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Differential Responses and Controls of Soil CO_2 and N_2O Fluxes to Experimental Warming and Nitrogen Fertilization in a Subalpine Coniferous Spruce (*Picea asperata* Mast.) Plantation Forest

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Abstract: Emissions of greenhouse gases (GHG) such as CO_2 and N_2O from soils are affected by many factors such as climate change, soil carbon content, and soil nutrient conditions. However, the response patterns and controls of soil CO_2 and N_2O fluxes to global warming and nitrogen (N) fertilization are still not clear in subalpine forests. To address this issue, we conducted an eight-year field experiment with warming and N fertilization treatments in a subalpine coniferous spruce (*Picea asperata* Mast.) plantation forest in China. Soil CO_2 and N_2O fluxes were measured using a static chamber method, and soils were sampled to analyze soil carbon and N contents, soil microbial substrate utilization (MSU) patterns, and microbial functional diversity. Results showed that the mean annual CO_2 and N_2O fluxes were 36.04 ± 3.77 mg C m^{-2} h^{-1} and 0.51 ± 0.11 μg N m^{-2} h^{-1}, respectively. Soil CO_2 flux was only affected by warming while soil N_2O flux was significantly enhanced by N fertilization and its interaction with warming. Warming enhanced dissolve organic carbon (DOC) and MSU, reduced soil organic carbon (SOC) and microbial biomass carbon (MBC), and constrained the microbial metabolic activity and microbial functional diversity, resulting in a decrease in soil CO_2 emission. The analysis of structural equation model indicated that MSU had dominant direct negative effect on soil CO_2 flux but had direct positive effect on soil N_2O flux. DOC and MBC had indirect positive effects on soil CO_2 flux while soil NH_4^+-N had direct negative effect on soil CO_2 and N_2O fluxes. This study revealed different response patterns and controlling factors of soil CO_2 and N_2O fluxes in the subalpine plantation forest, and highlighted the importance of soil microbial contributions to GHG fluxes under climate warming and N deposition.

Keywords: warming; nitrogen; greenhouse gas; soil characteristics; microbial properties

1. Introduction

Due to fossil fuel combustion and land use change, global air temperature has been increasing over the past decades [1]. The Qinghai–Tibet Plateau region (QTP) of China is experiencing a larger increase in temperature than other regions with an increasing rate of 0.2 °C per decade [2]. Accompanied with climate warming, nitrogen (N) deposition is increasing in many places on the Earth [3]. China has the third highest rate of nitrogen deposition, followed by North America and Western Europe due to the industrialization and intensive agricultural activities [3,4]. Additionally, in the QTP region, N

deposition continues to increase. The climate warming and N deposition are likely to have significant impacts on greenhouse gases (GHG) emissions in QTP ecosystems because the high-latitude regions are very sensitive to global change with large soil C pool, low inorganic N availability, and higher temperature sensitivity [3].

Carbon dioxide (CO_2) and nitrous oxide (N_2O) are two important GHGs, which contribute to about 60% and 6% of the global warming potential, respectively [2,5]. Many studies have investigated the effects of warming and N deposition on soil GHG fluxes, but large uncertainties still remain. For example, some studies found that warming leads to increase in soil CO_2 emission because it accelerates the decomposition of soil organic C (SOC) [6], but others reported that warming decreases or has no effect on soil CO_2 emission due to the loss of SOC in a long-term warming experiment [7]. Climate warming generally increases soil N_2O flux by enhancing decomposition and N mineralization [8,9], however, it may not influence soil N_2O flux or decrease it depending on the soil conditions [10]. Studies on the effect of N fertilization or deposition on GHG fluxes also showed various results. For example, Jassal et al. [11] found that the N application increases soil CO_2 and N_2O emissions in the first year, but shifts to soil N_2O uptake has no effect on soil CO_2 emission in the second year. Geng et al. [12] reported that the N addition at a low rate of 10 kg N ha^{-1} $year^{-1}$ significantly stimulated soil CO_2 emission, whereas the high rate of N addition (140 kg N ha^{-1} $year^{-1}$) significantly inhibits soil CO_2 emission in a temperate mixed forest.

The different responses of soil CO_2 and N_2O fluxes to global warming and N fertilization in different environments could be determined by soil physical–chemical properties such as soil temperature, soil inorganic nitrogen availability, and soil carbon content [13,14]. One study showed that soil CO_2 flux is positively related to soil dissolve organic carbon (DOC) and NO_3^--N, and soil N_2O flux is positively correlated with soil NH_4^+-N [14]. Another study found that soil CO_2 efflux is positively correlated with soil NH_4^+-N and negatively with soil NO_3^--N [12]. Thus, any changes in these soil properties caused by warming and N fertilization could have different impacts on GHG fluxes [15]. Indeed, Geng et al. [12] found high N addition enhances soil NO_3^--N and inhibits soil CO_2 emission, while low N addition does not affect soil NO_3^--N but stimulates soil CO_2 emission. Seo et al. [16] found warming increases the labile C pool, causes a loss of soil C, and increases soil CO_2 emission. Yin et al. [7] reported that warming decreases SOC and decreases soil CO_2 emission.

The influences of warming and N deposition on soil microbial activity and composition may have significant impacts on soil CO_2 and N_2O fluxes. Soil microorganisms are the major drivers in the biogeochemical processes such as soil C decomposition and N mineralization [17,18]. Any changes in soil microbial diversity and community structure may alter the C and N cycling [17]. For instance, the fungi to bacteria ratio is negatively correlated to soil N mineralization [19]. Furthermore, soil microorganisms can be affected by multiply factors such as climate, soil physical, and chemical properties, and substrate quantity and quality [20,21]. Several studies reported that soil microbial community structure and diversity are strongly impacted by warming and N fertilization, and play an important role on controlling soil CO_2 and N_2O fluxes [17,22,23]. However, convincing data about the direct link of soil GHG fluxes and soil microbial characteristics under warming and N fertilization are still scarce.

