralists and have a low grazing level for sika and a high grazing level for deer. Soil samples were collected in August 2021. Three 10 × 10 $m^2$ plots were randomly set at least 5 m apart for each land-use type, and three cores were randomly taken at a 20 cm depth from each plot and totally mixed into a soil sample, resulting in a total of 12 samples. The visible roots and rocks were removed at this step.

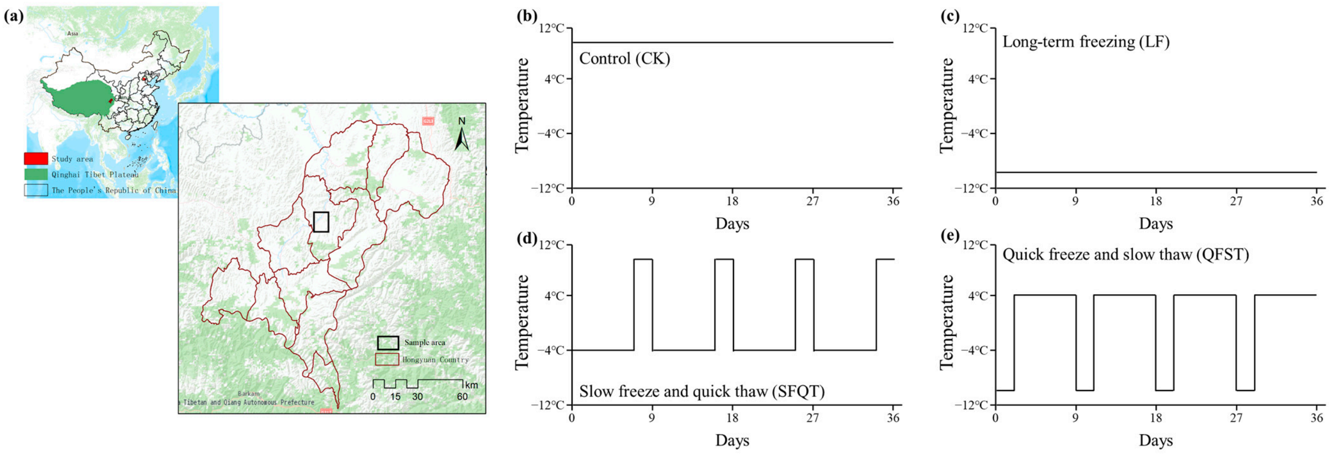


**Figure 1.** Sample site (**a**) and temperature and incubation time for different freeze–thaw treatments (**b**–**e**).

### 2.2. Microcosm Experiment

Soil samples were air-dried at 4 °C and sieved through a 2 mm mesh. To stimulate the soil water content and freeze–thaw cycles on the QTP, three soil water content levels combining four freeze–thaw types were established. We first stored 250 g air-dried soil in a 500 mL glass culture flask for 7 days under 25 °C to restore the activity of the soil microbes. Thereafter, sterile water was added to the soil by weighing it to establish the soil water content, which included 30%, 60%, and 100% of field capacity. Four different types of freeze–thaw treatments were used: control (CK), which was incubated at 10 °C for 36 days; long-term freezing (LF), which was incubated at −10 °C for 36 days; slowly freeze and quickly thaw (SFQT), which was incubated at −4 °C for 7 days then incubated at 10 °C for 2 days with four cycles; and quickly freeze and slowly thaw (QFST), which was incubated at −10 °C for 2 days then incubated at 4 °C for 7 days with four cycles (Figure 1b–d). A total of 144 pots were prepared for this experiment. For measuring the GHG fluxes, the air exchange through the microcosm flask was stopped on the 34th day. All the pots were transported at 4 °C and incubated for 24 h on the 36th day. Then, 50 mL of the air in the flask was drawn using a syringe and then transferred for storage into a 100 mL foil bag. After the soil incubation, microcosm soil was stored at 4 °C for enzyme activity measurements and freeze-dried for the analyses of the soil properties and microbial biomass.

### 2.3. Soil Properties, Enzyme Activities, and Soil Microbial Biomass

Soil pH was measured in water (1:5 *w*/*v*) using a pH electrode. Soil $NH_{4^+}$-N and $NO_{3^-}$-N were measured in 1 mol/L KCl (1:10 *w*/*v*) using an automatic continuous flow analyzer (AA3, Bran+Luebbe, Norderstedt, Germany). The soil was extracted in water (1:2.5 *w*/*v*) and filtered through a 0.45 µm Millipore filter. Then, the filtrate was assessed using a Multi N/C 3100 analyzer (Jena, Germany) to measure the concentration of total dissolved nitrogen (TDN) and dissolved organic carbon (DOC). Soil total nitrogen (TN) was measured using Kjeldahl methods, and soil organic carbon (SOC) was measured using the potassium dichromate sulfuric acid digestion method. The soil enzymes, including α-1,4-glucosidase (AG), β-1,4-glucosidase (BG), β-1,4-xylosidase (BX), cellobiohydrolase (CBH), leucine aminopeptidase (LA), and β-1,4-N-acetylglucosaminidase (NAG), were measured following the method of Saiya-Cork et al. [25]. The activity of urease was measured using the phenol-sodium hypochlorite colorimetric method. All the absorbance values within the measurement of soil enzyme activities were read using a multimode microplate reader (Fluoroskan ascent FL, Thermo Scientific, Boston, MA, USA). Soil microbes were extracted from the soil using the chloroform fumigation and extraction method [26], and then the microbial biomass carbon (MBC) and nitrogen (MBN) were measured using an elemental analyzer (Elementar Vario EL III CHNOS, Germany).

*2.4. Analysis of GHG Fluxes*

GHG concentrations were analyzed using a gas chromatograph (Varian CP-3800, Palo Alto, CA, USA) equipped with thermal conductivity (TCD), flame-ionization (FID), and electron capture (ECD) detectors, which assessed the carbon dioxide ($CO_2$), methane ($CH_4$), and nitrous oxide ($N_2O$) levels. The concentration of GHG was calculated using the following equation: $F = \beta \times (c/t) \times v \times 273/(W \times (273 + T))$, where F denotes $CO_2$ (mg $CO_2$-C $kg^{-1}$ $h^{-1}$), $CH_4$ (ug $CH_4$ $kg^{-1}$ $h^{-1}$), or $N_2O$ (ug $N_2O$-N $kg^{-1}$ $h^{-1}$); β denotes $CO_2$ (1.927), $CH_4$ (0.717), or $N_2O$ (1.250) density in a standard state; c/t denotes the $CO_2$ (ppm $h^{-1}$), $CH_4$ (ppm $h^{-1}$), or $N_2O$ (ppb $h^{-1}$) accumulation rate; v denotes the volume of the gas in the flask; W denotes the dry weight (Kg) of soil in the flask; T denotes the temperature inside the chamber during the sampling [27].

