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Soil Fungal Diversity and Functionality Changes Associated with Multispecies Restoration of *Pinus massoniana* Plantation in Subtropical China

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Abstract: Soil fungi play a critical role in the carbon and nutrient cycling of forest ecosystems. Identifying the composition of soil fungi in response to the broadleaf restoration of *Pinus massoniana* plantation is essential for exploring the mechanistic linkages between tree species and ecological processes, but remains unexplored. We compared the shifts in soil fungal diversity and guilds by high–throughput sequencing between two *P. massoniana* plantations at different stand ages, two modes of restoration with broadleaf trees, and a secondary forest in subtropical China. We found that soil fungal taxonomic and functional compositions significantly differed among forests. The highest Chao 1, Shannon, and phylogenetic diversity indices were consistently observed in the two *P. massoniana* monocultures, followed by the two modes of broadleaf mixing, and the secondary forests. Fungal communities transitioned from Ascomycota-dominated at *P. massoniana* plantations to Basidiomycota-dominated at other forests in the topsoil. Furthermore, saprotrophs and symbiotrophs were favoured in plantations and secondary forests, respectively. Soil pH exerted the most significant effect on the relative abundance of Ascomycota and Rozellomycota, as well as the saprotrophs. Moreover, the dominant phyla of Ascomycota, Mucoromycota, and Rozellomycota were negatively related to soil microbial biomass nitrogen, ammonium nitrogen, and total nitrogen contents; however, Mortierellomycota benefited from the elevated soil ammonium nitrogen content. On the other hand, soil nitrate nitrogen and available phosphorus contents strongly and negatively influenced the ectomycorrhizal fungi, while the other fungal guilds were mainly affected by soil pH. Our findings guide an evaluation of the consequences of forest restoration and contribute to an improved understanding of the mechanisms behind soil biogeochemical cycling in subtropical forest ecosystems.

Keywords: biogeochemical process; carbon cycling; diversity; guild; mycorrhizal fungi; tree species

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1. Introduction

Reforestation is widely used in practice in subtropical areas as an effective method to cope with global change and ecosystem degradation, which has greatly contributed to regional carbon sequestration over the past decades [1]. Monoculture plantations show an advantage in wood production. However, they also bear the ecological consequences of declines in biodiversity, soil fertility, and ecosystem stability [2,3]. In this context, ecological restoration of monoculture plantations has become the focus of study on biodiversity conservation and soil carbon sequestration [3]. Multispecies planting has become an effective strategy for promoting the reconstruction of plant communities and improving the carbon potential at a low cost [4].

Fungal communities are extremely diverse and normally dominated by the phyla of Basidiomycota, Ascomycota, Glomeromycota, Chytridiomycota, and Zygomycota in the forest soils [5]. Given the high taxonomical diversity and biomass, soil fungi play an important role in soil carbon cycling via organic substrate decomposition (e.g., lignin), forming

symbioses that are associated with roots, regulating forest productivity, and altering soil structure [6–8]. It is reported that soil fungi are superior to bacteria in converting litter into organic matter by hyphal growth and enzyme production, particularly in coniferous forests [5]. For instance, Agaricomycetes, a member of Basidiomycetes, are able to produce specific peroxidases to oxidize the phenolic macromolecules, such as lignin, tannins, and melanins [9]. In addition, the fungal communities shifted from Ascomycota-dominated to Basidiomycota-dominated after the grassland was converted to forests [10]. Furthermore, soil fungi are classified into saprotrophs, symbiotrophs, and pathotrophs [11], and these guilds act on nutrient cycling, plant community, and multiple plant-soil feedbacks, respectively [12–14]. The saprotrophs are free-living decomposers that obtain carbon and nutrients by decaying the organic matter; symbiotrophs facilitate the nutrient uptake to their host tree in exchange for required nutrients, and pathotrophs suppress the host recruitment and survival [6]. The altered proportion of saprotrophs, pathogens, and symbiotrophs markedly affected the ecological processes and ecosystem stability [8]. Consequently, understanding the changes in the soil fungal diversity and functionality is beneficial to predict soil health and carbon cycling after vegetation shift.

