Differential Responses and Controls of Soil CO₂ and N₂O 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 CO₂ and N₂O 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 CO₂ and N₂O 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 CO₂ and N₂O 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 CO₂ and N₂O 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 CO₂ flux was only affected by warming while soil N₂O 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 CO₂ emission. The analysis of structural equation model indicated that MSU had dominant direct negative effect on soil CO₂ flux but had direct positive effect on soil N₂O flux. DOC and MBC had indirect positive effects on soil CO₂ flux while soil NH₄⁺-N had direct negative effect on soil CO₂ and N₂O fluxes. This study revealed different response patterns and controlling factors of soil CO₂ and N₂O 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$_2$O) 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$_2$O flux by enhancing decomposition and N mineralization [8,9], however, it may not influence soil N$_2$O 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$_2$O emissions in the first year, but shifts to soil N$_2$O 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$_2$O 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$_2$O 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$_2$O 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$_2$O 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$_2$O 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$_2$O 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 CO₂, N₂O 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 CO₂ and N₂O 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 CO₂ and N₂O 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 CO₂ and N₂O Fluxes Measurements

Soil CO₂ and N₂O 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 CO₂ and N₂O 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:

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

where $F$ is the gas flux ($\mu$g N m$^{-2}$ h$^{-1}$ for N$_2$O 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$_2$Cr$_2$O$_7$-H$_2$SO$_4$ wet digestion method [31]. After digestion with K$_2$Cr$_2$O$_7$-H$_2$SO$_4$, FeSO$_4$ was used to titrate the remaining K$_2$Cr$_2$O$_7$ in the digestion solution and SOC was calculated based on the consumptions of the K$_2$Cr$_2$O$_7$. The dissolve organic C (DOC) was measured using the K$_2$Cr$_2$O$_7$-H$_2$SO$_4$ 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$_2$SO$_4$ 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$_2$SO$_4$ 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.
\[ H = -\sum p_i \ln p_i \]  

\[ U = \sqrt{\left(\sum n_i^2\right)} \]

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):

\[ F = ae^{bT} \]

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:

\[ Q_{10} = e^{10b} \]

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\(_2\)O (cumulative N\(_2\)O 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\(_2\)O 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\(_2\)O fluxes using the Amos 24.0 software package (IBM, New York, NY, USA). We first tested the relationships between the CO\(_2\) and N\(_2\)O 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\(_2\)O 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 (\( \chi^2 \)), \( P \)-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>
<thead>
<tr>
<th>Treatments</th>
<th>NO$_3^-$-N</th>
<th>NH$_4^+$-N</th>
<th>NO$_2^-$-N</th>
<th>TN</th>
<th>SOC</th>
<th>DOC</th>
<th>MBC</th>
<th>MBN</th>
<th>MBC/MBN</th>
<th>AWCD</th>
<th>H</th>
<th>U</th>
</tr>
</thead>
<tbody>
<tr>
<td>Season (S)</td>
<td>&lt;0.001</td>
<td>&lt;0.001</td>
<td>&lt;0.001</td>
<td>&lt;0.001</td>
<td>&lt;0.001</td>
<td>&lt;0.001</td>
<td>&lt;0.001</td>
<td>&lt;0.001</td>
<td>&lt;0.001</td>
<td>0.001</td>
<td>&lt;0.001</td>
<td>0.010</td>
</tr>
<tr>
<td>Warming (W)</td>
<td>&lt;0.001</td>
<td>0.756</td>
<td>&lt;0.001</td>
<td>&lt;0.001</td>
<td>&lt;0.001</td>
<td>&lt;0.001</td>
<td>&lt;0.001</td>
<td>&lt;0.001</td>
<td>&lt;0.001</td>
<td>0.009</td>
<td>0.461</td>
<td>0.006</td>
</tr>
<tr>
<td>Nitrogen (N)</td>
<td>&lt;0.001</td>
<td>0.103</td>
<td>&lt;0.001</td>
<td>0.009</td>
<td>&lt;0.001</td>
<td>&lt;0.001</td>
<td>&lt;0.001</td>
<td>&lt;0.001</td>
<td>&lt;0.001</td>
<td>0.237</td>
<td>0.953</td>
<td>0.276</td>
</tr>
<tr>
<td>S × W</td>
<td>&lt;0.001</td>
<td>0.322</td>
<td>&lt;0.001</td>
<td>&lt;0.001</td>
<td>&lt;0.001</td>
<td>&lt;0.001</td>
<td>&lt;0.001</td>
<td>&lt;0.001</td>
<td>&lt;0.001</td>
<td>&lt;0.001</td>
<td>0.032</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>S × N</td>
<td>&lt;0.001</td>
<td>0.008</td>
<td>0.040</td>
<td>0.365</td>
<td>&lt;0.001</td>
<td>&lt;0.001</td>
<td>&lt;0.001</td>
<td>&lt;0.001</td>
<td>&lt;0.001</td>
<td>0.002</td>
<td>0.013</td>
<td>0.006</td>
</tr>
<tr>
<td>W × N</td>
<td>0.565</td>
<td>0.011</td>
<td>0.059</td>
<td>0.015</td>
<td>&lt;0.001</td>
<td>0.002</td>
<td>0.003</td>
<td>&lt;0.001</td>
<td>&lt;0.001</td>
<td>0.016</td>
<td>0.115</td>
<td>0.032</td>
</tr>
<tr>
<td>S × W × N</td>
<td>&lt;0.001</td>
<td>0.209</td>
<td>&lt;0.001</td>
<td>0.080</td>
<td>&lt;0.001</td>
<td>&lt;0.001</td>
<td>&lt;0.001</td>
<td>&lt;0.001</td>
<td>&lt;0.001</td>
<td>0.001</td>
<td>0.002</td>
<td>0.004</td>
</tr>
</tbody>
</table>
The mean annual CO$_2$ and N$_2$O 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$_0$N$_0$), 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$_0$N treatment. Annual soil N$_2$O flux was increased by 8.2 times and 3.0 times in the W$_0$N and WN treatments. Soil CO$_2$ flux was mainly affected by warming, while soil N$_2$O flux was mainly affected by N fertilization and its interaction with warming. The cumulative GWP from CO$_2$ and N$_2$O were 9984 ± 321 and 20.31 ± 3.02 kg CO$_2$ hm$^{-2}$, respectively (Table 2).