The subalpine and alpine forest ecosystems in Eastern Tibetan Plateau, located at the high latitude of the transition zone from the QTP to Sichuan basin, constitute the second largest biome in China and are the main forest ecosystems in southwest China [24]. Spruce (*Picea asperata* Mast.) is the dominant tree species of the plantation, which is the major forest ecosystem in this region after deforestation in the 1950s. Past studies on climate warming and N fertilization in forests in this region mostly focused on the soil C pool and N pool and associated processes [7,25,26]. Although soil GHG fluxes are highly related to the soil C and N pool, these data are not directly reflecting the GHG magnitude. Direct evidence of variations of the responses of soil CO_2 and N_2O fluxes and their controls is needed.

We took advantage of an eight-year field experiment with warming and N fertilization in subalpine spruce plantation forest, and measured soil CO_2 and N_2O fluxes over one year using the static chamber

method. We also analyzed soil C and N contents, microbial substrate utilization patterns, and microbial functional diversity using BIOLOG microplates. We aimed to quantify the magnitude of soil CO_2, N_2O fluxes in the plantation forest and the effects of climate warming and applying N fertilization on the gas fluxes, and reveal influential factors that control soil CO_2 and N_2O fluxes.

2. Materials and Methods

2.1. Experimental Site

The experimental site is located at the Maoxian Ecological Station of the Chinese Academy of Sciences, Sichuan Province, China (31°41′ N, 103°53′ E, 1820 m a.s.l.). The site is in a subalpine canyon zone at the transition region from Qinghai–Tibet Plateau (QTP) to Sichuan basin, with the mean annual temperature, total annual precipitation, and evaporation of 8.9 °C, 920 mm, and 796 mm, respectively. The experiment started in March 2007 with warming and N fertilization treatments and ended in 2015. Soil CO_2 and N_2O fluxes were measured for one year from 14 June 2014 to 25 June 2015, eight years after the treatments were applied.

2.2. Experimental Set-Up and Design

To avoid the potential effects of soil heterogeneity on soil GHG fluxes, we collected the top 50 cm soil from a nearby spruce plantation forest and replaced the indigenous soil in all plots. In March 2007, 40 healthy four-year-old seedlings of spruce were randomly planted in each plot (2 m × 2 m). The seedlings were collected from a local nursery. The experiment included four treatments: Control, warming, N fertilization, and warming and N fertilization. A randomized block design with four replicates (blocks) was used in this study. Artificial warming and N application started in April 2007 and continued to the end of the experiment. The heating method were described in detail in published papers of our research team [27,28]. Ammonium nitrate solution (25 g N m^{-2} $year^{-1}$) was added weekly to the soil surface of fertilization treatment. The equivalent amount of water was added to the other four pairs of plots for unfertilized treatments. In order to eliminate the potential effects of difference in soil water on soil processes between the warmed and un-warmed plots, the warmed plots were watered as frequently as needed and were monitored with a hand-held probe (IMKO, Ettlingen, Germany).

2.3. Microclimate Measurements

Air temperature (20 cm above soil surface) and soil temperature (5 cm depth) were measured using the DS1923G temperature sensor with iButton data loggers (Maxim/Dallas Semiconductor Inc., Dallas, TX, USA) at 60 min intervals. The warming effect decreased with the trees growth and plant coverage. The monthly air temperature in the warmed plots was increased by an average of 2.1, 1.9, 0.3 °C in 2007, 2011, and 2014, respectively. The monthly soil temperature in the warmed plots was increased by an average of 2.6, 3.6, and 0.6 °C in 2007, 2011, and in 2014, respectively.

2.4. Soil CO_2 and N_2O Fluxes Measurements

Soil CO_2 and N_2O fluxes were measured monthly using the static chamber method and gas chromatography technique from 14 June 2014 to 25 June 2015 according to Cai et al. [29]. One PVC tube base with a groove outside but without top and bottom (20 cm inside diameter, and 15 cm height) was inserted into a 10 cm-depth soil in each plot. The removable chamber with a small silicon-sealed bent for gas sampling and a port for measuring chamber temperature at the top of the chamber (without bottom, 21 cm in diameter and 30 cm in height) was placed into the PVC tube base during sampling and removed afterwards. Litter and plants were removed around the tube base before fixing it and four replicates were set in each treatment.

Samples were taken between 10:00 a.m. and 1:00 p.m. in order to minimize diurnal variation in fluxes. Each time, four air samples of each chamber were manually pulled into 100 mL pre-evacuated

gas collecting bags (made in Dalian, China) at 0, 15, 30, and 45 min after enclosure of the chamber, and were taken to the laboratory for analysis using gas chromatography (Agilent 7890A, Santa Clara, CA, USA) within two weeks. Air temperature inside the chamber was measured with a mercury-in-glass thermometer at the time of gas sampling. Soil temperature and moisture were measured outside of each chamber with the DS1923G temperature sensor with iButton data loggers (Maxim/Dallas Semiconductor Inc., Dallas, TX, USA).