*2.5. Statistical Analyses*

All the statistical analyses were performed with the R language (version 4.1.3). For this experiment, as the data violated the normality, we used the nonparametric Wilcoxon paired test to compare the differences in the variables among the treatments [28]. We used the Scheirer–Ray–Hare test to calculate the significance of the effect of soil water content and freeze–thaw treatment [29]. To infer the direct and/or indirect impacts of the soil water content and freeze–thaw treatments on GHG fluxes, we analyzed the correlation between the 17 variables and the GHG fluxes using OLS regressions first, and then the significantly correlated variables combined with the water content and freeze–thaw treatments were used for variance partition based on the hierarchical partitioning theory using the rdacca.hp package [30]. To meet normality for these analyses, we transformed all the variables using the square root (sqrt), log, or BoxCox transformation method (Table S1).

## 3. Results

*3.1. Soil Properties and Enzyme Activities*

Our treatments had different influences on soil properties. Soil $NH_{4^+}$-N and TDN were significantly affected by both water content and freeze–thaw treatments, whereas DOC was only significantly affected by the soil water content, while soil pH, $NO_{3^-}$-N, and TN were only significantly affected by the freeze–thaw treatment. In addition, there was a significant effect of soil water content × freeze–thaw treatment interaction on $NO_{3^-}$-N. SOC was not affected by either soil water content or freeze–thaw treatment (Table 1). Across all the freeze–thaw treatments, TDN and DOC decreased with soil water content (Figure 2). As compared to other freeze–thaw treatments, LF had lower soil pH, $NH_{4^+}$-N, and TDN, but higher $NO_{3^-}$-N and TN under most of and across all the soil water content levels (Table 1; Figure 2).

Seven soil enzyme activities were investigated in this study. Soil water content had significant effects on all of them except the urease, and the AG, BG, BX, and CBH activities were increased with soil water content under each freeze–thaw treatment and across all freeze–thaw treatments (Table 2; Figure 3). The NAG activity was increased with soil water content under CK, SFQT, and QFST conditions (Table 2), and the trend between soil water content was positive across all freeze–thaw treatments. Freeze–thaw treatment had a significant effect on five types of soil enzyme activities but did not affect BX or urease (Table 2). Among the freeze–thaw treatments, the LF treatment had the most influence on the enzyme activities. LF had higher enzyme activities for AG, BG, and CBH under each soil water content and across all soil water content levels (Table 2; Figure 3), while it had lower NAG activity under 60% and 100% soil water contents and across all the water content levels (Table 2; Figure 3).

**Table 1.** The effects of water content and freeze–thaw treatment on the soil properties.

| WC | FTT | pH | $NH_4^+$-N (mg kg$^{-1}$) | $NO_3^-$-N (mg kg$^{-1}$) | TDN (mg kg$^{-1}$) | TN (g kg$^{-1}$) | DOC (mg kg$^{-1}$) | SOC (g kg$^{-1}$) |
|---|---|---|---|---|---|---|---|---|
| 30% | CK | 4.83 ± 0.09 ab | 19.1 ± 2.9 ab | 4.1 ± 0.4 abcd | 32.2 ± 1.6 a | 3.1 ± 0.2 ab | 57.5 ± 5.0 a | 50.4 ± 3.4 ab |
| | LF | 4.62 ± 0.09 ab | 2.6 ± 0.2 c | 32.0 ± 5.2 e | 22.7 ± 0.8 bc | 3.8 ± 0.2 c | 48.3 ± 3.9 b | 50.1 ± 2.9 a |
| | SFQT | 5.11 ± 0.08 a | 7.7 ± 1.2 d | 4.5 ± 0.6 abcd | 26.8 ± 1.0 d | 2.8 ± 0.2 ad | 48.4 ± 3.7 b | 49.2 ± 2.9 abc |
| | QFST | 4.90 ± 0.11 a | 8.9 ± 1.2 de | 3.6 ± 0.6 ab | 29.1 ± 0.8 ad | 3.0 ± 0.2 ad | 42.8 ± 2.9 bc | 49.5 ± 3.1 ab |
| 60% | CK | 4.71 ± 0.10 b | 18.7 ± 5.1 abe | 5.2 ± 0.6 cd | 26.0 ± 1.3 bd | 3.0 ± 0.2 abd | 39.5 ± 5.2 cd | 49.3 ± 3.5 abc |
| | LF | 4.58 ± 0.09 c | 2.7 ± 0.2 c | 25.1 ± 7.5 ace | 16.5 ± 0.8 e | 3.9 ± 0.2 c | 34.4 ± 2.5 d | 49.7 ± 2.7 ab |
| | SFQT | 5.10 ± 0.08 ab | 9.7 ± 2.1 ade | 4.8 ± 0.4 abcd | 19.8 ± 1.1 f | 3.3 ± 0.1 be | 32.1 ± 2.1 de | 48.1 ± 3.4 abc |
| | QFST | 4.81 ± 0.08 ab | 9.2 ± 1.7 de | 5.4 ± 0.6 cd | 22.1 ± 1.0 cf | 3.1 ± 0.1 abd | 29.4 ± 1.5 ef | 48.0 ± 3.8 abc |
| 100% | CK | 5.16 ± 0.10 a | 22.3 ± 7.1 abde | 3.3 ± 1.0 cbd | 7.1 ± 1.6 g | 3.0 ± 0.2 ad | 36.8 ± 4.7 cde | 47.8 ± 2.3 bc |
| | LF | 4.57 ± 0.10 c | 7.0 ± 1.5 de | 18.4 ± 7.4 abcde | 8.2 ± 1.1 g | 3.6 ± 0.3 e | 26.5 ± 1.9 f | 48.2 ± 3.2 abc |
| | SFQT | 4.78 ± 0.10 a | 15.7 ± 2.2 ab | 4.9 ± 0.6 cd | 8.9 ± 1.7 g | 2.9 ± 0.2 ad | 28.1 ± 2.0 ef | 45.7 ± 3.1 c |
| | QFST | 4.89 ± 0.08 a | 28.7 ± 4.9 b | 6.2 ± 0.9 ac | 8.8 ± 1.7 g | 2.8 ± 0.2 d | 29.7 ± 1.8 ef | 48.0 ± 3.0 abc |
| Effect of WC | | 0.214 | 0.003 | 0.641 | <0.001 | 0.490 | <0.001 | 0.388 |
| Effect of FTT | | 0.001 | <0.001 | 0.036 | 0.024 | <0.001 | 0.099 | 0.852 |
| Effect of WC × FTT | | 0.799 | 0.144 | 0.029 | 0.447 | 0.998 | 0.810 | 1.000 |

Data are means ± SE (n = 12). Different letters indicate significant ($p < 0.05$) differences tested by Wilcox test. The significant effect of water content, freeze-thaw type, and their interaction are tested by Scheirer–Ray–Hare test. Note: WC, water content; FTT, freeze–thaw treatment; CK, control; LF, long-term freezing; SFQT, slow freeze and quick thaw; QFST, quick freeze and slow thaw; TDN, total dissolved nitrogen; TN, total nitrogen; DOC, dissolved organic carbon; SOC, soil organic carbon.

