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Forest Gaps Slow the Humification Process of Fir (*Abies faxoniana* Rehder & E.H.Wilson) Twig Litter during Eight Years of Decomposition in an Alpine Forest

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Abstract: Litter humification plays a crucial role in organic matter formation and soil carbon sequestration in forest ecosystems. However, how forest gap formation and gap size variation affect the litter humification process remains poorly understood. An eight-year in situ decomposition experiment was conducted to evaluate humus accumulation (humic substances, humic and fulvic acid), humification degrees, humification ratios and optical properties (ΔlogK, E4/E6 and A600/C) of Minjiang fir (*Abies faxoniana* Rehder & E.H.Wilson) twig litter in four gap size treatments in an alpine primitive forest on the eastern Tibetan Plateau, including (1) closed canopies, (2) small gaps (38–46 m^2 in size), (3) medium gaps (153–176 m^2 in size),and (4) large gaps (255–290 m^2 in size). The results indicated that the accumulation of humic substances and humic acid in the closed canopies was significantly higher than that in the large gaps during the first two years of decomposition. After eight years of decomposition, there were significant differences in the humic substance accumulations and the values of ΔlogK and A_{600}/C among the different gap sizes. Furthermore, twig litter was humified in the first 2 years of incubation, and the net accumulation of humic substances was ranged from −23.46% to −44.04% of the initial level at the end of the experiment. The newly accumulated humus was young (mature (type Rp) humus) and transformed to mature (type A) humus after 4–6 years of decomposition. Partial least squares (PLS) suggested that gap-induced variations in twig litter chemistry (i.e., contents of cellulose, lignin, nitrogen (N) and phosphorus (P), and the ratios of C/N N/P) mainly drove the process of twig litter humification. Our results presented here denote that the formation of forest gaps retard twig litter humification process, which might be detrimental to carbon sequestration in the alpine forest ecosystems.

Keywords: gap size; twig litter; humification; humic substances; optical properties; alpine forest

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1. Introduction

Litter humification is a crucial process for the soil organic matter (SOM) formation and fertility, which is important to soil carbon sequestration in terrestrial ecosystems [1–3]. Similar to litter decomposition process, litter humification is suggested to be regulated by climatic factors (i.e., moisture and temperature), vegetation community (i.e., species composition and community structure), substrate quality (i.e., litter chemistry), soil properties (i.e., pH and parent material) and soil decomposers (fauna and microorganisms) [4–7]. Among them, climate conditions, vegetation community and soil properties are distal factors affecting the formation of humus forms, while plant roots and litter and soil decomposers are the agents and sources [8–10]. In forest ecosystems, gaps formation may have critical repercussions on litter humification [11–14]. Compared to the climatic factors in forest opening areas, lower wind speed and less direct solar radiation lead to temperature and moisture variations that are dampened under or within tree canopies [15]. These alterations influence the specific environments controlling the interactions between plants and soil organisms [9], and thereby alter the functional communities of soil invertebrates and microbes, litter chemistry and soil nutrient availability to a considerable extent [11,16]. As a result, gap formation has the potential to not only directly affect the degree and rate of litter humification at a relatively small spatial scale by modulating the climatic conditions [3,17], but also indirectly alter the humification process by modifying the litter quality, decomposing organisms and soil nutrient availability [9,18–20].

In the alpine forests, litter humification is limited by low temperatures [21]. Forest gap formation can change litterfall input, solar radiation, and precipitation in the growing season and the distribution of snow cover in winter, forming heterogeneous microclimates from the gap centre areas to the closed canopies [22,23]. These changes may result in different patterns of litter humification between the gap centres and closed canopies in different seasons [24,25]. In winter, snow cover and freeze-thaw cycles are believed to be vital factors affecting the entire litter humification process [3,20]. A thicker snowpack has a relatively better thermal insulation effect in forest gaps without canopy shelter, providing suitable conditions for soil organism activities under snow [26]. The higher temperature and moisture in soil after snow melting is conducive to increases in the abundance and activity of microorganisms, which may stimulate the rapid release of labile components and lead to variations in chemical properties in litter [27,28]. Litter with more refractory substances remained in gaps, as the main precursors of humus [2,29], polymerized to humic substances with more complex conjugate structures and promoted the accumulation of stable SOM [9,24]. In contrast, the low temperature in closed canopy is generally not conducive to decomposer activities [21,30], while the high freezing-thawing cycle frequency may promote the physical degradation of formed humus [3]. In the subsequent growing season, more solar radiation in forest gap centres can lead to heat stress or desiccation of decomposers and may restrict litter humification mediated via microbial metabolic activity [5,31]. In addition, humus undergoes photodegradation upon the effect of UV radiation, and the molecular weights decrease with the destruction of the aromatic structure [32,33]. Therefore, the formation of gaps and the increase in size may promote humus accumulation and stabilization in winter and inhibit them in the growing season. However, the long-term humification dynamics of plant litter in gaps and closed canopies are still unclear.

Litter humification is a complex process for forming a mixture of microbial and plant polymers and their degradation products under the combined action of various biotic and abiotic factors [34–36]. Litter quality, especially the litter chemistry (e.g., N content and the radios of C/N and lignin/N), can affect the humification process and regulate the type and formation rate of humus at regional and local scales [3,37,38]. The accumulation of humus and the formation of stable organic matter have been demonstrated to be associated with litter degradability. In recent years, labile compounds (i.e., dissolved organic carbon and acid hydrolysable fiber) in litter have been shown to promote humic substance formation by improving microbial carbon-use efficiency and bonding to mineral surfaces, which challenged the traditional assumption that SOM forms from the fragmentation and condensation of partially decomposed recalcitrant litter material (e.g., acid unhydrolyzable fiber) [39]. Furthermore, some studies have indicated that SOM consists of particulate and mineral-associated organic matter and that different components of litter drive SOM formation via biochemical and physical pathways [34]. Nonstructural compound depolymerization and sorption or the microbial products associated with minerals may form SOM and reside in soil for a longer time [10,39]. Therefore, litter with high labile compounds may be more conducive to humus formation and stabilization [40,41]. A previous study showed that forest gap formation accelerated foliar litter humification [24]. Compared to leaf litter, twig litter contains more recalcitrant components (i.e., lignin) and less nutrient (i.e., N and P) material [29], which may contribute to the lower accumulation of humus.

Generally, more than 50% of the aboveground net primary productivity of forest ecosystems is returned to the soil in the form of plant litter [42]. Twig litter is the second major component of plant litter and accounts for approximately 26% of the total litterfall in forest ecosystems [2]. In the alpine forests of Southwest China, the forest canopy is mainly composed of fir (*Abies faxoniana* Rehder & E.H.Wilson), and approximately 363 kg·hm^{-2} of twig litter is returned to the forest floor each year [23,43], constituting an important source of SOM. To understand twig litter humification in different gap sizes, an eight-year field experiment based on the litterbag method was conducted in an original fir forest on the eastern Tibetan Plateau of China. We placed litterbags at four gap size treatments, including (1) closed canopies, (2) small gaps (38–46 m^2 in size), (3) medium gaps (153–176 m^2 in size), and (4) large gaps (255–290 m^2 in size). We hypothesized that: (i) forest gaps formation might accelerate twig litter humification due to the more suitable microclimate and biological environment in forest gaps and (ii) the accumulation and stabilization of twig litter humic substances may decrease with an increase from the small gap to the large gap.

2. Materials and Methods

2.1. Study Site

This study was conducted in a primitive fir (*Abies faxoniana*) forest at the Long-term Research Station of Alpine Forest Ecosystems in Miyaluo Nature Reserve (31°15.88′ N, 102°54.72′ E, 3582 m a.s.l.) in Southwest China (Figure 1A). The climate in this region is a temperate mountain monsoon climate with a mean annual air temperature ranging from 2 to 4 °C and a mean annual precipitation of 850 mm [23,44]. The snow cover typically starts in late October and lasts 5–6 months, with a maximum snow depth of approximately 50 cm [23,44]. The forest canopies are mainly composed of fir (Figure 1B), and the understorey vegetation includes azalea (*Rhododendron lapponicum*), bamboo (*Fargesia nitida*) and dwarf willow (*Salix paraplesia*) as well as some herbs (e.g., *Carex* spp., *Cystopteris montana*, and *Cacalia* spp.). The soil is classified as Cambic Umbrisol [45]. The soil pH and average concentrations of total C, N and P are 6.1, 160.24 g/kg, 58.02 g/kg and 1.70 g/kg in the organic layer, and 5.7, 45.2 g/kg, 1.9 g/kg and 0.7 g/kg in the mineral layer, respectively [46] (Figure 1C).