Soil CO_2 and N_2O fluxes were calculated as the slope of linear regression between gases concentration and time with an average chamber temperature [30]. All the coefficients of the linear regression (r^2) were greater than 0.80 in this study. Flux was calculated as:

$$F = \frac{dc}{dt} \times \frac{p}{0.082T} \times M \times \frac{V}{A} \quad (1)$$

where F is the gas flux (μg N m^{-2} h^{-1} for N_2O and mg C m^{-2} h^{-1} for CO_2), $\frac{dc}{dt}$ is the rate of change in gas concentration inside the chamber, p is barometric pressure at temperature T (atm), T is the air temperature inside the chamber in K, M is the molecular weight of the gas, 0.082 is the universal gas constant, V is the chamber volume (m^3) and A is the chamber area (m^2).

2.5. Soil Samples and Analysis

Soil samples (n = 4) in each treatment were collected in August and November of 2014, and February and May of 2015. At each sampling date, we took five topsoil (0–15 cm) cores (2.5 cm diameter) close to each chamber and then combined into one composite sample. Soil samples were sieved through 2 mm mesh to remove visible living plant and rock, stored in an icebox at 4 °C, and delivered to the laboratory for analysis.

Soil organic C (SOC) was determined using the $K_2Cr_2O_7$-H_2SO_4 wet digestion method [31]. After digestion with $K_2Cr_2O_7$-H_2SO_4, $FeSO_4$ was used to titrate the remaining $K_2Cr_2O_7$ in the digestion solution and SOC was calculated based on the consumptions of the $K_2Cr_2O_7$. The dissolve organic C (DOC) was measured using the $K_2Cr_2O_7$-H_2SO_4 wet digestion method after extracted by deionized water [32]. Total soil N (TN) was determined by semi-micro Kjeldahl digestion using Se, $CuSO_4$, and K_2SO_4 as catalysts [33]. Soil ammonium (NH_4^+), nitrate (NO_3^-), and nitrite (NO_2^-)-N concentrations were determined using Auto Analyzer 3 (AA3, Bran Luebbe, Norderstedt, Germany) after being extracted with 2 M KCl solution (soil:water = 1:5) for 1 h [34]. Microbial biomass C (MBC) and N (MBN) concentrations were measured with the chloroform fumigation extraction method [35]. MBC and MBN were calculated as the difference between the C and N concentrations extracted with 2 M K_2SO_4 solution of the fumigated and non-fumigated soil, respectively, and then divided an efficiency factor K = 0.45. All the concentrations were calculated based on soil dry weight.

Microbial substrate utilization (MSU) patterns were analyzed using BIOLOG ECO plates (Biolog, Inc., Hayward, CA, USA). Equivalent to 1.0 g dry soil from each fresh sample was first added into 99 mL distilled autoclaved water and was shaken for 20 min to ensure that all the fungal spores are well mixed. Then, the soil solutions were settled for 30 min at 4 °C to remove suspended clay particles. 150 μL supernatant was transferred to the plates and then was incubated at 25 °C for up to 168 h. The OD values (absorbance at 590 nm and 750 nm, respectively) were measured at each 24 h from 48 to 168 h with a microtiter-plate reader (Biolog GenIII Microstation, Biolog company, Hayward, CA, USA). The OD value at 590 nm subtracting the OD value at 750 nm, and then the difference in the control was subtracted from each well's OD to correct for background activity. To minimize the effects of different inoculation densities, data from the 96 h reading were normalized by h dividing the absorbency of each well by the average absorbency for the whole plate (average well color development, AWCD) [17]. AWCD reflect the metabolic activity of soil microbes. Moreover, the Shannon diversity index (H) and diversity index (U) were calculated to represent the diversity and uniformity of the microbial communities.

$$H = -\sum p_i ln p_i \quad (2)$$

$$U = \sqrt{\left(n_i^2\right)} \quad (3)$$

where $p_i = \frac{OD(i,j,t)}{\sum OD(i,j,t)}$; and $n_{i=OD(i,j,t)}$

2.6. Data Analysis

The exponential model was used to determine the sensitivity of soil GHG fluxes to soil temperature (T):

$$F = ae^{bT} \quad (4)$$

where F is the GHG flux, a is the value of flux at 0 °C, and b is the sensitivity of flux to temperature.

The flux sensitivity to temperature (Q_{10}) was calculated as:

$$Q_{10} = e^{10b} \quad (5)$$

The cumulative global warming potential (GWP, kg CO_2 hm^{-2}) was calculated by adding cumulative soil CO_2 flux, and the cumulative GWP from N_2O (cumulative N_2O flux multiplied by 298) [36].

The repeated measure-ANOVA was used to analyze the effects of warming and N fertilization on soil CO_2 and N_2O fluxes. A three-way analysis of variance (ANOVA) was used to test the effects of warming, N fertilization, and sample time (season) on TOC, DOC, TN, inorganic N, AWCD, H, and U. The ECO plates contained 31 types of carbon substrates. The microbial substrates utilization patterns were analyzed to identify the effects of treatments and soil environment factors such as soil water, temperature, soil DOC, SOC, and inorganic nitrogen using Canonical Correspondence Analysis (CCA) in the CANOCO 4.5 software (Microcomputer Power, Ithaca, NY, USA).

Structural equation modelling (SEM) was performed to determine the relative importance of soil variables to soil CO_2 and N_2O fluxes using the Amos 24.0 software package (IBM, New York, NY, USA). We first tested the relationships between the CO_2 and N_2O fluxes and soil properties before the SEM analysis. If the correlation was significant, that variable was put into the SEM. As microbial substrate utilization patterns included 31 types of carbon source utilization, we selected the significant correlations of the carbon source utilization with soil CO_2 and N_2O fluxes, and then used the Principal Component Analysis (PCA) to create a multivariate functional index. The best-fit model was derived using maximum likelihood and a chi-square test (χ^2), P-values, df, and root mean square errors of approximation (RSMEA) were used to evaluate model fitting.