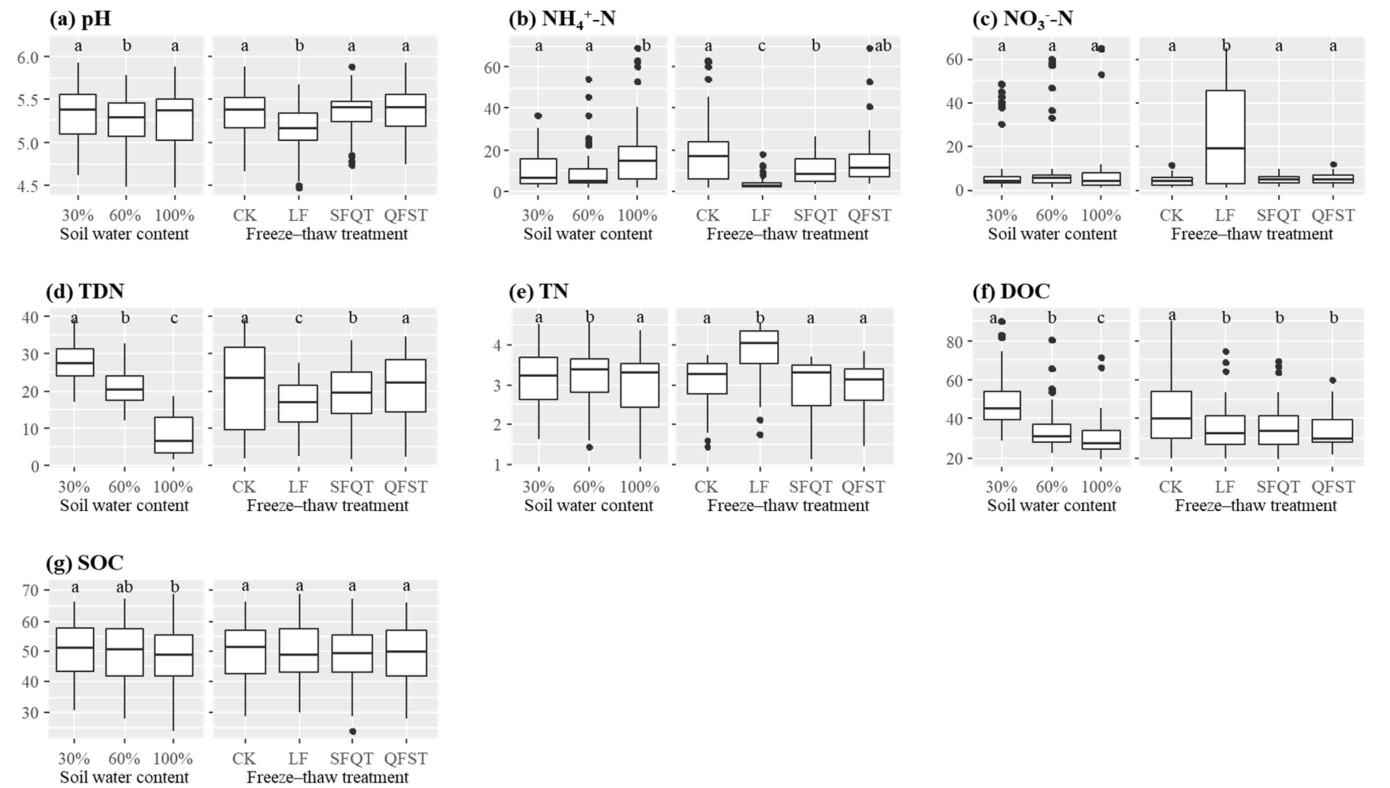


**Figure 2.** The soil properties under different soil water content or freeze–thaw condition. Different letters indicate significant ($p < 0.05$) differences tested by Wilcox test. CK, control; LF, long-term freezing; SFQT, slow freeze and quick thaw; QFST, quick freeze and slow thaw. DON, dissolved organic nitrogen; TN, total nitrogen; DOC, dissolved organic carbon; SOC, soil organic carbon.

**Table 2.** The effects of soil water content and freeze–thaw treatment on the soil enzyme activities.

| WC | FTT | AG | BG | BX | CBH | LA | NAG | Urease |
|---|---|---|---|---|---|---|---|---|
| 30% | CK | 0.6 ± 0.1 a | 11.6 ± 2.0 ab | 15.3 ± 4.1 abcd | 6.1 ± 0.8 a | 10.2 ± 1.0 abc | 14.8 ± 1.6 a | 37.2 ± 1.0 abcde |
| | LF | 3.3 ± 0.7 b | 21. 6 ± 2.7 cd | 23.4 ± 1.9 abcd | 32.4 ± 4.5 bc | 14.0 ± 0.6 d | 19.1 ± 1.9 ab | 38.6 ± 1.7 ab |
| | SFQT | 0.7 ± 0.1 ac | 9.7 ± 1.8 ab | 18.0 ± 1.7 ab | 8.1 ± 0.7 d | 12.2 ± 1.0 ade | 15.2 ± 1.5 ab | 36.5 ± 1.5 acde |
| | QFST | 0.8 ± 0.1 acd | 9.0 ± 1.4 ab | 17.9 ± 2.0 abcd | 8.6 ± 1.3 ade | 11.6 ± 1.0 abd | 16.6 ± 1.6 ab | 37.8 ± 1.5 abcd |