2.2. Experimental Design

The forest gaps in this experiment were defined as the concept of forest expanded gaps, the sizes of which were determined by the area of the trunk bases of border trees, and the maximum size was approximately 280 m^2 [23,44,47]. Therefore, four gap size classes were chosen in a fir forest, including (1) closed canopies, (2) small gaps (38–46 m^2 in size) with a diameter < 10 m, (3) medium gaps (153–176 m^2 in size) with a diameter of 10–15 m, and (4) large gaps (255–290 m^2 in size) with a diameter of 15–20 m, to explore the effects of forest gaps on the humification process of fir twig litter (Figure 1B). Additional details on the original fir forest can be found in [23,44].

An in situ litterbag incubation study was conducted in three replicated plots (25 m × 25 m in size) with similar topographies and patterns and durations of gap formation in the fir forest [23,44]. From May to September 2012, newly fallen fir twig litter was collected by litter traps with a diameter of 1 m, and then twigs were air-dried at room temperature and uniformly cut into pieces with a length of 5 cm [23,44] (Figure 1A). Ten ± 0.05 g of air-dried twig litter was transferred into nylon litterbags measuring 20 × 25 cm with mesh sizes of 1.0 mm on the top and 0.5 mm on the bottom [23,44]. A total of 800 litterbags were prepared and moved in the forest gaps and the closed canopies floors on November 15 of that year [23]. Litterbags were in the gap centres and in the closed canopies to avoid decomposition microenvironment differences between the southern and northern edges of the gap centre [23,44]. These litterbags were strung together at 2–5 cm intervals in each subplot and placed on the forest floor. We collected five of them randomly to determine the initial twig litter chemistry during sample establishment [23]. Moreover, twig litter surface temperatures were measured by data loggers (iButton DS1923-F5, Sunnyvale, CA, USA). The snow thickness was manually measured by a steel measuring tape in winter [23].

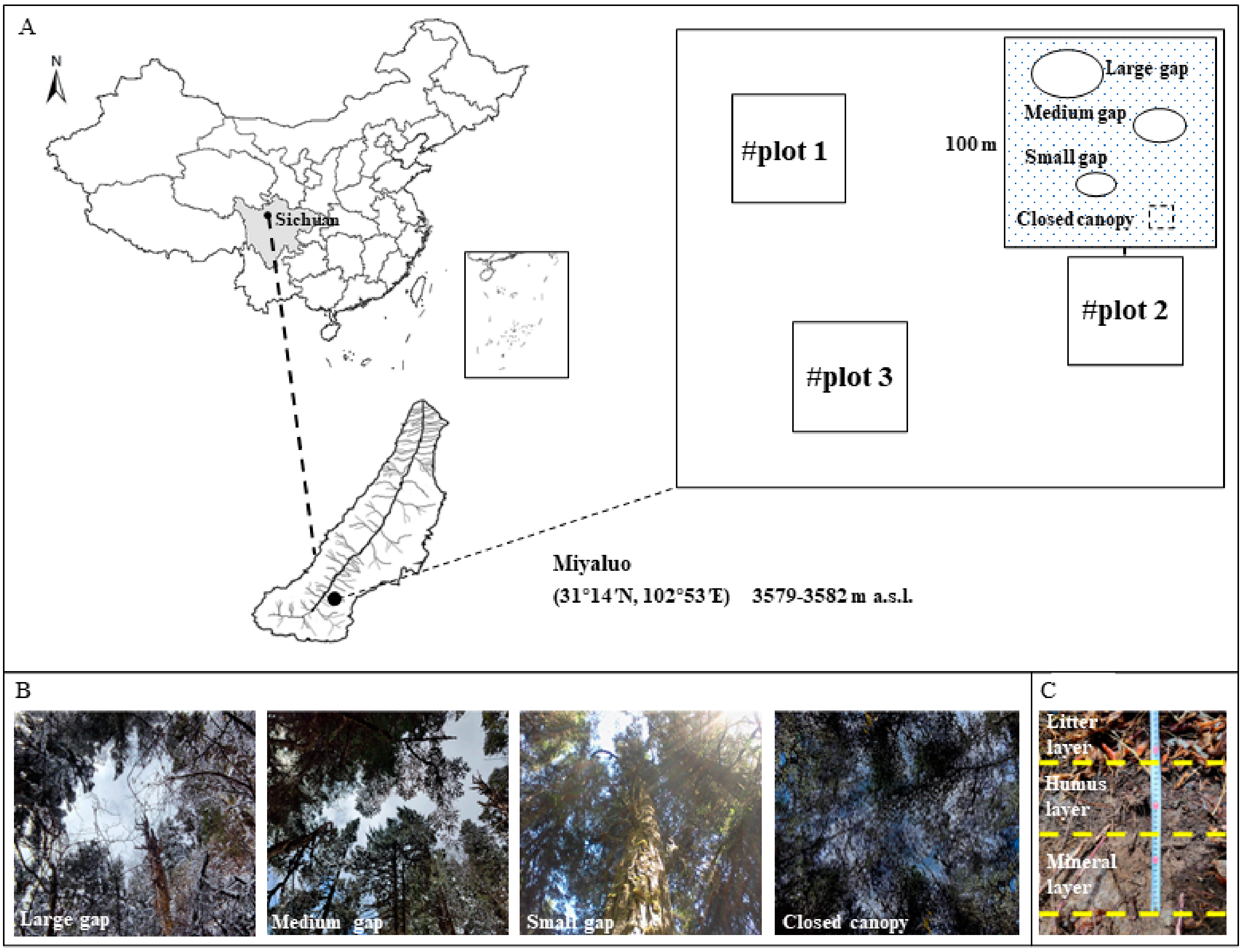

Figure 1. Schematic map of the study site. (**A**) displays the location of our study area and sampling sites. (**B**) shows photos of four gap size treatments in the experiment design, including large gap with a size of 255–290 m^2, medium gaps with a size of 153–176 m^2, small gaps with a size of 38–46 m^2 and closed canopies, and (**C**) shows the soil photo of the sampling plot.

2.3. Sample Collection and Chemical Analysis

From November 2012 to October 2020, the field decomposition experiment had completed 13 sampling events and was run for 8 years. The sampling schedule is described in elsewhere [23]. On each sampling date, we collected four litterbags randomly from each subplot and transferred them to the laboratory. In this experiment, the humus accumulation, humification degrees, humification ratios and optical properties of twig litter were evaluated over a two-year sampling interval.

For each subplot, three litterbags were oven-dried (105 °C, 48 h) to determine the remaining mass after removing soils, fine roots and debris from the litterbags at each specific sampling event [44]. The oven-dried twig litters were milled and screened through a 60 mesh sieve for subsequent chemical analyses. The humic substances and humic acid and fulvic acid concentrations were determined according to the alkali method [48]. Although the method itself has some limitations [49], it can quantitatively evaluate humus accumulation in the litter decomposition process to some extent [17]. In brief, the dried samples (1.0 g) were extracted with sodium pyrophosphate and NaOH. Humic substance, humic and fulvic acid contents were measured by a TOC analyser (multi N/C 2100; Analytik Jena, Th€uringen, Germany). Moreover, humus substances can decolorize during degradation, and the compositions of humic substances with a darker colour indicate greater stability and a high resistance to biodegradation [25,50]. In general, the E4/E6 ratio (465/665 nm ratio of absorption) is related to the degree of condensation of the aromatic carbon (C) composition, and ΔlogK (400/ 600 nm ratio of absorption on a logarithmic scale) is almost linear [13]. A_{600}/C (absorbance at 600 nm per mg of C per ml of extraction) could also indicate the humification degree and aromaticity degree [51]. Hence, the optical properties of the alkaline solutions extracted were measured at the four bands of 400, 600, 465, and 665 nm with an ultraviolet spectrophotometer (TU-1901, Puxi, Beijing, China) to assess the conjugation structural changes in the macromolecules of alkaline extracted humic substances [52,53]. The lower ΔlogK and E4/E6 values and the higher A_{600}/C suggested a higher degree of humification [13]. Furthermore, the ratio of A_{600}/C and the ΔlogK values were used to evaluate humic substance types, and A_{600}/C values of 2.5 and 5.0 were used as thresholds to determine type Rp (Rp1, Rp2), P, B and A [14,25,51], with the humification degree increasing in this order. In addition, the concentrations of total C, N and P were determined using dichromate oxidation ferrous sulphate titration, the Kjeldahl method and the molybdenum-blue colorimetric method, respectively [54]. Lignin and cellulose concentrations were determined by the acid detergent lignin method [55]. All litter samples were analysed in triplicate.