3. Results

3.1. Soil Carbon, Nitrogen and Microbial Properties

Warming significantly increased soil NO_3^--N, NO_2^--N, DOC, and the ratio of MBC/MBN, but decreased TN, SOC, MBC, and MBN (Table 1). Nitrogen fertilization significantly increased soil NO_3^--N, TN, TOC, and the ratio of MBC/MBN, but decreased soil NO_2^--N, DOC, MBC, and MBN. The metabolic activity of soil microbes measured as the average absorbency for the whole BIOLOG ECO plate (AWCD), the Shannon diversity index (H), and uniformity index (U) varied seasonally (Table 1). Warming decreased AWCD and U. Nitrogen fertilization alone had no effect on AWCD and U but significantly affected these variables with warming. The CCA analysis identified 21 substrates that were the most important variables in separating plots along the environmental axes among the 31 carbon substrates (Figure 1). Most of these MSU patterns were correlated with temperature, soil DOC, and soil water. The correlation coefficient were 0.68, 0.72, −0.72 in CCA1 and −0.62, −0.18, 0.32 in CCA2 for temperature, soil DOC, and soil water, respectively.

Table 1. Factorial ANOVA results (*p*-values) of the effects of season, warming, nitrogen fertilization, as well as their interactions on the soil properties and microbial function diversity. MBC: Microbial biomass C, MBN: Microbial biomass N, SOC: Soil organic C, DOC: Dissolved organic C, TN: Total N, AWCD: Average well color development, H: Shannon diversity index, U: Uniformity.

Treatments	NO_3^--N	NH_4^+-N	NO_2^--N	TN	SOC	DOC	MBC	MBN	MBC/MBN	AWCD	H	U
Season (S)	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	0.001	<0.001	0.010
Warming (W)	<0.001	0.756	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	0.009	0.461	0.006
Nitrogen (N)	<0.001	0.103	<0.001	0.009	<0.001	<0.001	<0.001	<0.001	<0.001	0.237	0.953	0.276
S × W	<0.001	0.322	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	0.032	<0.001
S × N	<0.001	0.008	0.040	0.365	<0.001	<0.001	<0.001	<0.001	<0.001	0.002	0.013	0.006
W ×N	0.565	0.011	0.059	0.015	<0.001	0.002	0.003	<0.001	<0.001	0.016	0.115	0.032
S × W × N	<0.001	0.209	<0.001	0.080	<0.001	<0.001	<0.001	<0.001	<0.001	0.001	0.002	0.004

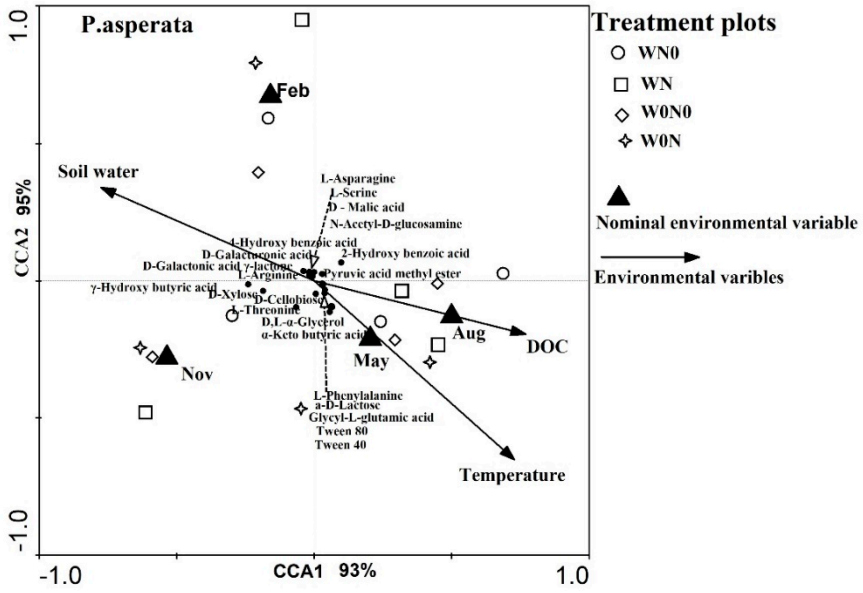

Figure 1. Canonical correspondence analysis (CCA) ordination biplot of treatment plot scores, Biolog substrates, and significant environmental variables. Arrows indicate the direction and relative importance (arrow length) of the environmental variable. Substrates with approximate correlation coefficient >0.20 to the environmental variables are labelled. W_0N_0: Ambient temperature without nitrogen fertilization; W_0N: Ambient temperature with nitrogen fertilization; WN0: Warming without nitrogen fertilization; WN: Warming with nitrogen fertilization. Environmental variables in CCA1 and CCA2 explain 93% and 95%, respectively.

3.2. Soil CO_2 and N_2O Fluxes

The highest soil CO_2 and N_2O fluxes occurred in August and the lowest in January (Figure 2). The mean annual CO_2 and N_2O fluxes were 36.04 ± 3.77 mg C m^{-2} h^{-1} and 0.51 ± 0.11 μg N m^{-2} h^{-1}, respectively (Table 2). Compared to the control (W_0N_0), the annual soil CO_2 flux was slightly decreased in the WN_0 and WN treatments but was increased by 27.8% in the W_0N treatment. Annual soil N_2O flux was increased by 8.2 times and 3.0 times in the W_0N and WN treatments. Soil CO_2 flux was mainly affected by warming, while soil N_2O flux was mainly affected by N fertilization and its interaction with warming. The cumulative GWP from CO_2 and N_2O were 9984 ± 321 and 20.31 ± 3.02 kg CO_2 hm^{-2}, respectively (Table 2).