3.2. Soil CO$_2$ and N$_2$O Fluxes

The highest soil CO$_2$ and N$_2$O fluxes occurred in August and the lowest in January (Figure 2). The mean annual CO$_2$ and N$_2$O 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$_0$N$_0$), 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$_0$N treatment. Annual soil N$_2$O flux was increased by 8.2 times and 3.0 times in the W$_0$N and WN treatments. Soil CO$_2$ flux was mainly affected by warming, while soil N$_2$O flux was mainly affected by N fertilization and its interaction with warming. The cumulative GWP from CO$_2$ and N$_2$O were 9984 ± 321 and 20.31 ± 3.02 kg CO$_2$ hm$^{-2}$, respectively (Table 2).

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$_0$N$_0$: Ambient temperature without nitrogen fertilization; W$_0$N: Ambient temperature with nitrogen fertilization; WN$_0$: Warming without nitrogen fertilization; WN: Warming with nitrogen fertilization. Environmental variables in CCA1 and CCA2 explain 93% and 95%, respectively.

Figure 2. Seasonal changes of soil CO$_2$ (a) and N$_2$O fluxes (b) affected by warming and nitrogen fertilization. W$_0$N$_0$: Ambient temperature without nitrogen fertilization; W$_0$N: 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$_2$O (µg N m$^{-2}$ h$^{-1}$) (means ± SE) and the cumulative global warming potential (GWP) from CO$_2$ and N$_2$O fluxes (kg CO$_2$ hm$^{-2}$ year$^{-1}$) as affected by treatments.

<table>
<thead>
<tr>
<th>Variables</th>
<th>Treatment</th>
<th>CO$_2$</th>
<th>N$_2$O</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fluxes</td>
<td>$W_0N_0$</td>
<td>36.04 ± 3.77$^a$</td>
<td>0.51 ± 0.11$^a$</td>
</tr>
<tr>
<td></td>
<td>$WN$</td>
<td>27.90 ± 3.14$^a$</td>
<td>0.65 ± 0.27$^a$</td>
</tr>
<tr>
<td></td>
<td>$W_0N$</td>
<td>46.08 ± 5.39$^b$</td>
<td>4.68 ± 1.61$^b$</td>
</tr>
<tr>
<td></td>
<td>$WN$</td>
<td>29.07 ± 3.29$^a$</td>
<td>2.02 ± 0.32$^b$</td>
</tr>
<tr>
<td>GWP</td>
<td>$W_0N_0$</td>
<td>9984 ± 321$^a$</td>
<td>20.31 ± 3.02$^a$</td>
</tr>
<tr>
<td></td>
<td>$WN$</td>
<td>7800 ± 844$^a$</td>
<td>25.63 ± 10.33$^a$</td>
</tr>
<tr>
<td></td>
<td>$W_0N$</td>
<td>12748 ± 2110$^b$</td>
<td>208.8 ± 56.37$^b$</td>
</tr>
<tr>
<td></td>
<td>$WN$</td>
<td>8002 ± 282$^a$</td>
<td>79.88 ± 8.90$^b$</td>
</tr>
</tbody>
</table>

ANOVA (F values)

<p>| | | | |</p>
<table>
<thead>
<tr>
<th></th>
<th></th>
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<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>N fertilization</td>
<td>1.64</td>
<td>17.47**</td>
<td></td>
</tr>
<tr>
<td>Warming × N fertilization</td>
<td>1.27</td>
<td>5.34*</td>
<td></td>
</tr>
</tbody>
</table>

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$_2$O Fluxes and Environmental Factors

Soil CO$_2$ and N$_2$O 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 $WN$ 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$_2$O 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$_2$O 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$_2$O Fluxes