2.4. Data Calculations and Statistical Analyses Statistical Analyses and Calculations

The accumulation of humic substances (HS, g), humic acid (HA, g) and fulvic acid (FA, g), humification degree (HD, %) and humification ratio (HR, %) were calculated for the twig litter as follows [13,48]:

$$\mathrm{FA_C} = \mathrm{HS_C} - \mathrm{HA_C} \tag{1}$$

$$\mathrm{HS} = \mathrm{HS_C} \times \mathrm{M} \tag{2}$$

$$\mathrm{HA} = \mathrm{HA_C} \times \mathrm{M} \tag{3}$$

$$\mathrm{FA} = \mathrm{FA_C} \times \mathrm{M} \tag{4}$$

$$\mathrm{HD} = \mathrm{HS_C}/\mathrm{OC} \tag{5}$$

$$\mathrm{HR} = \mathrm{HD}/\mathrm{D_t} \tag{6}$$

where HS_C, HA_C and FA_C are the concentrations of humic substance, humic acid and fulvic acid at each sampling period, respectively; M indicates the remaining litter mass at each sampling time; OC is the organic carbon concentration at each sampling time; and D_t is the year of the sampling interval.

To determine the net accumulation of humic substance dynamics of decomposing twig litter, the accumulation of humic substance (HS_{acc}), humic acid (HA_{acc}), and fulvic acid (FA_{acc}) were calculated for the litter as follows [3]:

$$\mathrm{H_{acc}}(\%) = \mathrm{H_t}/\mathrm{H_0} \times 100 \tag{7}$$

where H_0 and H_t are the initial accumulation of the humic substances, humic and fulvic acid at each sampling period, respectively.

The values of ΔlogK, E4/E6 and A_{600}/C of the alkaline solutions extracted for the litter were calculated as follows [51]:

$$\Delta\mathrm{logK} = \mathrm{logA_{400}}/\mathrm{A_{600}} \tag{8}$$

$$E4/E6 = A_{465}/A_{665} \quad (9)$$

where A_{400}, A_{600}, A_{465} and A_{665} represent the absorbance values at 400, 600, 465 and 665 nm, respectively. The C of A_{600}/C is the mg carbon per ml extraction.

The snow depth (SD), mean temperature (MT), positive and negative accumulated temperature (PAT & NAT) and freeze-thaw cycle (FTC) as important climate characteristics in this study area were assessed in real-time during winter (W) and the growing season (GS) [23,56].

Two-way analysis of variance (ANOVA) was used to analyse the individual and interactive influences of forest gap sizes and sampling times on the contents and net accumulation of humus accumulation (humic substances, humic and fulvic acid), and the optical properties (ΔlogK, E4/E6 and A_{600}/C) of the alkaline solutions. In addition, one-way ANOVA was applied to understand the significant variations within the variables at each sampling time, and post hoc Tukey's HSD test was used if multiple comparisons were performed. We analysed the equality of variances by Levene's test and performed log transformation on the data before ANOVA if needed. All analyses above were carried out in SPSS 20.0 (IBM SPP Statistics Inc., Chicago, IL, USA). Furthermore, partial least squares (PLS) regression with PLS coefficients and variable importance (VIP) was used to explore the effect of litter chemistry (i.e., the contents of N and P and C/N, N/P, C/P and lignin/N ratios) and climate conditions (i.e., the freeze-thaw cycle frequency, the snow depth and the mean and accumulated temperature) on twig litter humification in forest gaps and closed canopies [23,44] (Appendix Table A1). Based on the linear conversion of numerous predictors to a few orthogonal factors, PLS can eliminate multicollinearity between predictors and achieve a small sample size [3]. We used PLS coefficients to determine the magnitudes and directions of the effects of these factors on litter humification, and the VIP to estimate the relative importance of each factor, with VIP > 1 suggesting that the factor significantly affects humification [57]. PLS analyses were carried out by SIMCA 14.1 (Umetrics, Umeå, Sweden) and the graphs were generated by Origin 2018 (OriginLab Inc., Northampton, MA, USA).

3. Results

3.1. Accumulation of Humus

Regardless of gap size classes, the humus rapidly accumulated in the first 2 years of decomposition but gradually mineralized in the following 6 years of decomposition (Figure 2A–C). Compared to the initial values, the humus decreased by 26.10% to 37.00%, 7.28% to 21.37% and 38.01% to 48.31%, respectively, across gap size classes after eight years of decomposition. Moreover, the net accumulations of humus in the closed canopies were significantly higher ($p < 0.001$, $p < 0.001$ and $p = 0.013$) than that in the forest gaps, although the differences between the gap centres and the closed canopies gradually decreased as litter decomposition proceeded (Figure 3A–C). The accumulation of humus decreased with increasing gap-size at the end of the decomposition experiment (Figure 2A–C). Furthermore, the humic acid/fulvic acid ratio increased with incubation time during the study (Figure 2D). Overall, humic substances and humic acid accumulation were significantly affected by forest gap size, with fulvic acid accumulation affected by the interaction of the incubation time and gap size (Table 1).

Table 1. Two-way ANOVA results for the influences of sampling time and forest gap sizes on twig litter accumulation of humus, humic acid/fulvic acid ratio, humification degree and humification rate, and the E4/E6, ΔlogK and A_{600}/C values of humification.

Variables	Gap Size (GS)			Sampling Time (T)			GS × ST		
	df	*F*	*p*	*df*	*F*	*p*	*df*	*F*	*p*
Accumulation of humic substances	2	4.790	0.011 *	4	105.457	<0.001 **	8	1.038	0.406
Accumulation of humic acid	2	7.772	0.001 **	4	38.379	<0.001 **	8	1.460	0.202
Accumulation of fulvic acid	2	2.732	0.071	4	198.146	<0.001 **	8	2.421	0.033 *
Humic acid to fulvic acid ratio	2	0.164	0.849	4	55.822	<0.001 **	8	1.054	0.404
Humification degree	2	3.222	0.045 *	4	350.901	<0.001 **	8	0.577	0.794
Humification rate	2	0.715	0.493	4	176.572	<0.001 **	8	2.395	0.039 *
E4/E6	2	3.841	0.026 *	4	1037.761	<0.001 **	8	0.680	0.708
ΔlogK	2	6.863	0.002 **	4	700.596	<0.001 **	8	2.218	0.035 *
A_{600}/C	2	4.612	0.013 *	4	435.932	<0.001 **	8	0.586	0.786

Notes: Asterisks denote significant (* $p < 0.05$, ** $p < 0.01$) differences between forest gaps and closed canopies over the whole experiment.