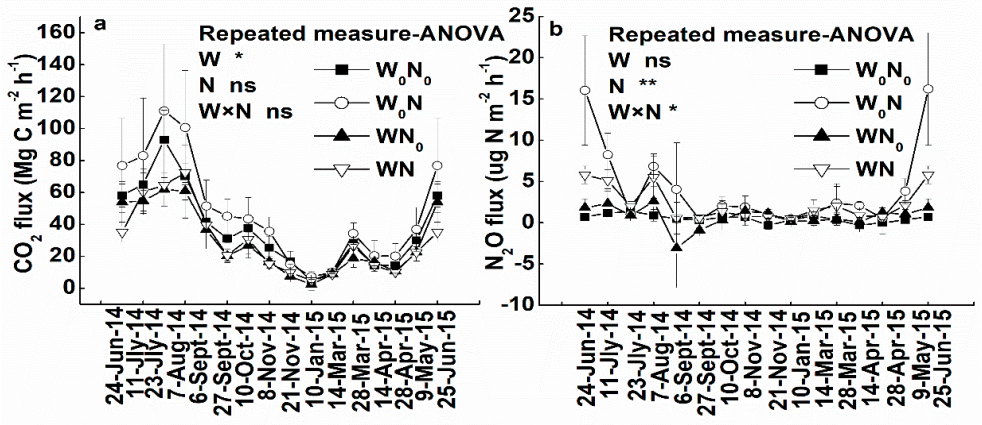

Figure 2. Seasonal changes of soil CO_2 (**a**) and N_2O fluxes (**b**) affected by warming and nitrogen fertilization. W_0N_0: Ambient temperature without nitrogen fertilization; W_0N: Ambient temperature with nitrogen fertilization; WN_0: Warming without nitrogen fertilization; WN: Warming with nitrogen fertilization.

Table 2. Mean annual fluxes of CO_2 (mg m^{-2} h^{-1}), N_2O (μg N m^{-2} h^{-1}) (means + SE) and the cumulative global warming potential (GWP) from CO_2 and N_2O fluxes (kg CO_2 hm^{-2} $year^{-1}$) as affected by treatments.

Variables	Treatment	CO_2	N_2O
Fluxes	W_0N_0	36.04 ± 3.77 ^{ab}	0.51 ± 0.11 ^{a}
	WN_0	27.90 ± 3.14 ^{a}	0.65 ± 0.27 ^{a}
	W_0N	46.08 ± 5.39 ^{b}	4.68 ± 1.61 ^{b}
	WN	29.07 ± 3.29 ^{a}	2.02 ± 0.32 ^{b}
GWP	W_0N_0	9984 ± 321 ^{ab}	20.31 ± 3.02 ^{a}
	WN_0	7800 ± 844 ^{a}	25.63 ± 10.33 ^{a}
	W_0N	12748 ± 2110 ^{b}	208.8 ± 56.37 ^{b}
	WN	8002 ± 282 ^{a}	79.88 ± 8.90 ^{b}
ANOVA (F values)	Warming	8.97 *	4.52
	N fertilization	1.64	17.47 **
	Warming * N fertilization	1.27	5.34 *

Different lowercase letters represent significant differences ($p < 0.05$) between the treatments analyzed by least-significant difference (LSD). Significant * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

3.3. Relationship between the Soil CO_2 and N_2O Fluxes and Environmental Factors

Soil CO_2 and N_2O fluxes increased exponentially with soil temperature across all treatments (Figure 3). The Q_{10} values for CO_2 flux were not significantly different among the control, W_0N, and WN_0 treatments, while Q_{10} in the WN treatment was increased to 5.54 compared to the control (3.94). The Q_{10} values for soil N_2O flux was increased by N fertilization without warming but was decreased by N fertilization with warming.

Soil CO_2 flux was positively correlated with soil MBC, DOC and the microbial substrates utilization, and negatively correlated with soil NH_4^+-N. Soil N_2O flux was positively correlated with the MSU and negatively correlated with soil NH_4^+-N (Figure 3).

3.4. Contributions of Soil Variables to Soil CO_2 and N_2O Fluxes

To quantify the relative importance of the different controlling factors on soil CO_2 and N_2O fluxes, two structural equation modellings (SEMs) were constructed based on the known relationships between soil CO_2 and N_2O fluxes and their key drivers in soil. The SEM showed a better fit to our hypothesized causal relationships ($\chi^2 = 2.82$, $p = 0.59$, RMSEA) = 0.000, Figure 4a; $\chi^2 = 0.81$, $p = 0.94$, RMSEA = 0.000, Figure 4b). The models accounted for 63% and 22% of the variance of soil CO_2 and N_2O fluxes, respectively. Microbial substrates utilization patterns had dominant direct negative effect on soil CO_2 flux and positive effect on N_2O (Figure 5). Soil NH_4^+-N had negative effects on soil CO_2 and N_2O fluxes. DOC and MBC had indirect positive effects on soil CO_2. In addition, soil NO_3^--N and NO_2^--N had indirect effects on soil N_2O.