**Table 2.** *Cont.*

| WC | FTT | AG | BG | BX | CBH | LA | NAG | Urease |
|---|---|---|---|---|---|---|---|---|
| 60% | CK | 1.0 ± 0.1 acd | 14.2 ± 4.0 ac | 27.1 ± 8.7 acef | 24.3 ± 4.2 b | 8.7 ± 0.8 efh | 73.0 ± 16.2 c | 40.8 ± 1.8 b |
| | LF | 45.2 ± 5.6 e | 35.6 ± 4.8 ef | 26.8 ± 4.2 cdeg | 45.5 ± 5.5 cfg | 3.5 ± 0.3 h | 1.6 ± 0.3 d | 38.0 ± 1.8 abcd |
| | SFQT | 1.3 ± 0.2 df | 12.5 ± 3.7 ab | 16.9 ± 7.6 bd | 21.5 ± 5.9 bdeh | 9.5 ± 1.5 abcefg | 54.8 ± 14.0 ce | 37.0 ± 1.8 acde |
| | QFST | 1.1 ± 0.2 cd | 10.7 ± 3.0 b | 21.1 ± 7.9 bdg | 21.2 ± 5.9 beh | 8.8 ± 1.1 bcfg | 34.2 ± 10.2 bf | 35.3 ± 1.3 cde |
| 100% | CK | 2.1 ± 0.2 bf | 27.1 ± 2.4 de | 36.6 ± 3.1 efg | 50.9 ± 8.8 cfg | 11.3 ± 0.7 abef | 49.8 ± 13.0 e | 33.4 ± 1.5 e |
| | LF | 79.1 ± 6.6 g | 58.7 ± 10.2 f | 61.4 ± 8.8 h | 67.8 ± 8.0 f | 7.3 ± 1.0 cg | 11.4 ± 5.5 ad | 39.0 ± 2.5 abc |
| | SFQT | 2.1 ± 0.3 bf | 29.7 ± 3.4 e | 47.3 ± 4.8 fh | 38.6 ± 3.8 gh | 11.4 ± 1.0 abef | 48.8 ± 10.2 cef | 35.0 ± 1.6 de |
| | QFST | 2.0 ± 0.2 bf | 31.6 ± 3.5 e | 54.6 ± 5.3 h | 43.5 ± 6.1 gh | 12.1 ± 0.5 ab | 73.3 ± 11.3 ce | 35.3 ± 0.8 cde |
| Effect of WC | | <0.001 | <0.001 | <0.001 | <0.001 | <0.001 | 0.002 | 0.186 |
| Effect of FTT | | <0.001 | <0.001 | 0.249 | <0.001 | 0.046 | <0.001 | 0.447 |
| Effect of WC × FTT | | 0.861 | 0.712 | 0.698 | 0.717 | 0.002 | <0.001 | 0.296 |

Data are means ± SE (n = 12). Different letters indicate significant ($p$ < 0.05) differences tested by Wilcox test. The significant effect of water content, freeze-thaw type, and their interaction are tested by Scheirer–Ray–Hare test. Note: AG, α-1,4-glucosidase; BG, β-1,4-glucosidase; BX, β-1,4-xylosidase; CBH, cellobiohydrolase; LA, leucine aminopeptidase; NAG, β-1,4-N-acetylglucosaminidase.

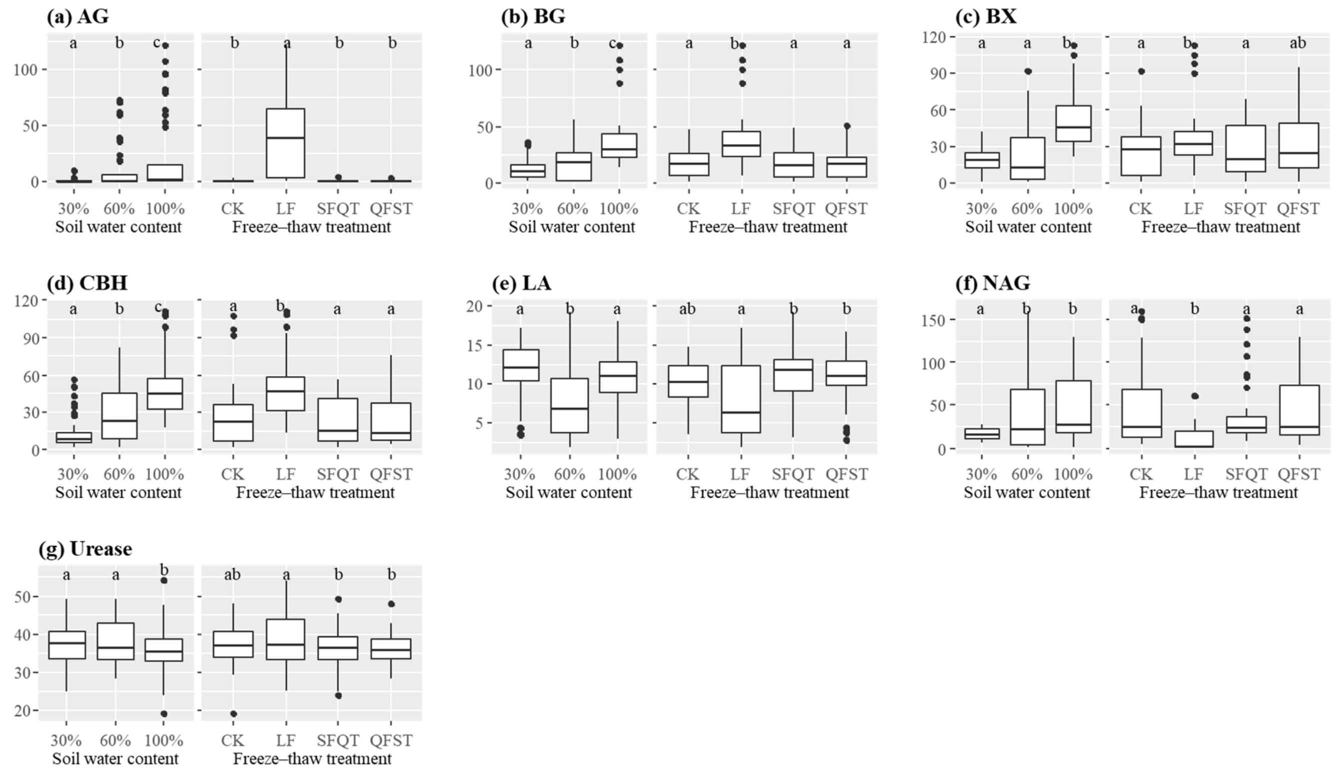


**Figure 3.** The soil enzyme activities under different soil water content or freeze–thaw condition. Different letters indicate significant ($p$ < 0.05) differences tested by Wilcox test. AG, α-1,4-glucosidase; BG, β-1,4-glucosidase; BX, β-1,4-xylosidase; CBH, cellobiohydrolase; LA, leucine amiopeptidase; NAG, β-1,4-N-acetylglucosaminidase.

### *3.2. Soil Microbial Biomass and Soil GHG Fluxes*

The soil water content had significant influences on the MBC and MBN and the microbial C/N (Table 3). The MBC decreased with the soil water content under each freeze–thaw treatment and across all the freeze–thaw treatments (Table 3; Figure 4), and MBN content was significantly higher under 30% water content across all the freeze–thaw treatments. The freeze–thaw treatment only had a significant influence on the MBN (Table 4). The soil water content × freeze–thaw treatment interaction significantly affected the MBN. Moreover, the MBN decreased with soil water content under the QFST condition but was significantly reduced by 60% soil water content under the CK condition (Table 3).