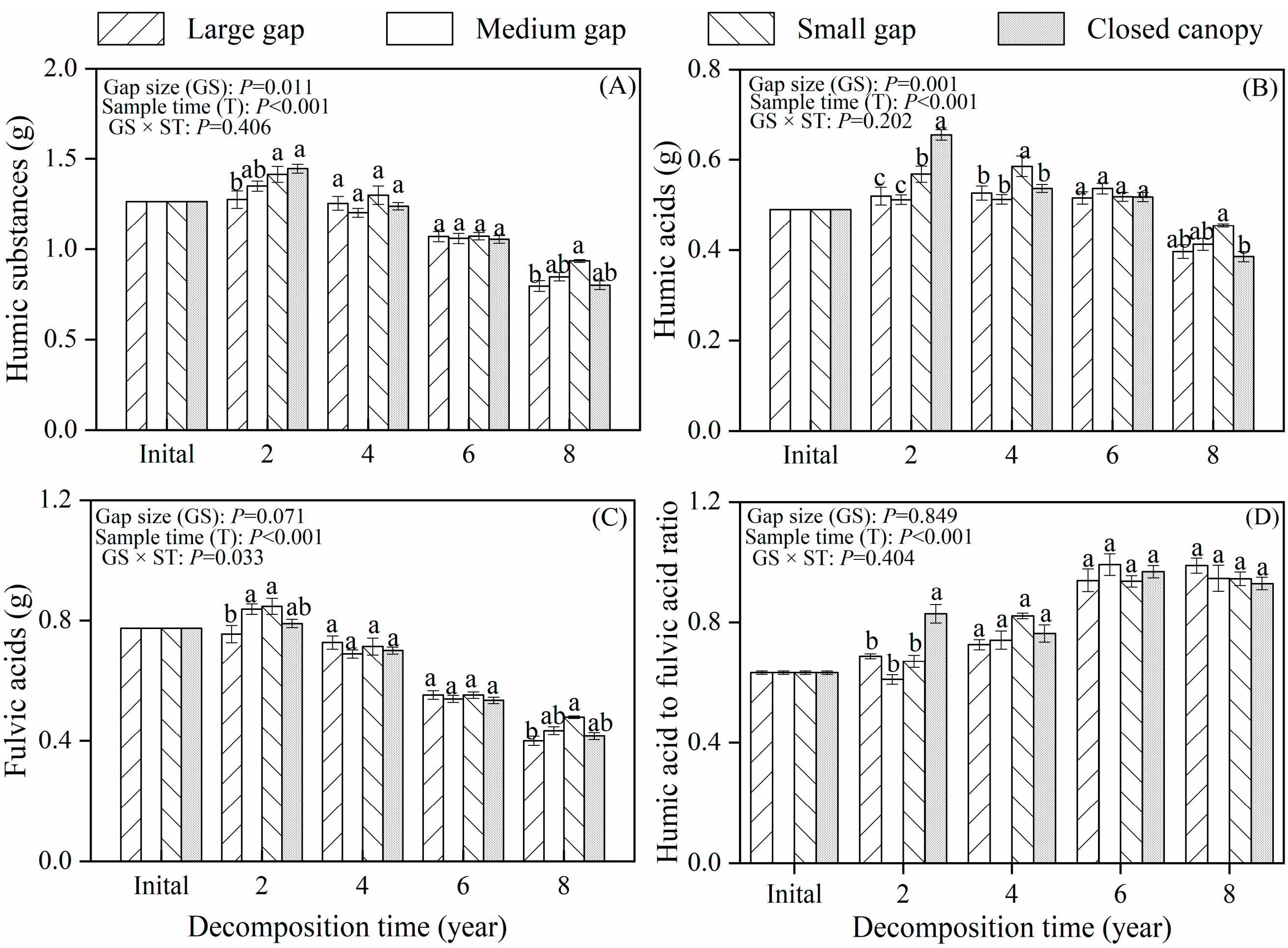

Figure 2. Humus accumulations (**A**–**C**) and humic acid/fulvic acid ratio (**D**) of twig litter in four gap classes during the eight-year decomposition (mean ± SE, $n = 6$). Different lowercase letters denote statistically significant ($p < 0.05$) differences between the three gap classes and the closed canopies at the same decomposition time. The repeated-measures ANOVA results for the effects of sampling time and forest gap sizes on the accumulation of humus and humic acid/fulvic acid radios in litter.

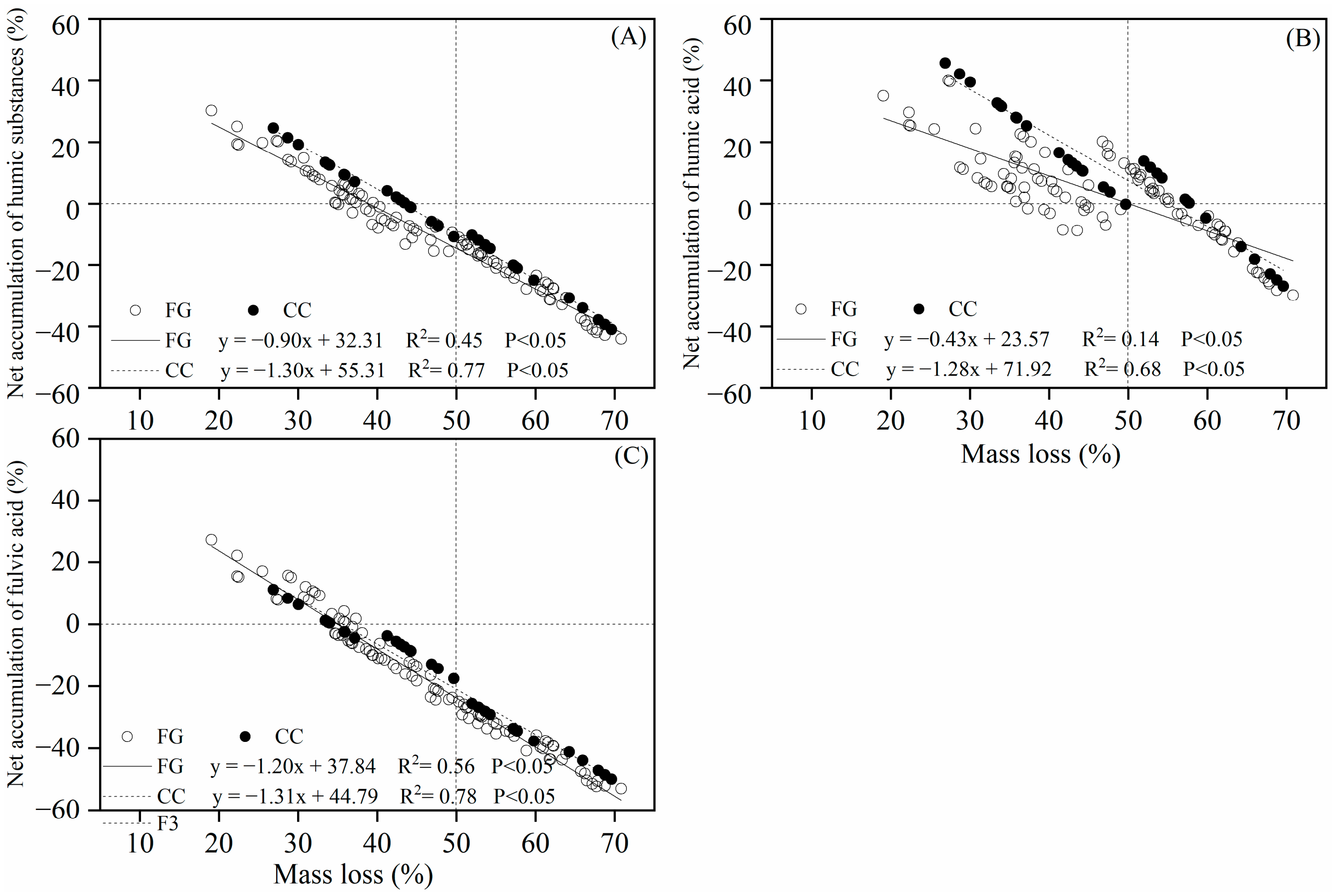

Figure 3. Net accumulation of humus accumulations (**A**–**C**) versus mass loss. p values between the closed canopy and the gap from the covariance analysis are <0.001, <0.001 and 0.013 for each panel (**A**–**C**), respectively. FG, forest gap; CC, closed canopy.

3.2. Humification Degree and Humification Rate

An increasing tendency in humification degree was observed for the gap centres and closed canopies over eight years of incubation, and the humification degrees ranged from 56.81% to 60.79% at the end of the experiment (Figure 4A). Moreover, the highest humification rates were observed in the first two years of incubation across the four gap-size classes (Figure 4B). Furthermore, no significant differences in the humification degree and rate or humic acid/fulvic acid ratio were found among the four gap-size treatments after 8 years of decomposition (Figure 4). Overall, the humification degree and humification rate varied remarkably along the decomposition time and the humification rate was significantly influenced by the interaction of gap size and sampling time (Table 1).