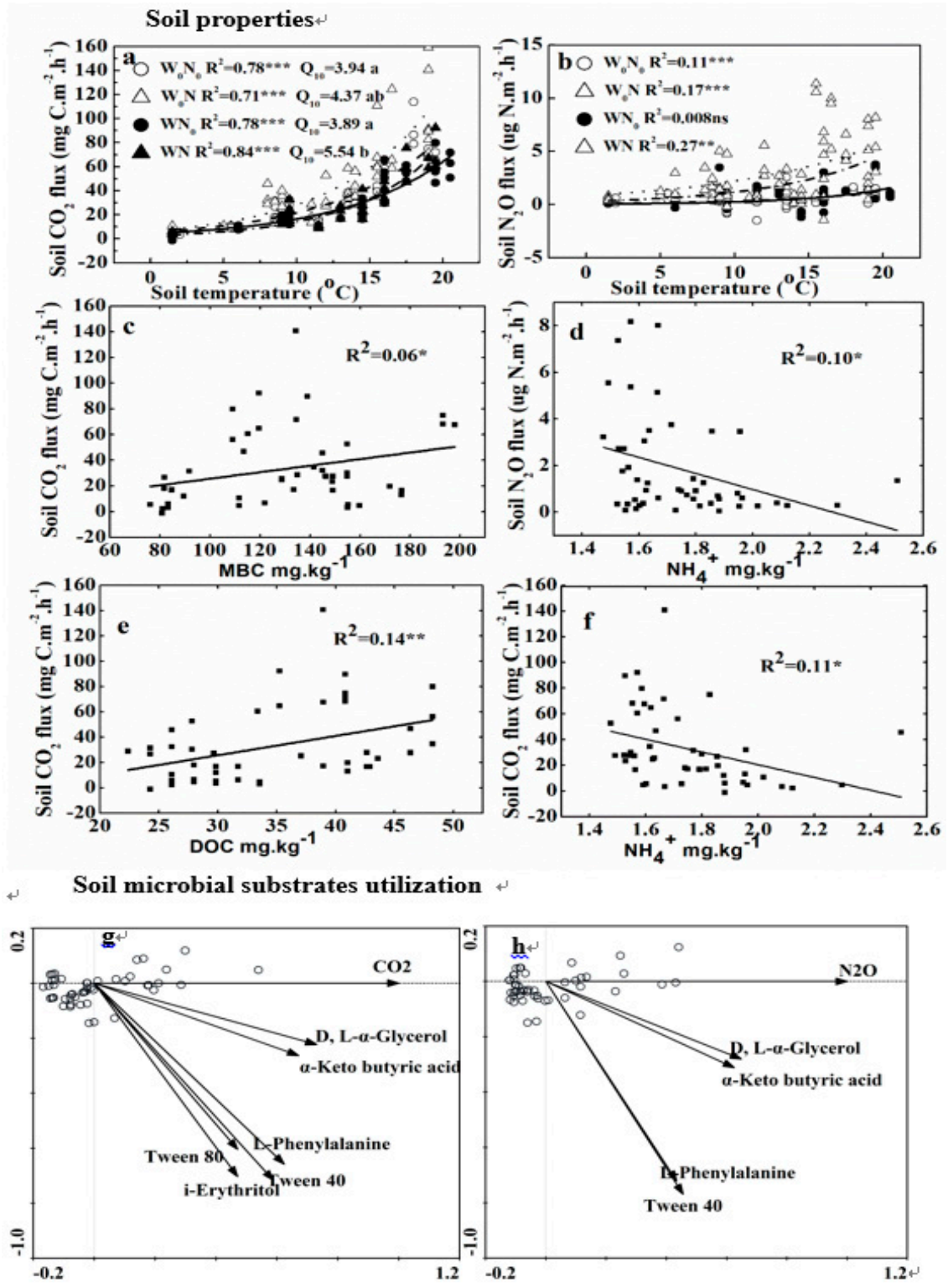

Figure 3. Relationships between the fluxes of soil CO_2 and soil temperature (**a**), MBC (**c**), DOC (**e**), soil NH_4^+ (**f**) and soil microbial substrates utilization (**g**), and between the fluxes of soil N_2O and soil temperature (**b**), soil NH_4^+ (**d**), and carbon utilization of microbial communities (**h**) in the different treatments. Q_{10} values with different lowercase letters indicate significant difference at $p < 0.05$. W_0N_0: Ambient temperature without nitrogen fertilization; W_0N: Ambient temperature with nitrogen fertilization; WN_0: Warming without nitrogen fertilization; WN: Warming with nitrogen fertilization. Different lowercase letters in Figure 3a represent significant differences ($p < 0.05$) between the treatments using least square difference (LSD) method. Significant * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

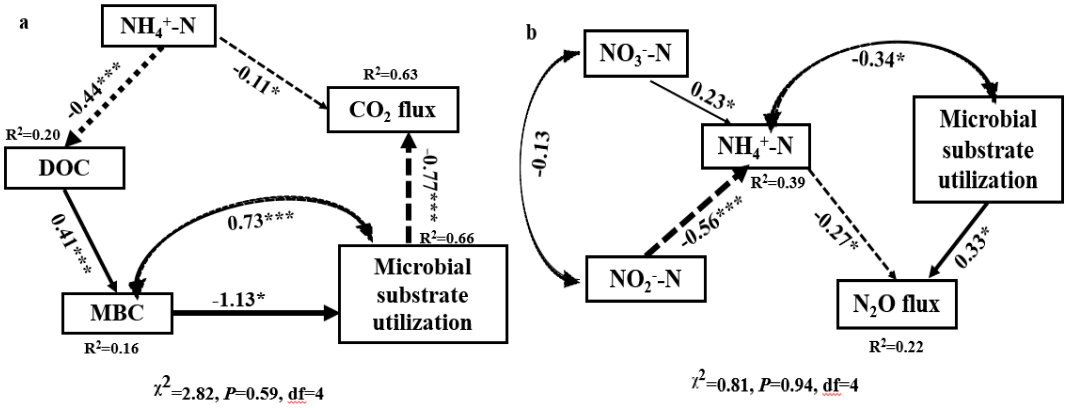

Figure 4. Result of structural equation modelling (SEM) to assess the direct and indirect effects of soil carbon, nitrogen, and microbial properties on soil CO_2 (**a**) and N_2O fluxes (**b**). Single-headed arrows indicate the hypothesized direction of causation. Double-headed arrows represent covariance between related variables. Arrow width is proportional to the strength of the relationship. The numbers adjacent to arrows are standardized path coefficient, which reflect the effect size of the relationship. R^2 value represent the proportion of variance explained for each endogenous variable. Significant * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