Understanding the taxonomic composition and functionality of soil fungi is fundamental for soil functions [15]. Prior studies showed that soil microbial communities varied with vegetation shift [14]. Multispecies planting affects litter quality and edaphic biogeochemistry such as soil moisture, pH, nutrient contents, and bulk density, which will likely have consequences for soil fungi and functions of the subtropical forest ecosystems [7,12]. A previous study revealed that the dominant fungal guilds shifted from ectomycorrhizal to saprotrophic fungi after aboveground biomass removal in a Canadian forest [7]. However, some researchers also illustrated that the microbial communities were not affected by the vegetation types [16,17], because the effects of specific vegetation traits on the microbial taxa rely on the soil category [18]. Similarly, recent global data revealed that the soil carbon to nitrogen ratio strongly controlled the global pattern of fungal biomass, abundance and genetic functions [15]; soil pH was negatively associated but bulk density and soil organic carbon positively related to the fungal diversity. Moreover, the fungal symbiotrophs responded more sensitively to variations in soil fertility compared with pathotrophs and saprotrophs [19]. Given that the microbiota controls the ecological processes and in turn, responds to the substrate supply, it remains a challenge to predict the correlation between fungal communities and edaphic properties.

Pinus massoniana was a pioneer afforestation species in subtropical China [20]. However, in recent years, the ecological service of *P. massoniana* monocultures declined due to the pine wood nematode disease [21]. Mixing local broad-leaved trees under the coniferous canopy and transforming the coniferous plantation into local broad-leaved trees are prevalent means to restore the degraded coniferous plantations. It was reported that the transformation of *P. massoniana* plantation to local broad-leaved trees increased soil organic carbon content, microbial carbon use efficiency and arbuscular mycorrhizal fungal biomass by 37%, 54%, and 26%, respectively [21]. In addition, multispecies restoration with native trees increased wood production but enhanced the water limitation in the *Eucalyptus* plantations [22]. Multispecies planting was reported to alter the litter productivity and quality and created habit heterogeneity, which proved highly complementary to resource supply for underground biota [23]. On the other hand, the changes in functional traits of trees (such as mycorrhizal types) are expected to alter the relative proportion of saprotrophs and symbiotrophs due to the competition for nutrient availability after multispecies restoration. Given that the decomposition of needles is usually associated with soil acidification via the leaching of organic acids [14], the multispecies restoration may suppress the growth of a specific species or guild by changing the soil pH [12,14]. However, it remains unknown how multispecies restoration affects the diversity and functionality of soil fungi which has wide-ranging effects on forest health, biogeochemical processes, and carbon budget [6,7]. In this study, we aimed to test the hypothesis that (1) multispecies restoration would alter the dominant taxonomic phyla and increase the diversity of soil fungi in *P. massoniana* plan-

tation to a level similar to the secondary forests; (2) the relative percentage of saprotrophs and symbiotrophs would change after the multispecies restoration; and (3) the dominant phyla and functional guilds would be primarily regulated by soil pH and nitrogen content.

2. Materials and Methods

2.1. Study Area and Sample Collection

Soil samples were collected from five forests in Shanghang County, Fujian Province in subtropical China (25.1°–25.3° N and 116.5°–116.6° E) in June 2021 (Figure 1). The study area has a typical subtropical monsoon climate with an annual temperature of 20.1 °C and an annual precipitation of 1646 mm. The soil was classified as Oxisol based on the United States Department of Agriculture (USDA) Soil Taxonomy [24].