**Table 3.** The effects of water content and freeze–thaw treatment on the soil microbial biomass of C, N, C: N, and GHG fluxes.

| WC | FTT | MBC | MBN | Microbial C/N | $N_2O$ | $CH_4$ | $CO_2$ |
|---|---|---|---|---|---|---|---|
| 30% | CK | 490.1 ± 31.2 a | 25.4 ± 2.5 ab | 21.6 ± 2.6 abc | 0.81 ± 0.08 abcd | 0.90 ± 0.05 abc | 0.77 ± 0.07 abc |
| | LF | 603.0 ± 27.3 b | 27.4 ± 3.1 a | 24.7 ± 2.4 abc | 24.03 ± 10.12 efg | 0.81 ± 0.02 abdef | 0.55 ± 0.01 de |
| | SFQT | 565.8 ± 21.7 b | 17.8 ± 2.4 cd | 40.5 ± 6.8 d | 1.60 ± 0.55 abe | 0.81 ± 0.02 def | 1.29 ± 0.08 f |
| | QFST | 600.7 ± 18.0 b | 29.6 ± 3.0 a | 22.7 ± 2.3 ab | 2.71 ± 0.77 cdfh | 0.82 ± 0.02 ade | 0.67 ± 0.04 a |
| 60% | CK | 382.6 ± 29.1 cde | 15 ± 2.9.0 bcde | 34.3 ± 5.1 ad | 0.71 ± 0.15 ac | 0.84 ± 0.01 abcdf | 1.06 ± 0.11 fgh |
| | LF | 458.6 ± 13.9 acd | 20.6 ± 3.0 abcde | 27.9 ± 3.9 abd | 14.16 ± 7.01 bdefhgi | 0.81 ± 0.02 de | 0.55 ± 0.01 d |
| | SFQT | 438.4 ± 17.5 acd | 18.4 ± 1.1 ce | 24.5 ± 1.4 a | 9.18 ± 3.04 gi | 0.79 ± 0.03 def | 1.34 ± 0.17 fg |
| | QFST | 442.4 ± 28.3 ac | 22.7 ± 2.1 abe | 21.2 ± 2.2 abc | 6.74 ± 2.31 fghi | 0.79 ± 0.02 de | 0.92 ± 0.06 bh |
| 100% | CK | 366.6 ± 38.9 def | 23.0 ± 2.8 abce | 17.7 ± 1.8 ce | 5.63 ± 3.25 abcdhi | 0.86 ± 0.01 bc | 0.88 ± 0.10 abch |
| | LF | 359.5 ± 17.4 ef | 27.5 ± 3.1 a | 14.8 ± 1.6 e | 1.46 ± 0.38 abcd | 0.80 ± 0.01 e | 0.53 ± 0.01 d |
| | SFQT | 341.1 ± 20.8 ef | 17.8 ± 1.1 cde | 19.5 ± 1.1 bc | 2.22 ± 0.84 abcdefh | 0.86 ± 0.03 c | 0.87 ± 0.13 abcegh |
| | QFST | 311.2 ± 27.9 f | 15.2 ± 1.6 d | 21.3 ± 1.7 bc | 0.81 ± 0.11 ac | 0.85 ± 0.01 bcf | 0.78 ± 0.03 c |
| Effect of WC | | <0.001 | 0.009 | <0.001 | 0.036 | 0.106 | 0.035 |
| Effect of FTT | | 0.271 | 0.044 | 0.103 | 0.028 | 0.010 | <0.001 |
| Effect of WC × FTT | | 0.487 | 0.007 | 0.051 | 0.009 | 0.857 | 0.074 |

Data are means ± SE (n = 12). Different letters indicate significant ($p < 0.05$) differences tested by Wilcox test. The significant effect of water content, freeze-thaw type, and their interaction are tested by Scheirer–Ray–Hare test. Note: MBC, microbial biomass carbon; MBN, microbial biomass nitrogen.

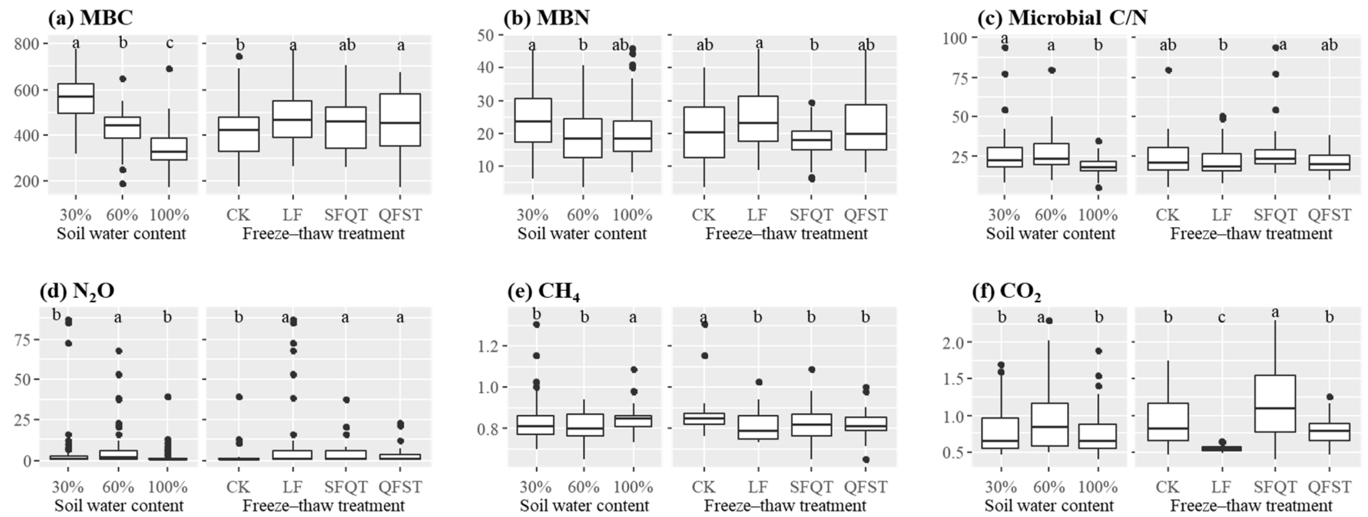


**Figure 4.** The soil microbial biomass and GHG fluxes under different soil water content or freeze–thaw condition. Different letters indicate significant ($p < 0.05$) differences tested by Wilcox test.