3.3. Optical Properties of Extracted Alkaline Solutions (Humic Acid-like)

The E4/E6 and ΔlogK values of twig litter continued to decrease regardless of the gap-size class during the e8-year decomposition (Figure 5A,B). At the end of the experiment, the E4/E6 and ΔlogK values ranged from 7.57 to 7.96 and 0.77 to 0.80 across the gap-size treatments, respectively. Compared with the closed canopies, litter in the large gaps had significantly higher values of E4/E6 (p = 0.029, F = 1.995) and ΔlogK (p = 0.016, F = 2.941) after 8 years of decomposition. Furthermore, the E4/E6 and ΔlogK values had decreasing trends from large gaps to small gaps (Figure 5A,B). Overall, the E4/E6 and ΔlogK values were significantly affected by gap size and sampling time (Table 1).

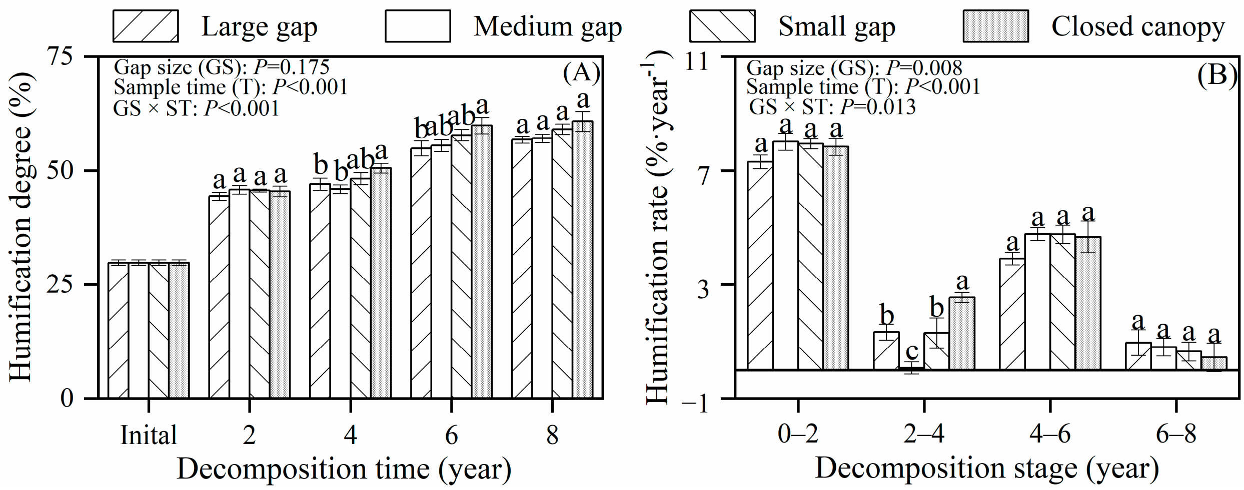

Figure 4. Humification degree (**A**) and humification rate (**B**) in different forest gap size classes of the Minjiang fir twig litter during the eight-year incubation (mean ± SE, $n = 6$). Different lowercase letters denote significant differences among the four forest gap types at the same decomposition time ($p < 0.05$). Results of the repeated-measures ANOVA for the effects of sampling time and forest gap sizes on the degree of litter humification and humification rate.

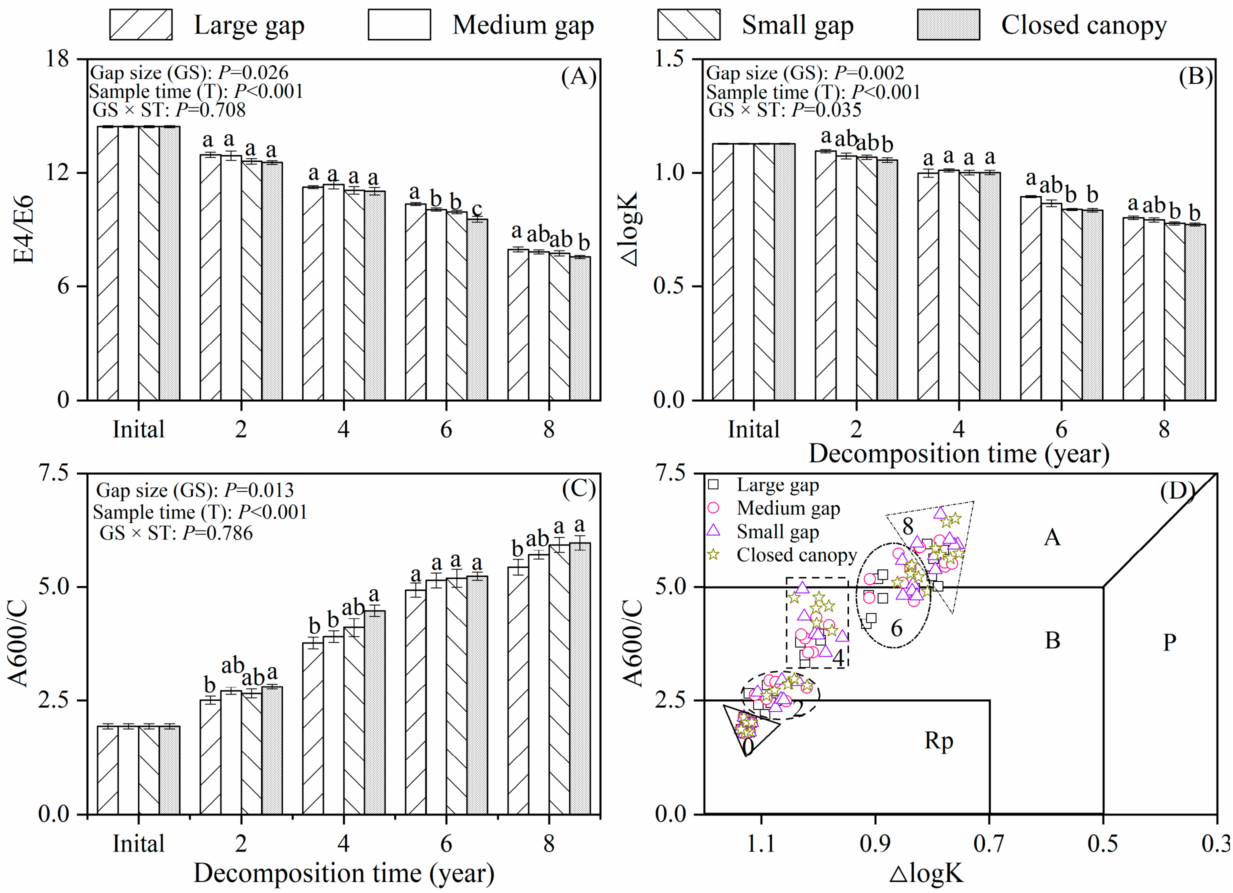

Figure 5. The E4/E6 (**A**), ΔlogK (**B**) and A_{600}/C values (**C**) of humification and the accumulative humic substance types (**D**) based on a modified Kumada classification in different forest gap sizes and the closed canopies for the fir twig litter during the eight-year decomposition period. Different lowercase letters denote significant differences among the four forest gap types at the same decomposition time ($p < 0.05$). Results of the repeated-measures ANOVA for the effects of sampling time and forest gap size on the E4/E6, ΔlogK and A_{600}/C values of humification.

Contrary to the values of ΔlogK and E4/E6, the A_{600}/C values of twig litter tended to increase and ranged from 5.43 to 5.97 regardless of gap size treatments during the eight-year decomposition period (Figure 5C). The A_{600}/C values in the closed canopies were significantly ($F = 2.657$, $p = 0.020$) higher than those in the large gaps after 8 years of incubation. Overall, the gap size had a significant effect on the A_{600}/C values over time (Table 1). Furthermore, according to the values of ΔlogK and A_{600}/C, the accumulated humic substance of the twig litter was determined to be type Rp (i.e., young) during the first 2 years of decomposition and turned to type A (i.e., mature) after 4–6 years of decomposition (Figure 5D).