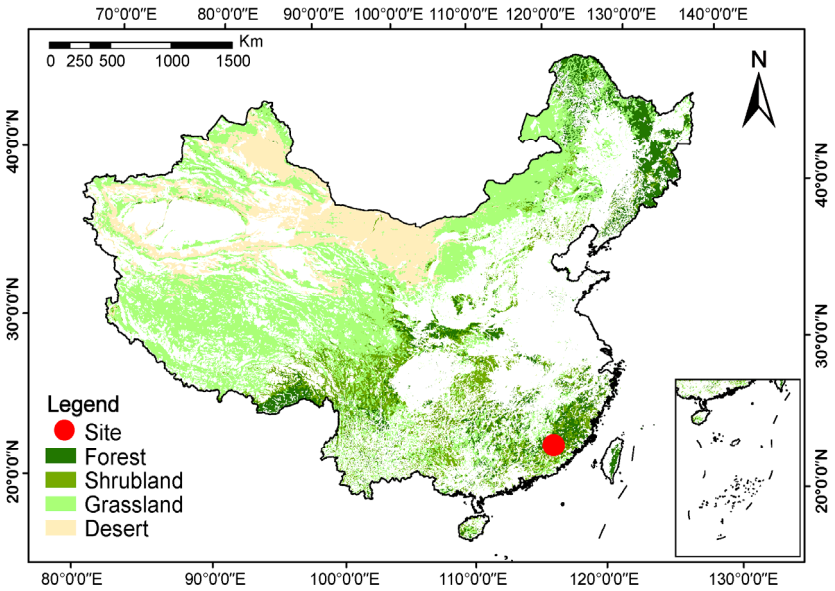

Figure 1. Map of the study area.

We sampled from two multispecies restoration modes, two *P. massoniana* plantations (20-year plantation, PM, and 40-year overmature plantation, PO) and a 40-year secondary forest (SF). One restoration mode is the transformation of the coniferous plantation using local broadleaf trees including *Mytilaria laosensis*, *Liquidambar formosana*, *Quercus acutissima*, *Michelia macclurei*, *Castanopsis hystrix*, and *Ilex chinensis* (BF). The other mode is replenishment with broadleaf trees (*M. laosenis*, *L. formosana*, *M. macclurei*, and *C. hyperix*) under the coniferous canopy (PB). *Schima superba*, *L. formosana*, *Syzygium buxifolium*, and *I. pubescens* are the common trees in the SF.

We established three sites consisting of three 20 m × 20 m plots in each forest type. To reduce the heterogeneity, we randomly selected nine cores in each plot and then pooled the cores to obtain a composite sample by depth (topsoil, 0–10 cm and subsoil, 30–50 cm). Furthermore, litter was also collected from each plot and air-dried for element analysis. Each fresh soil sample was sieved through a 2 mm sieve, and the visible roots and rocks were removed carefully. Aliquots of soil samples were immediately stored at −80 °C, 4 °C, or air-dried for the subsequent molecular and physicochemical analyses.

2.2. DNA Extraction and Bioinformatics

Soil microbial DNA was extracted using the Fast DNA Spin Kit (MP Biomedicals, Santa Ana, CA, USA) following the manufacturer's instructions. The quality and quantity of the DNA were determined using a NanoDrop 2000 (Thermo Scientific, Wilmington, NC, USA). We used the primer sets internal transcribed spacer (ITS) 1F/ITS2R to amplify the fungal ITS rRNA genes on the Illumina MiSeq platform (Illumina, San Diego, CA, USA) of the Majorbio Bio-Pharm Technology Co., Ltd. (Shanghai, China) [25]. PCR was run in

20 µL reactions, containing 10 ng of template DNA. The PCR denaturation was as follows: at 95 °C for 3 min, followed by 35 cycles at 95 °C for 30 s, 55 °C for 30 s, and 72 °C for 45 s, with a final extension of 72 °C for 10 min.

The high-quality sample sequences were denoised using the 'DADA2' plugin to resolve amplicon sequence variants (ASVs) which permits robust downstream analyses [26]. The ASVs were identified taxonomically based on the UNITE v8.0 database for fungi [13]. The fungal ASVs were rarefied to 29787 reads per sample. Furthermore, fungal guilds were identified using the FUNguild database as described in Nguyen et al. [27].

To avoid over-interpretation of the fungal guilds, we only retained the taxa assigned to a single guild (e.g., symbiotrophs, pathogens, and saprotrophs) with probable or highly probable confidence ranking [7].