The soil water content significantly affected the fluxes of $N_2O$ and $CO_2$. However, the trend between soil water content and GHG fluxes was not significant across all freeze–thaw treatments (Table 3; Figure 3). The freeze–thaw treatment significantly affected the fluxes of all the $N_2O$, $CH_4$, and $CO_2$. Across all soil water contents and as compared to the CK, the LF, SFQT, and QFST treatments increased the $N_2O$ flux, the LF and QFST reduced the $CH_4$ flux, and the LF decreased but SFQT increased the $CO_2$ flux (Figure 4). The soil water content × freeze–thaw treatment interaction significantly affected the $N_2O$ flux (Table 3; Figure 4).

There were no significantly linear relationships found among GHG fluxes (Figure 5), but each GHG had different significantly related factors (Table 4). The $N_2O$ flux was negatively related to pH, $NH_{4^+}$-N, BG, and BX and positively related to $NO_{3^-}$-N and MBC. The $CH_4$ flux was negatively related to pH, BG, and CBH and positively related to $NH_{4^+}$-N, SOC, LA, and urease. The $CO_2$ flux was negatively related to TN, AG, BG, CBH, and MBN and positively related to $NH_{4^+}$-N, NAG, and microbial C/N. Considering these significant factors and the effects of the soil water content and freeze–thaw treatments, the variance partition model found that only freeze–thaw treatment, BX, $NH_{4^+}$-N, and BG were significant in terms of predicting the variance of $N_2O$ flux, and freeze–thaw treatment was the most important individual predictor (Figure 6a). The pH, LA, $NH_{4^+}$-N, and freeze–thaw treatment were significant in terms of predicting the variance of $CH_4$ flux, and pH was the most important individual predictor (Figure 6b). For $CO_2$ flux, there were eight significant prediction factors, excepting the microbial C/N, soil water content, and LA, and among these eight factors, the freeze–thaw treatment was the most important predictor (Figure 6c).

**Table 4.** Correlation between GHG fluxes and soil properties, enzymes activities, and soil microbes using Pearson's correlation coefficient.

| Variables | $N_2O$ | | $CH_4$ | | $CO_2$ | |
|---|---|---|---|---|---|---|
| | *r* | *p*-Value | *r* | *p*-Value | *r* | *p*-Value |
| pH | **−0.173** | **0.039** | **−0.423** | **<0.001** | −0.012 | 0.891 |
| $NH_{4^+}$-N | **−0.211** | **0.011** | **0.278** | **0.001** | **0.372** | **<0.001** |
| $NO_{3^-}$-N | **0.205** | **0.014** | 0.076 | 0.367 | −0.142 | 0.089 |
| TDN | 0.095 | 0.258 | 0.020 | 0.814 | 0.106 | 0.204 |
| TN | 0.051 | 0.543 | −0.146 | 0.080 | **−0.311** | **<0.001** |
| DOC | 0.061 | 0.469 | 0.082 | 0.328 | 0.103 | 0.221 |
| SOC | 0.075 | 0.370 | **0.201** | **0.016** | 0.012 | 0.886 |
| AG | −0.019 | 0.824 | −0.032 | 0.705 | **−0.425** | **<0.001** |
| BG | **−0.216** | **0.009** | **−0.207** | **0.013** | **−0.422** | **<0.001** |
| BX | **−0.265** | **0.001** | −0.016 | 0.847 | −0.114 | 0.172 |
| CBH | −0.132 | 0.114 | **−0.180** | **0.031** | **−0.396** | **<0.001** |
| LA | 0.056 | 0.504 | **0.281** | **0.001** | **0.180** | **0.031** |
| NAG | −0.009 | 0.917 | 0.111 | 0.187 | **0.379** | **<0.001** |
| Urease | 0.068 | 0.421 | **0.191** | **0.022** | 0.032 | 0.703 |
| MBC | **0.213** | **0.010** | −0.051 | 0.547 | -0.096 | 0.253 |
| MBN | 0.017 | 0.836 | −0.078 | 0.356 | **−0.304** | **<0.001** |
| Microbial C/N | 0.121 | 0.147 | 0.031 | 0.716 | **0.257** | **0.002** |

Bold values denote statistical significance at the $p < 0.05$ level.

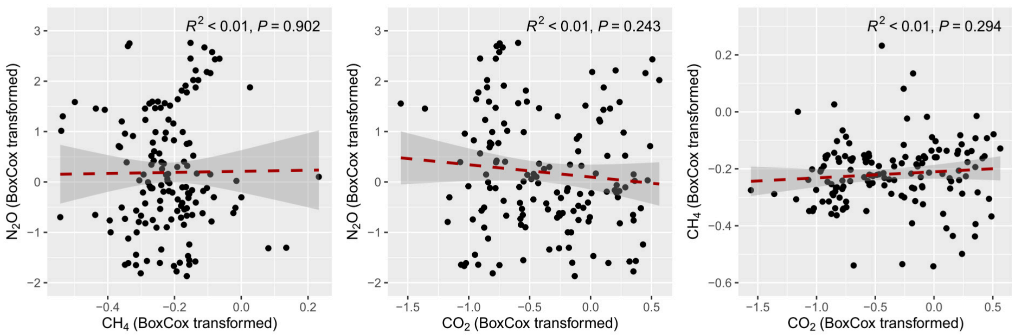


**Figure 5.** Linear relationships among GHG fluxes. The dark red fitted lines are from OLS regression. Shaded areas show 95% confidence interval of the fit.

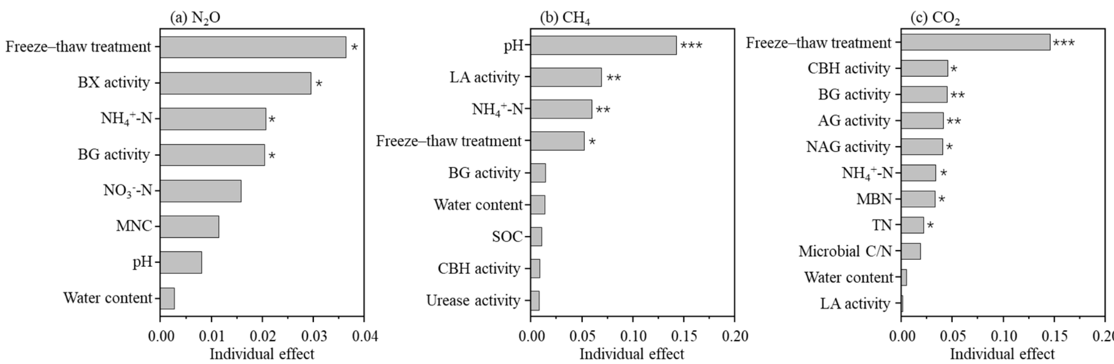


**Figure 6.** The percentage of variance of GHGs explained by correlated variables using variation partition analysis (VPA). Significance levels are indicated by asterisks: *** $p < 0.001$, ** $p < 0.01$, * $p < 0.05$.