3.4. Controlling Factors

The PLS analysis suggested that the accumulations of humus were dominantly controlled by the cellulose content, C/N ratio and N content (Figure 6A–C). Moreover, the accumulation of HA and FA in the closed canopies was significantly inversely related to the N/P. Climate factors (i.e., FTCW, FTCGS, NAT-GS, PAT-GS, and PAT-W) acted on humic acid accumulation in the forest gaps to some extent (Figure 6B). In addition, optical properties (i.e., the values of ΔlogK, E4/E6 and A_{600}/C) in the forest gap and the closed canopy were dominantly influenced by twig litter chemistry (i.e., cellulose content, lignin content, P content and N/P lignin/N) (Figure 7A–C). Overall, there were distinct variations in the direction and degree of the influence of micro-environmental factors and litter chemistry on the humus accumulation and optical properties in twig litter between gaps and the closed canopies (Figures 6 and 7D–F).

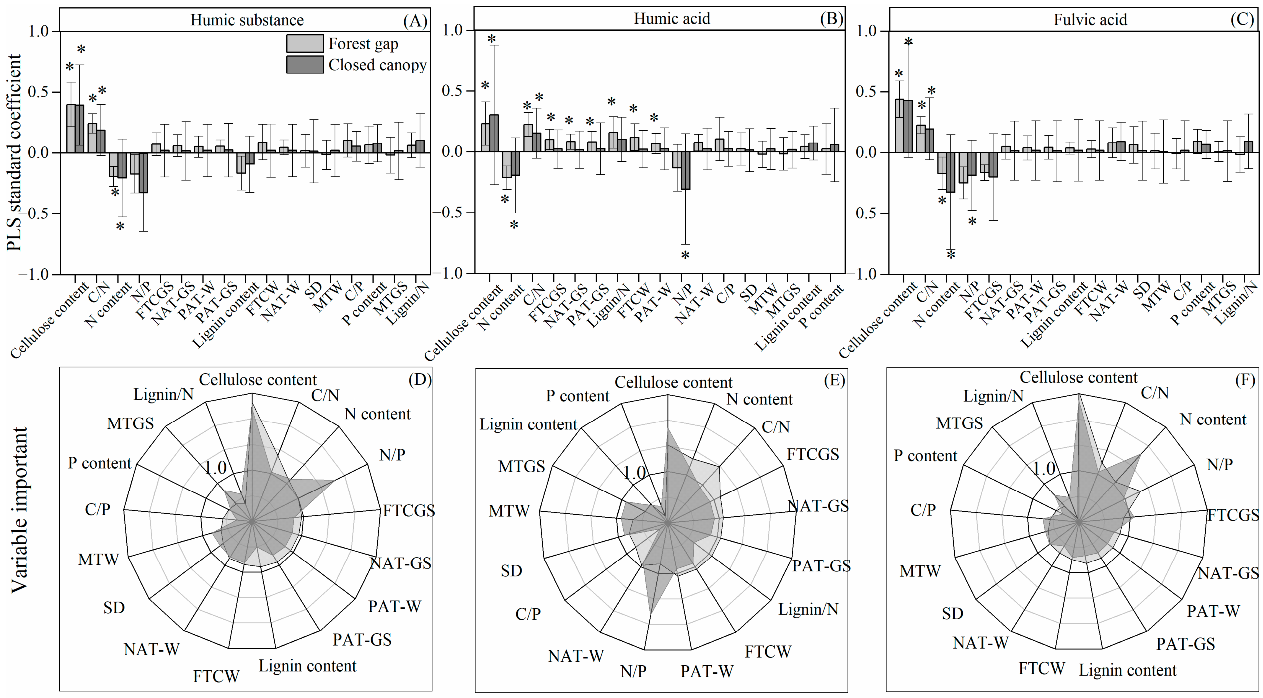

Figure 6. PLS of the influences of litter chemistry and environmental factors on humic substances (**A**,**D**), humic acid (**B**,**E**) and fulvic acid (**C**,**F**) accumulation in twig litter. The PLS coefficient values less than 0 denote negative effects, and those greater than 0 denote positive effects. "*" denotes a significant effect ($p < 0.05$) based on VIP > 1. MTW and MTGS, mean temperature in winter and the growing season; PAT-W and -GS, the positive accumulated temperature in winter and the growing season; NAT-W and -GS, the negative accumulated temperature in winter and the growing season; FTCGS and FTCW, the frequency of freeze–thaw cycle in the growing season and in winter; SD, snow depth.

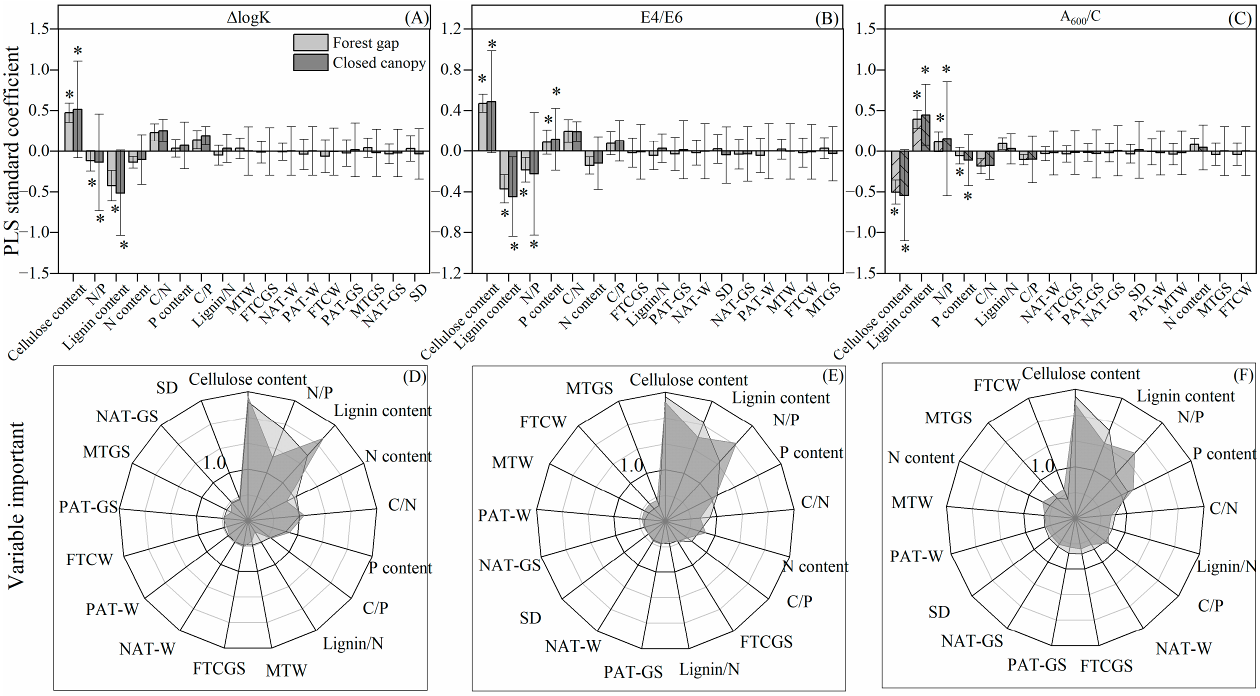

Figure 7. PLS of the effects of litter chemistry and environmental factors on the ΔlogK (**A**,**D**), E4/E6 (**B**,**E**) and A_{600}/C (**C**,**F**) values in twig litter. The PLS coefficient values less than 0 denote negative effects, and those greater than 0 denote positive effects. "*" denotes a significant effect (p < 0.05) based on VIP > 1. MTW and MTGS, mean temperature in winter and the growing season; PAT-W and -GS, the positive accumulated temperature in winter and the growing season; NAT-W and -GS, the negative accumulated temperature in winter and the growing season; FTCGS and FTCW, the frequency of freeze–thaw cycle in the growing season and in winter; SD, snow depth.

4. Discussion

4.1. Effect of Forest Gaps on Twig Litter Humification Accumulation

Litter chemistry is considered one of the most crucial factors in the formation of humus processes and is affected by the specific environment. The formation of forest gaps induces changes in climate, and the soil microenvironment might affect litter quality and soil organism activity and thereby alter litter humification processes in high altitude and high-latitude regions [20,21,46]. Inconsistent with the first hypothesis, a higher accumulation of humic substances and lower values of ΔlogK and E4/E6 were found in the twig litter from closed canopies. Moreover, the accumulation of humic substances increased from large gaps to small gaps, and the values of ΔlogK and E4/E6 showed the opposite trend, which supported the second hypothesis. These results indicated that forest gaps formation slowed twig litter humification and that larger gaps might play a negative role in the form and stability of humic substances.