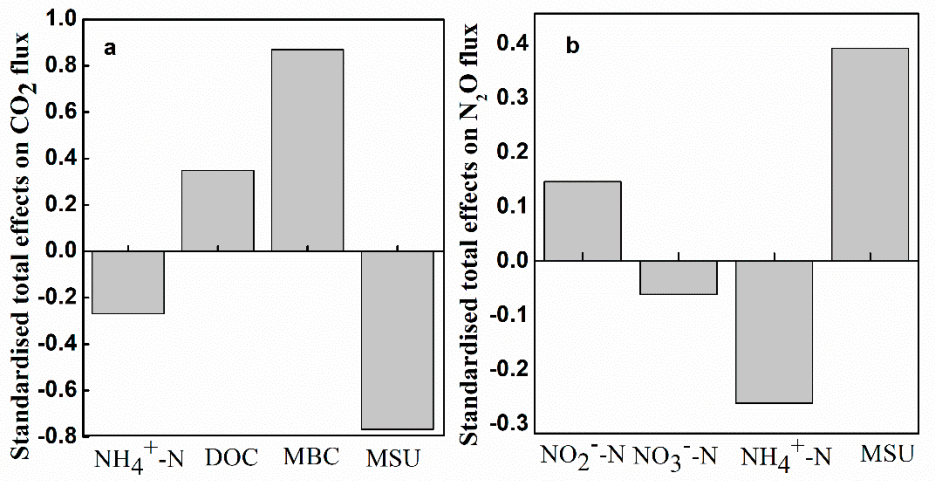

Figure 5. Standardized total effects of soil variables on soil CO_2 (**a**) and N_2O fluxes (**b**) derived from structural equation modelling (SEM). MSU: Microbial substrate utilizations; DOC: Dissolved organic C; MBC: Microbial biomass C.

4. Discussion

4.1. Effects of Warming and Nitrogen Fertilization on Soil CO_2 Flux

We found that warming decreased soil CO_2 flux, while the N fertilization and its interaction with warming had no significant effect on soil CO_2 flux (Figure 2a, Table 2). These results were quite different to some previous studies. For example, Zou et al. [5] and Xu et al. [37] found warming increases soil CO_2 flux in spruce forests, and the effect of N fertilization on soil CO_2 flux varied in forest plantations [12,38,39]. These differences were a consequence of the different soil properties and experimental conditions among these sites, as the interactions among climate, soil organisms, and vegetation, and the duration of experiment could influence soil CO_2 flux [40].

Carbon quality and quantity could regulate the responses of soil CO_2 flux to temperature. The decrease in soil respiration could be due to the consuming of labile C [41,42]. In this study, the experimental plots were filled with forest soil and spruce seedlings were planted in the plots. There was very limited C input compared to the forest plantation with mature trees. Consequently, the CO_2

emission could be restricted by less carbon in the soil [7]. Indeed, SOC and MBC at the site were lower after eight years of warming, although soil DOC was enhanced in this study. Bossio et al. [43] also found similar results. Although SEM analysis showed that soil DOC had a positive effect on soil CO_2 emission, the decreases in MBC had larger effect on soil CO_2 emission than the increases in DOC (Figures 4a and 5a). Overall, warming decreased soil CO_2 emission.

Climate warming and N fertilization studies have mostly focused on the changes of microbial processes (respiration and N mineralization) [25,26]. Few studies have investigated the direct link of soil microbial community with soil CO_2 flux. In this study, we found that microbial substrate utilization patterns had a direct negative effect on soil CO_2 flux (Figures 4a and 5a). This result suggested that there is an association of soil microbial community composition with the response of soil CO_2 flux to warming and N fertilization, as different microbial communities had different sole substrate utilization patterns in the BIOLOG ECO-plate analysis [44]. The CCA analysis further showed that the MSU patterns were positively correlated with soil DOC and soil temperature in the CCA1 although they had contrast effects in the CCA2 (Figure 3). It suggested that climate warming could enhance the activity of the microbial community and the DOC, then reduce the quantity of SOC, and finally decrease soil CO_2 emission. A similar result was reported by Walker et al. [43] who found that permanent warming accelerates microbial activity and causes more carbon loss from soil, and the soil carbon loss in return reduces soil microbial biomass and constrains the influence of microbes on the ecosystem. In this study, warming decreased the microbial metabolic activity represented by AWCD and uniformity of microbial community. The result further suggested that warming induced a shift of microbial community structure from bacteria to fungi. Since fungi have lower growth rates than bacteria on BIOLOG plates, higher fungal dominance may have lower color development rate, resulting in lower AWCD [17]. Consistently, the higher ratio of MBC/MBN in the warmed plots indicated that warming enhanced the fungi as the microbial biomass C/N ratio has been used as an indicator of changes in microbial community structure [45]. Since fungi have greater C assimilation efficiency compared to bacteria, warming decreased the CO_2 release [17,46]. These findings highlighted the important contribution of soil microbial community to soil CO_2 emission.