## 4. Discussion

### *4.1. Freeze–Thaw Treatments Increase $N_2O$ Flux*

In the variation partition analysis, we found that freeze–thaw treatments had the highest explanatory power. It revealed that irregular temperature changes around zero affect $N_2O$ flux more than irregular rainfall. Across all the soil water content levels, the freeze–thaw had increased the $N_2O$ flux, which is in accordance with the results of Libby et al. [31]. The traditional $N_2O$ emission pathways mainly exist in nitrification and denitrification, the substrates of which are $NH_{4^+}$-N and $NO_{3^-}$-N, respectively [32]. However, we found a negative correlation for $N_2O$ flux with $NH_{4^+}$-N, which is contrary to the findings of Horák et al. [33], while the positive correlation for $N_2O$ flux with $NO_{3^-}$-N is consistent with the findings of Horák et al. [33]. A reasonable explanation may be that the freeze–thaw treatment enhances the nitrification but weakens denitrification. Furthermore, this could also explain the significantly lower $NH_{4^+}$-N level and higher $NO_{3^-}$-N level under LF condition, which was most intense freezing treatment.

In this study, the $N_2O$ fluxes of the LF and QFST treatments were significantly higher than that of the CK treatment under 30% and 60% soil water contents, but not 100% soil water content. These results demonstrate that a high soil water content can reduce the effect of freeze–thaw cycles on $N_2O$ flux. We suppose the main reason for this is the fact that a high water content in soil can reduce nitrification rates by limiting the oxygen supply to nitrifiers, which may reduce difference among freeze–thaw treatments. This could also explain why the $N_2O$ flux for 100% soil water content was smaller than that of 60% soil water content. Moreover, the $N_2O$ flux for 30% soil water content was also lower than that of 60% soil water content. This may be because water films in under 30% soil water content conditions are discontinuous around soil particles, which makes substrate diffusion more difficult and limits the nitrification rates [32].

The enzymes BG and BX were significant in individually predicting $N_2O$ flux, and both were negatively correlated with $N_2O$ flux. These results were quite different to those in the previous studies [34–36]. This may be because the microbes were stimulated to produce the enzymes by low temperature [37], rather than being spontaneously produced for nutrient absorption as in field conditions. The more stimulation on microbes by low temperature, the more soil enzymes are produced, which is in contrast to the mechanism in which the $N_2O$ flux is increased by enhancing the nitrifiers' activity. Thus, the correlations of $N_2O$ flux with BG and BX activities are negative. Furthermore, the explanation involving lower temperature stimulation could also explain why the activities of AG, BG, BX, CBH, and even urease were all higher for the LF treatment in most of or across all the soil water content levels (Table 2; Figure 3), because the LF treatment had the strongest stimulation on soil microbes.

### *4.2. Freeze–Thaw Treatments Reduce $CH_4$ Flux*

In this study, soil pH was the strongest individual predictor for $CH_4$ flux in the variation partition analysis (Figure 5b), and the $CH_4$ flux was negatively correlated with pH (Table 4). This result has been reported in other ecosystems [38–40] and is explained by the fact that microbial methanogenic activity is strongly affected by soil pH [38]. LA activity was positively correlated with $CH_4$ flux and demonstrated significance in the variation partition analysis in explaining $CH_4$ flux. This is because leucine aminopeptidase (LA) is important in carbon degradation [41], which might be significant for $CH_4$ flux in this study.

As compared to soil water content, we found that freeze–thaw treatments had more significant effects on $CH_4$ flux (Table 3; Figure 5b). However, across all the freeze–thaw treatments, $CH_4$ flux was higher at 100% soil water content due to the higher $CH_4$ flux under SFQT and QFST at 100% soil water content (Table 3). This result indicates that high soil water content could enhance $CH_4$ flux during freeze–thaw cycling. This might be due to the soil water, which has a high specific heat capacity [42], and could alleviate the freeze–thaw cycles' effect on methanogenic activity. Across all the soil water contents, the LF, SFQT, and QFST treatments had significantly lower $CH_4$ fluxes than CK. This may be because the low temperature weakens the methanogenic activity [43,44], as methanogenic microbes are more sensitive to freeze–thaw cycles as compared to other microbes [45].

### *4.3. Freeze–Thaw Treatments Have Different Impacts on $CO_2$ Flux*

Soil $CO_2$ is normally produced by soil respiration and represents the most important component of GHGs. Moreover, it has a much longer lifetime than other GHGs [46]. The variation partition analysis showed that the freeze–thaw treatment had the highest individual effect in predicting the $CO_2$ flux (Figure 6c). The effect of different freeze–thaw treatments on $CO_2$ flux was different to the effect on $N_2O$ and $CH_4$ fluxes. Among the freeze–thaw treatments, LF had a significantly lower $CO_2$ flux than the other treatments under each soil water content and across all the soil water content levels, and SFQT had a higher $CO_2$ flux across all the soil water content levels. It is obvious that LF, as the long-term freezing treatment at $-10$ °C, reduces soil respiration [47,48]; however, this cannot properly explain the $CO_2$ flux of SFQT, which also underwent a freezing incubation period during the experiment and was higher than that of CK, LF, and QFST across all the water content levels. Elberling [49] found that certain groups of soil microbes demonstrated activity around freezing; thus, the produced $CO_2$ might be accumulated in freezing soil and, in our experimental setting, could be emitted after warming [50]. Despite QFST being a similar treatment to SFQT, we did not find its $CO_2$ flux to be higher than that of CK. The main reason for that is the fact that the QFST treatment comprised lower temperature at freezing and warmer temperature at thawing, and the thawing had a much longer experimental duration. Hence, $CO_2$ accumulation was low, and the emission time lasted long after thawing for QFST treatment.

We found that the higher $CO_2$ flux for SFQT was obvious under 30% water content (higher than all other freeze–thaw treatments) and 60% water content (higher than LF and QFST); however, it was not obvious under 100% water content. This result demonstrates that a high water soil content can reduce the effect of SFQT on $CO_2$ flux. We also found that across all freeze–thaw treatments, the $CO_2$ flux under 60% water content was higher than that under 30% and 100% soil water contents. The explanation for this is similar to that of the $N_2O$ flux, i.e., a reduced substrate diffusion under 30% soil water content and a reduced oxygen supply to decomposers under 100% soil water content.