It is likely that a variety of potential mechanisms might act in combination rather than independently to impact the litter humification process in alpine forests [29]. Generally, multiple ecosystem processes in alpine forests are constrained by low temperatures [20], and the increase in temperature under the snowpack in the gap centre is favorable to the rapid release of labile substances and enhances microbial activity by providing nutrient substrates [19,27], thus stimulating the formation of humus [58–60]. However, thinner or no snowpack with lower temperature in the closed canopies resulted in a higher humic substances accumulation and humification degrees in twig litter than that in gap centres in our study, which was in contrast to some findings in leaf litter [24]. PLS showed that gap-induced variations in litter chemistry, including the contents of lignin, cellulose, N and P, and C/N and N/P ratios, dominated the humification process, which was consistent with other studies [3,38]. On the one hand, cellulose content was found to be a key factor in twig litter humus accumulation, while lignin content was found to affect the structural stabilization of litter humus to some extent in this study (Figures 6 and 7). Decomposers, especially bacteria and fungi, have been shown to degrade dead plant matter (e.g., lignin and cellulose) by the action of a range of extracellular enzymes and further form high-molecular-weight humic substances [1,26,61], which was confirmed by the results of previous studies showing that soil microbial biomass carbon and degradation of lignin and cellulose were higher in the closed canopies than in gap centres [22,23]. A large amount of plant-derived carbon input in closed canopies, such as fresh plant materials and dead roots caused by freeze-thaw events in closed canopies, can provide exogenous carbon to microbial activities, resulting in an increased decomposition rate of lignin and its polymers and thus forming more complex and stable macromolecular organic compounds [62,63]. Moreover, live roots and root exudates directly affect the rhizosphere microbiome community under canopies and increase extracellular enzyme production to accelerate transformation of organic compound [7,64–66]. Some mineral element oxides may interact with humus during litter decomposition and form organic-mineral conjugates, thus protecting soil organic matter from depolymerization by extracellular enzymes by being spatially inaccessible to microbes [1,39]. Therefore, in the closed canopy, twig litter humification may be driven by cellulose and lignin-like products associated with microbial metabolism [1,29]. On the other hand, labile litter materials can bond with minerals and promote the accumulation of humus [67]. Litter humification was found to be largely limited by the N and/or P contents in the same regions due to nutrient availability and microbial activity was suppressed by the low N and P contents in the cold environments [13]. Our previous results indicated that litter N and P releases were higher in closed canopies than in gaps [45], which can supply nutrients to soil microbial communities and accelerate the process of humification [10,68]. Meanwhile, the higher N and P contents of litter can favour cellulose and lignin loss in gaps, resulting in lower lignin-derived compound contents and thus reducing the stabilization of humus [23,69,70]. This could be proven by the significant negative correlation between humic substances and N and P contents in the closed canopy and forest gap (Figures 6 and 7). Therefore, forest gap formation that slows the release of C, N, and P in twig litter may not promote its humification process [45].

In addition, litter humification may vary with the components of plant litter and chemical quality during the decomposition process [2,3]. In our previous studies based-on leaves and roots, higher humic substance accumulation and humification degrees were found in broadleaf (birch) litter and fine roots with higher nutrients than in coniferous litter (fir, cypress and larch) and coarse roots after 1 to 2 years of decomposition, respectively [7,13,24]. Compared with fir foliar litter, twig litter contained lower initial N and P contents and labile components and higher lignin contents, C/N ratios, N/P ratios and lignin/N ratios in this study, and a lower accumulation of humic substances was found in twig litter [7,23,44]. These results suggested that high quality characterized by litters with high nutrient contents and low stoichiometric ratios, might contribute to forming humus. In addition, forest gaps accelerate nutrient release from foliar litter but inhibit nutrient release from twig litter [44,46]. Meanwhile, a lower humic substance content and accumulation and a lower humification degree were found in twig litter in the gaps [21]. Therefore, differences in litter substrate might cause variations in litter humification responses to forest gap effects.

Apart from litter quality, the significant negative effect of gap sizes on litter humus accumulation and humification degree to some extent was affected by climate factors [3] (Figure 5). In winter, the snow depth increased from small gaps to large gaps, with higher temperatures and lower oxygen conditions [20]. Some anaerobic microorganisms may mainly promote humic substance degradation in litter under thick snowpack, especially after fresh litter is returned as a source of energy-rich substrates [71–73]. This process is not conducive to the stability of humus and causes a decrease in the humification degree [25]. In the growing season, higher temperatures and more precipitation were found in the larger gap size. Although more suitable temperatures and moisture may be beneficial to the abundance and activity of decomposers [5], higher solar radiation with decreasing soil humidity may reduce decomposer activities and thus slow the accumulation of litter humification in large gaps [68]. Furthermore, the humus formed in large forest gaps may be degraded by photomineralization [32,74], which causes a higher humification degree in twig litter and more complex molecular structures were observed in the smaller gaps and the closed canopies.

4.2. Temporal Dynamics of Twig Litter Humification

The formation period of organic substances in litter decomposition has long been debated. Traditional views hold that humic substances form in the later stage of decomposition and mainly transform and condense into recalcitrant substances [2,75]. In this study, we observed the humification degree of approximately 30% in the newly fallen twig litter, which supports the results that litter humification may occur in the early stage of litter decomposition in recent years [1,13,17]. This process was significantly affected by the cellulose content, which partly confirmed the microbial efficiency-matrix stabilization framework [1]. Fresh litter typically contains relatively more labile-components and unshielded cellulose [2,15], which can improve microbial use efficiency and promote the accumulation of humus [34,40]. However, humus accumulation showed a dynamic change over long-term time scale. Specifically, a net accumulation of humus occurred in the first 2 years of decomposition and was followed by a net release to 29.31%–37% at the end of the experiment. This might be related to the decrease in litter labile components, which can promote the decomposition of formed humus [46,72,76]. Numerous studies indicate that microbes such as fungi and bacteria effectively degrade synthesized humus [73,77,78]. Recalcitrant humic substances can be efficiently degraded via co-metabolism processes when carbon sources are plentiful, while humic acid can be decomposed by some fungi to supply energy (C or N sources) for their activities [36,73,79]. Furthermore, the humic acid/fulvic acid ratios first increased quickly and then levelled off and were less than 1 during the 8 years of decomposition, which suggested that the formation of fulvic acid may occur earlier than that of humic acid in the early stage of twig litter humification and is then mutually transformed and reaches a dynamic equilibrium as decomposition proceeds due to the instability of fulvic acid [35,80].

A higher humification degree and more mature humus type were found in the gaps and closed canopies in the late stage of decomposition. Labile components were consistently reduced, lignin was transformed to humic substances with a predominant aliphatic character under the action of fungi and more formed macromolecular organic compounds were maintained in the litter as decomposition progressed [29,56]. Meanwhile, we observed that the difference in humus accumulation between forest gaps and closed canopies decreased gradually with mass loss, and converged when the mass loss reached approximately 60%. This partially conformed with the results of Zhou et al. [81], and previous findings that litter stoichiometric traits, including contents of lignin, cellulose, N and P, and C/N, N/P and lignin/N ratios, regulate humus formation [7,38]. Studies have indicated that litter with different initial chemical compositions finally tends to have similar chemistry when the mass loss reaches 75%–80% [3,37]. Common and limited biochemical pathways and physiological abilities are shared by decomposers and may further affect the chemical composition of decomposing organic matter [37,41]. In general, soil microbes are the key factor affecting litter humification and are clearly affected by litter quality [40,82,83]. Litter with nutrient-rich conditions and low C/N ratios and lignin/N ratios are beneficial for enhancing microbial biomass and activity [72], attracting the consumption of soil fauna and promoting microbial colonization [26,44]. Unfortunately, our study did not validate the role of decomposers in twig litter humification, but it needs to be considered in the future. In addition, it is noteworthy that despite the widespread use of litterbag methodology to study litter decomposition rates, nutrient release, and the influences of biotic and abiotic factors on decomposition and humification rates, these results may have certain biases compared to the actual decomposition process in natural ecosystems [8]. The use of litterbags may alter the microenvironment around the litter, which can affect the decomposition rate [84].