Moreover, soil carbon quality and quantity and microbes, soil N had a significant effect on soil CO_2 flux. Previous studies showed that the soil N availability affects the soil C turnover by modifying microbial composition and activity or through its limitation on plant growth [47,48]. With sufficient C supply, an increase in N availability could stimulate the microbial activity, and accelerate SOC mineralization [49]. In this study, there was relatively a lack of soil C and no effect on microbial community induced by N fertilization. As a result, N fertilization did not affect the soil CO_2 flux. One surprising finding was that soil NH_4^+-N had a negative effect on soil CO_2 flux in this study (Figure 4a). The positive effect of soil NH_4^+ on soil CO_2 flux had been reported in temperate and subtropical forests [12,50]. The difference between our study and the previous studies may be attributed to the following two reasons. One reason was that spruce prefers to absorb soil NO_3^--N than soil NH_4^+-N [51]. As NH_4^+ was strongly absorbed and held to cation exchange sites of SOC and clay minerals, it would lead to declines in labile C compounds and increases in complex C compounds [50,52]. Thus, soil NH_4^+ had a negative effect on soil DOC as shown in the SEM (Figure 4a). The second reason was that soil NH_4^+ had a negative relationship with the microbial substrate utilization (Figure 4), tended to inhibit soil microbial activity and community composition, and resulted in a decrease in the decomposition of SOC [50]. Therefore, soil NH_4^+ had a negative effect on soil CO_2 flux in this study.

4.2. Effects of Warming and Nitrogen Fertilization on Soil N_2O Flux

Previous studies showed strong positive correlations between soil temperature and N_2O emission in temperate forests [53,54], but quite weak correlations in tropical forests [55,56]. In this study, we found that the soil N_2O emission was slightly positively correlated with the soil temperature and warming did not significantly affect soil N_2O flux in the subalpine plantation forest. However, applying N fertilization had a positive effect on soil N_2O emission. These results suggested that the soil N

condition rather than the temperature controls soil N_2O emission. Consistent with our study, other studies also found that soil N_2O emission increased with N addition in forests [57,58]. The reasons could be that high NO_3^- deposition provided additional N for denitrification and thus increased soil N_2O emission. In this study, the fertilizer as NH_4NO_3 was added into the soil and resulted in an increase in soil NO_3^-, but the SEM indicated that the soil NH_4^+ and NO_2^- were the key factors controlling soil N_2O emission and soil NO_3^- had little effect on soil N_2O. Furthermore, N fertilization had no effect on soil NH_4^+ and decreased soil NO_2^- which may result from enhanced nitrification of soil NH_4^+ and denitrification of soil NO_2^- by nitrifier. The resulting increase in soil N_2O emission, with the depletion of soil NH_4^+, was probably not due to plant uptake as spruce prefers to uptake soil NO_3^- than NH_4^+ [51]. In theory, inorganic N, as the substrate for nitrification and denitrification processes, should be positively correlated with soil N_2O emission regardless of N forms [14,57]. However, more soil NH_4^+ decreased the soil DOC (Figure 4a) and inhibited the soil microbiomes activity (Figure 4b). Since soil N_2O emission was positively correlated with soil CO_2 flux, soil NH_4^+ had the negative effect on soil N_2O emission.

Soil microbe is another factor controlling soil N_2O emission (Figure 4b). The analysis of SEM showed that the soil microbial substrate utilization pattern had a positive effect on soil N_2O emission, which provided direct information that the soil microbial activity controls the soil N_2O emission under global change. Several previous studies showed that climate change can impact N transformations and N_2O emissions via indirect effects on the abundance of different microbial populations and microbial community structure [9,59]. For instance, Cantarel et al. [9] showed a stronger correlation of N_2O fluxes with the soil denitrification activity and the nirK denitrifiers community. In this study, the method of the BIOLOG ECO plates identified soil microbial community and functional diversity mainly through carbon substrates, which may not be sensitive to N addition and may not directly reflect N transformation. Thus, MSU patterns was not affected by N fertilization in this study. Future study is needed to determine the relative importance of the specific microbial activities in nitrification and denitrification.

Furthermore, N condition and microbes, many other soil environmental factors such as soil moisture and soil pH may influence soil N_2O emission [60]. In this study, soil moisture was not influenced by treatments as plots were monitored and watered as frequently as needed to eliminate the effects of soil moisture induced by warming. Seasonal variation of soil N_2O flux could be influenced by soil moisture change. Soil pH varied slightly seasonally and among different treatments, and might not have a large influence on soil N_2O emission. In addition, soil moisture and soil pH mainly affect the soil N availability and soil microbial activity and then indirectly influence soil N_2O emission [60]. Thus, soil N condition and soil microbes were the main factors controlling soil N_2O emission.

5. Conclusions

Eight years after continuous warming and N fertilization in a subalpine spruce plantation forest, we found that soil CO_2 flux was decreased by warming while soil N_2O flux was significantly increased by N fertilization and its interaction with warming. Warming enhanced the DOC and MSU pattern, reduced SOC and MBC, and further constrained the metabolic potential of soil microbes, uniformity index of microbial communities, and finally resulted in a decrease in soil CO_2 emission. For soil N_2O emission, the MSU pattern and soil NO_2^- had positive effects on soil N_2O flux, while the soil NH_4^+ had a negative effect on soil N_2O emission. Both for soil CO_2 flux and N_2O flux, the microbes played a more important role than other factors. This study revealed different response patterns and controls of soil CO_2 and N_2O fluxes in the subalpine plantation forest under climate warming and N deposition, and further highlighted the important contributions of soil microbes to GHG fluxes.

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