$CO_2$ flux had eight significant individual explanation factors in the variation partition analysis, including the activities of four soil enzymes. Among these four enzymes, $CO_2$ flux was negatively correlated with AG, BG, and CBH but positively correlated with NAG. NAG is an enzyme that degrades chitin, which is the main component of fungal cell walls and the second most abundant polysaccharide in natural soil [51]. A previous study found that the activity of NAG was positively correlated with fungal biomass [52] and that fungal biomass was also positively correlated with $CO_2$ flux [53]. This can explain why the $CO_2$ flux had a positive correlation with NAG. AG, BG, and CBH had a negative correlation with $CO_2$ flux, as reported in the freeze–thaw experiment of Gao et al. [54]. The main reason for this is that freezing or low temperatures can stimulate the microbes to produce enzymes [37]; the stronger the stimulation, the more enzymes are produced by microbes, and the more microbial respiration is inhibited. This was why there was a negative correlation between $CO_2$ flux and these three enzyme activities.

### *4.4. The Changes in Soil Nutrients, Enzyme Activities, and Soil Microbes*

In this study, we found that soil TDN and DOC were decreased with soil water content. This was previously reported in Niboyet et al. [55] and Zhang et al. [56]. Because both TDN and DOC are dissolved matter, they are normally lost from the soil via leaching [57], and that leads to increasing soil water contents and reduces the TDN and DOC concentrations. The other three freeze–thaw treatments exhibited the same trend as CK, i.e., reduced DOC across all soil water content levels (Table 1; Figure 2). This might be because the freeze–thaw cycle can stimulate the soil carbon decomposition by carbon-source-decomposing bacteria, which accelerates the DOC consumption and adsorption [58,59].

Soil water content also significantly affected the $NH_{4^+}$-N concentration. The $NH_{4^+}$-N concentration was higher under 100% soil water content across all freeze–thaw treatments. This result is contrary to the results reported by Wu et al. [60], who found that precipitation decreased the soil $NH_{4^+}$-N. However, the increase in the $NH_{4^+}$-N concentration under 100% soil water content did not happen under CK, but was observed under the LF, SFQT, and QFST treatments, wherein the mean concentration increased approximately twofold as compared to under 30% and 60% soil water content levels. We supposed that a high water content enhances the positive effect of freezing on the $NH_{4^+}$-N concentration. Across all soil water content levels, LF reduced the $NH_{4^+}$-N and TDN concentrations as compared to other freeze–thaw treatments; however, this was to a lesser extent and was not significant as compared to SFQT. Moreover, LF increased the $NO_{3^-}$-N and TN concentrations. These results demonstrated that long-term freezing (LF) soil had a lower mineralization, which resulted in TN not being mineralized and lower TDN and $NH_{4^+}$-N concentrations. Furthermore, we also supposed that the denitrification of $NO_{3^-}$-N was weakened under the LF condition due to the extent of freezing leading to a higher $NO_{3^-}$-N concentration [61].

The activities of AG, BG, BX, and CBH increased with soil water content under most of and across all freeze–thaw treatments, and NAG also exhibited a similar trend. All these five enzymes are involved in the degradation of cellulose [62], which is the major component of plant litter. The QTP is characterized as having low amounts of available soil nutrients [63], which indicates that these soil enzyme activities are important in maintaining nutrient cycling in this ecosystem. Previous studies have found that water exclusion weakens soil enzyme activities [64,65], which is consistent with our results. The MBC decreased with soil water content under each freeze–thaw treatment and across all freeze–thaw treatments in this study (Table 3; Figure 4). This result conflicts with the field experiment results of Dietrich et al. [66] and Bhanwaria et al. [67]. A possible reason for this may be that our incubation experiment did not involve growing plants, which breaks the water–plant–microbe linkages under increasing soil water, causing no carbon resources to be secreted by plant roots for microbial growth when water-enriched [68]. Moreover, an excessive water content in soil can suppress the microbial oxygen supply for microbe and nutrient uptake, thus causing the microbial biomass carbon to decrease in this study.

## 5. Conclusions

This study has important implications for predicting the future impact of irregular climate change on the eastern edge of the QTP. Our findings demonstrated that both $N_2O$ and $CO_2$ fluxes were significantly influenced by soil water content and freeze–thaw treatments, wherein the 60% soil water content level exhibited higher GHG fluxes. As compared to the control treatment, the LF, SFQT, and QFST freeze–thaw treatments exhibited higher $N_2O$ flux, and the LF treatment reduced but the SFQT increased the $CO_2$ flux. $CH_4$ flux was only significantly influenced by the freeze–thaw treatments, i.e., the LF, SFQT, and QFST freeze–thaw treatments reduced $CH_4$ flux. We also found that soil water content and freeze–thaw treatment impacted various soil properties, enzyme activities, and microbial biomass values; however, the variation partition analysis revealed that the variance in $N_2O$ and $CO_2$ fluxes could, for the most part, be individually predicted by freeze–thaw treatment, and the variance in $CH_4$ flux could mostly be individually predicted by soil pH, which was also only significantly influenced by the freeze–thaw treatments. Therefore, our results demonstrate that the freeze–thaw cycles have more of an effect on GHG flux than soil water content on the eastern edge of the QTP, while the effects of freeze–thaw cycles on $N_2O$, $CH_4$, and $CO_2$ were shown to be quite different.

**Supplementary Materials:** The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/su15020928/s1, Table S1: The method for data transformation for each variable in this study.

**Author Contributions:** Conceptualization, S.Z. and Y.Y.; methodology, S.Z. and J.W.; software, W.B.; validation, S.Z., J.W. and M.Q.; formal analysis, S.Z. and X.Y.; investigation, S.Z. and W.B.; resources, S.Z. and X.Y.; data curation, S.Z. and J.W.; writing—original draft preparation, S.Z.; writing—review and editing, M.Q; visualization, S.Z. and M.Q.; supervision, Y.Y.; project administration, J.W.; funding acquisition, J.W. All authors have read and agreed to the published version of the manuscript.

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## Abbreviations

| | |
|---|---|
| QTP | Qinghai-Tibetan Plateau |
| GHG | greenhouse gas |
| $N_2O$ | nitrous oxide |
| $CH_4$ | methane |
| $CO_2$ | carbon dioxide |
| WC | water content |
| FTT | freeze–thaw treatment |
| CK | control |
| LF | long-term freezing |
| SFQT | slow freeze and quick thaw |
| QFST | quick freeze and slow thaw |
| TDN | total dissolved nitrogen |
| TN | total nitrogen |
| DOC | dissolved organic carbon |
| SOC | soil organic carbon |
| AG | αG1,4-glucosidase |
| BG | βG1,4-glucosidase |
| BX | βX1,4-xylosidase |
| CBH | cellobiohydrolase |
| LA | leucine aminopeptidase |
| NAG | βAG,4-N-acetylglucosaminidase |
| MBC | microbial biomass carbon |
| MBN | microbial biomass nitrogen |
| OLS | ordinary least squares |

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