The mesh size and shape of the litter bag may affect the composition and activity of soil biota, as well as the escape of decomposed litter fragments [85]. Therefore, it is necessary to consider and compensate for these biases through other methods and approaches to fully understand the complex process of litter decomposition in natural ecosystems.

5. Conclusions

Our study investigated twig litter humification process across four gap classes over 8 years in an alpine forest. Although the humus of twig litter accumulated in the early stages of decomposition and subsequently mineralized regardless of the forest gaps or closed canopy conditions, the humification degree increases in the later stage of decomposition and formed more mature humus. Compared with closed canopies, litter humification was slowed by forest gap formation, the effects decreased from small gaps to large gaps, and the gap-effect gradually dissipated as decomposition proceeded. Moreover, the temporal dynamics of gap-induced variations in twig litter chemistry (i.e., the contents of lignin, cellulose, N and P and the ratios of C/N, N/P and lignin/N) strongly regulated the humification process. Our results suggest that forest gaps formation retard the conversion of twig debris to stable soil organic matter during forest regeneration. Compared with large gaps, smaller gaps might contribute to carbon sequestration and soil security in these alpine forests. Finally, we note that this study concentrated on litter humification and neglected the soil carbon sequestration process and contribution from plant debris to SOM, which future work should focus on.

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Appendix A

Table A1. Contents of carbon (C), nitrogen (N) and phosphorus (P) contents, lignin and cellulose and ratios of C, N, P and lignin /N in the closed canopy and forest gaps during decomposition (±SE, *n* = 9).

Variables	Decomposition Time (Year)	Large Gap	Medium Gap	Small Gap	Closed Canopy
Carbon content (g/kg)	Initial	441.88 ± 10.77	441.88 ± 10.77	441.88 ± 10.77	441.88 ± 10.77
	2	455.47 ± 2.92b	460.29 ± 5.59b	463.63 ± 11.28b	493.28 ± 0.93a
	4	463.55 ± 6.63a	458.15 ± 8.39a	451.90 ± 14.82a	461.47 ± 6.36a
	6	417.10 ± 10.99a	395.82 ± 10.46ab	391.96 ± 11.00ab	382.73 ± 7.71b
	8	350.27 ± 9.84a	356.40 ± 44.56a	382.59 ± 24.88a	396.22 ± 37.34a

Table A1. *Cont.*

Variables	Decomposition Time (Year)	Large Gap	Medium Gap	Small Gap	Closed Canopy
Nitrogen content (g/kg)	Initial	8.11 ± 0.16	8.11 ± 0.16	8.11 ± 0.16	8.11 ± 0.16
	2	6.86 ± 0.36a	6.39 ± 0.20a	7.53 ± 0.72a	6.45 ± 0.35a
	4	6.00 ± 0.42a	4.76 ± 0.35b	4.6 ± 0.24bc	3.72 ± 0.08c
	6	6.66 ± 0.28ab	6.13 ± 0.30b	6.67 ± 0.21ab	7.19 ± 0.36a
	8	10.41 ± 0.44a	9.24 ± 0.64ab	8.44 ± 0.42b	9.99 ± 0.27ab
Phosphorus content (g/kg)	Initial	0.67 ± 0.03	0.67 ± 0.03	0.67 ± 0.03	0.67 ± 0.03
	2	0.68 ± 0.04a	0.71 ± 0.09a	0.62 ± 0.03a	0.58 ± 0.04a
	4	0.27 ± 0.01a	0.24 ± 0.00ab	0.25 ± 0.00ab	0.23 ± 0.00b
	6	0.55 ± 0.02bc	0.68 ± 0.02a	0.60 ± 0.01ab	0.54 ± 0.01c
	8	0.47 ± 0.00a	0.47 ± 0.00a	0.47 ± 0.00a	0.47 ± 0.00a
Lignin content (%)	Initial	32.40 ± 0.00	32.40 ± 0.00	32.40 ± 0.00	32.40 ± 0.00
	2	35.82 ± 0.60b	36.70 ± 0.13 b	39.38 ± 0.39a	38.70 ± 0.32a
	4	43.44 ± 1.50a	44.28 ± 0.75a	39.59 ± 0.86b	42.61 ± 0.58ab
	6	44.96 ± 0.58ab	45.32 ± 1.09a	44.80 ± 1.07ab	43.12 ± 0.60b
	8	41.28 ± 1.55ab	39.66 ± 0.68b	43.83 ± 0.64a	39.44 ± 0.92b
Cellulose content (%)	Initial	23.81 ± 0.00	23.81 ± 0.00	23.81 ± 0.00	23.81 ± 0.00
	2	21.96 ± 0.68b	23.58 ± 1.24b	21.00 ± 1.59a	22.06 ± 0.76ab
	4	18.83 ± 0.85ab	18.33 ± 0.47a	19.51 ± 0.39ab	17.21 ± 1.31b
	6	17.22 ± 0.90ab	16.72 ± 1.04a	17.96 ± 0.90ab	16.32 ± 0.80b
	8	13.59 ± 0.23ab	12.72 ± 0.89ab	13.79 ± 0.60a	11.48 ± 0.64b
C/N	Initial	54.46 ± 0.87	54.46 ± 0.87	54.46 ± 0.87	54.46 ± 0.87
	2	66.73 ± 2.96a	72.13 ± 1.66a	62.4 ± 4.33a	66.97 ± 4.05a
	4	77.84 ± 4.16c	97.06 ± 6.05b	98.57 ± 2.45b	120.99 ± 5.31a
	6	63.38 ± 3.00ab	65.97 ± 3.45a	57.8 ± 2.06ab	54.72 ± 3.04b
	8	33.74 ± 1.39b	35.23 ± 3.56ab	47.29 ± 5.77a	42.34 ± 3.16ab
C/P	Initial	657.42 ± 21.36	657.42 ± 21.36	657.42 ± 21.36	657.42 ± 21.36
	2	678.57 ± 36.23a	672.24 ± 78.15a	749.54 ± 22.77a	865.20 ± 69.57a
	4	1724.22 ± 46.61c	1919.53 ± 16.03ab	1837.05 ± 54.31bc	2012.04 ± 16.65a
	6	776.92 ± 43.14a	574.08 ± 14.98b	649.81 ± 24.70ab	711.37 ± 19.50a
	8	747.19 ± 20.32a	698.8 ± 101.45a	843.48 ± 67.52a	902.88 ± 79.67a
N/P	Initial	12.07 ± 0.37	12.07 ± 0.37	12.07 ± 0.37	12.07 ± 0.37
	2	10.18 ± 0.39ab	9.29 ± 0.94b	12.10 ± 0.69ab	12.93 ± 0.70a
	4	22.22 ± 0.62a	19.94 ± 1.31ab	18.68 ± 0.9ab	16.70 ± 0.79b
	6	12.34 ± 0.83ab	9.13 ± 0.54c	11.05 ± 0.28b	13.39 ± 0.82a
	8	22.20 ± 0.91a	19.75 ± 1.38ab	18.05 ± 0.88b	21.3 ± 0.61ab
Lignin/N	Initial	39.96 ± 0.76	39.96 ± 0.76	39.96 ± 0.76	39.96 ± 0.76
	2	51.90 ± 1.36a	57.38 ± 1.42a	52.90 ± 3.98a	52.31 ± 2.66a
	4	71.36 ± 1.41b	93.34 ± 6.16ab	85.36 ± 1.6ab	110.93 ± 4.26a
	6	66.93 ± 3.42ab	72.31 ± 3.26a	66.06 ± 1.76ab	59.44 ± 3.98b
	8	38.86 ± 0.76b	42.94 ± 1.99b	51.73 ± 2.51a	39.00 ± 0.74b

Different lowercase letters within a line indicate statistically (one-way ANOVA) significant ($p < 0.05$) differences in the contents of C, N, P, lignin and cellulose and ratios of C, N, P, and lignin/N among the gap size classes.

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