To quantify the relative importance of the different controlling factors on soil CO$_2$ and N$_2$O fluxes, two structural equation modellings (SEMs) were constructed based on the known relationships between soil CO$_2$ and N$_2$O 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$_2$O fluxes, respectively. Microbial substrates utilization patterns had dominant direct negative effect on soil CO$_2$ flux and positive effect on N$_2$O (Figure 5). Soil NH$_4^+$-N had negative effects on soil CO$_2$ and N$_2$O 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$_2$O.
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$_2$O 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 $p < 0.05$. W0N0: Ambient temperature without nitrogen fertilization; W0N: Ambient temperature with nitrogen fertilization; WN0: 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 * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$. 
Forests 2019, 10, 808

Figure 4. Result of structural equation modelling (SEM) to assess the direct and indirect effects of soil carbon, nitrogen, and microbial properties on soil CO2 (a) and N2O 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 CO2 (a) and N2O 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 CO2 Flux

We found that warming decreased soil CO2 flux, while the N fertilization and its interaction with warming had no significant effect on soil CO2 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 CO2 flux in spruce forests, and the effect of N fertilization on soil CO2 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 CO2 flux [40].

Carbon quality and quantity could regulate the responses of soil CO2 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 CO2
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 CO2 emission, the decreases in MBC had larger effect on soil CO2 emission than the increases in DOC (Figures 4a and 5a). Overall, warming decreased soil CO2 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 CO2 flux. In this study, we found that microbial substrate utilization patterns had a direct negative effect on soil CO2 flux (Figures 4a and 5a). This result suggested that there is an association of soil microbial community composition with the response of soil CO2 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 CO2 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 CO2 release [17,46]. These findings highlighted the important contribution of soil microbial community to soil CO2 emission.

Moreover, soil carbon quality and quantity and microbes, soil N had a significant effect on soil CO2 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 CO2 flux. One surprising finding was that soil NH4+-N had a negative effect on soil CO2 flux in this study (Figure 4a). The positive effect of soil NH4+ on soil CO2 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 NO3−-N than soil NH4+-N [51]. As NH4+ 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 NH4+ had a negative effect on soil DOC as shown in the SEM (Figure 4a). The second reason was that soil NH4+ 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 NH4+ had a negative effect on soil CO2 flux in this study.

4.2. Effects of Warming and Nitrogen Fertilization on Soil N2O Flux

Previous studies showed strong positive correlations between soil temperature and N2O emission in temperate forests [53,54], but quite weak correlations in tropical forests [55,56]. In this study, we found that the soil N2O emission was slightly positively correlated with the soil temperature and warming did not significantly affect soil N2O flux in the subalpine plantation forest. However, applying N fertilization had a positive effect on soil N2O emission. These results suggested that the soil N
condition rather than the temperature controls soil \( \text{N}_2\text{O} \) emission. Consistent with our study, other studies also found that soil \( \text{N}_2\text{O} \) 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 \( \text{N}_2\text{O} \) emission. In this study, the fertilizer as NH\(_4\)NO\(_3\) 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 \( \text{N}_2\text{O} \) emission and soil NO\(_3^-\) had little effect on soil \( \text{N}_2\text{O} \). 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\(_3^-\) by nitrifier. The resulting increase in soil \( \text{N}_2\text{O} \) 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 \( \text{N}_2\text{O} \) 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 \( \text{N}_2\text{O} \) emission was positively correlated with soil CO\(_2\) flux, soil NH\(_4^+\) had the negative effect on soil \( \text{N}_2\text{O} \) emission.

Soil microbes are another factor controlling soil \( \text{N}_2\text{O} \) emission (Figure 4b). The analysis of SEM showed that the soil microbial substrate utilization pattern had a positive effect on soil \( \text{N}_2\text{O} \) emission, which provided direct information that the soil microbial activity controls the soil \( \text{N}_2\text{O} \) emission under global change. Several previous studies showed that climate change can impact N transformations and \( \text{N}_2\text{O} \) 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 \( \text{N}_2\text{O} \) 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 \( \text{N}_2\text{O} \) 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 \( \text{N}_2\text{O} \) 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 \( \text{N}_2\text{O} \) emission. In addition, soil moisture and soil pH mainly affect the soil N availability and soil microbial activity and then indirectly influence soil \( \text{N}_2\text{O} \) emission [60]. Thus, soil N condition and soil microbes were the main factors controlling soil \( \text{N}_2\text{O} \) 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 \( \text{N}_2\text{O} \) 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 \( \text{N}_2\text{O} \) emission, the MSU pattern and soil NO\(_2^-\) had positive effects on soil \( \text{N}_2\text{O} \) flux, while the soil NH\(_4^+\) had a negative effect on soil \( \text{N}_2\text{O} \) emission. Both for soil CO\(_2\) flux and \( \text{N}_2\text{O} \) flux, the microbes played a more important role than other factors. This study revealed different response patterns and controls of soil CO\(_2\) and \( \text{N}_2\text{O} \) 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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