2.3. Soil Properties

Soil pH value was determined from a soil slurry in a 1:2.5 (*w*:*v*) and moisture was measured by oven-drying the fresh soil at 105 °C. Soil and plant total carbon and nitrogen were determined using an Elemental Combustion Analyzer (ElementarVario, Max, Hanau, Germany). Microbial biomass carbon (MBC) and nitrogen (MBN) were measured according to the chloroform fumigation method [28], and ammonium nitrogen (NH_4^+-N), nitrate nitrogen (NO_3^--N), and available phosphorus (AP) were measured with a flow analyser (San++, Skalar, Breda, The Netherlands) [29,30]. Soil clay content was determined using a laser diffraction particle size analyser (Mastersizer 3000, Malvern, England).

2.4. Statistical Analysis

In this study, the widely used Chao 1, Shannon and Phylogenetic diversity (PD) indices were calculated from ASV abundance tables to indicate the alpha diversity. A two-way ANOVA test was implemented to examine the significant effects of forest type and soil depth on the fungal communities. The different taxa among forest types were further identified by the Kruskal–Wallis H test. Multiple comparisons were performed with the "agricolae" package in the R 4.1.2 (R Development Core Team, 2021) [31]. The principal analysis (PCA) illustrated the compositional shifts of fungi. Moreover, redundancy analysis (RDA) was conducted to examine the effects of litter and edaphic properties on fungal guilds with the "vegan" package [32]. Correlation analysis was used to test relationships between fungal alpha diversity, dominant fungal phyla, and litter and soil properties. Random Forest analysis was conducted to identify the main explanatory factors of the dominant fungal phyla using "randomForest" package [33]. The significance level of all analyses was set at $p < 0.05$.

3. Results

3.1. Soil Fungal Diversity and Composition

A total of 10306 fungal ASVs remained from the complete data set after quality trimming and removal of chimeras, which were clustered into 12 phyla, 121 orders, 293 families, 658 genera and 1090 species (Table A1). The dominant fungal phyla were Basidiomycota, Ascomycota, Mortierellomycota, and Rozellomycota (Figure 2), which remarkably differed among forests according to the Kruskal–Wallis H test (Table 1; $p < 0.05$). In the topsoil, the relative abundance of Basidiomycota, Ascomycota and Mortierellomycota was significantly highest in PB, followed by PM, and SF (Figure 2; $p < 0.05$). With increasing stand age in *P. massoniana* monocultures, the relative abundance of Basidiomycota in topsoil increased, while the relative abundance of Basidiomycota in subsoil and Ascomycota in topsoil and subsoil decreased.

Two-way ANOVA showed that fungal diversity was significantly affected by forest types ($p < 0.001$), but soil depth had no significant effects (Table A2; Figure 3; $p > 0.05$). The indices of Chao 1, Shannon and Pd were highest in *P. massoniana* plantations (PM and PO), followed by the two modes of multispecies restoration (BM and PB) and SF in different soil layers (Figure 3). Additionally, PCA results showed that the composition of fungal

communities differed in forest types in both topsoil and subsoil (Figure 4; $p = 0.001$). The first axis separated the two *P. massoniana* monocultures from other forests.

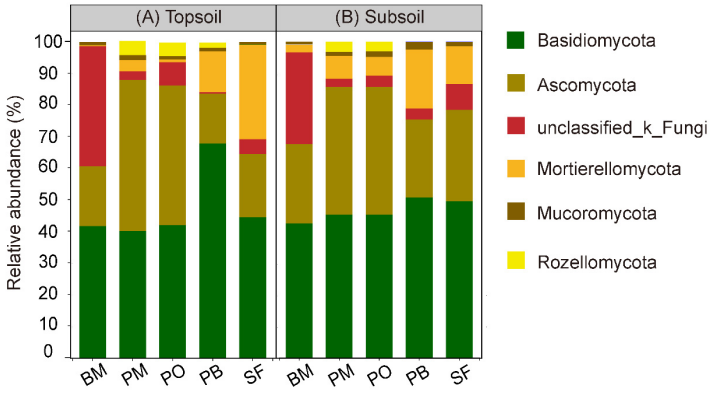

Figure 2. Relative abundance of main fungal phyla in different forest types. Broadleaf-oriented transformation, BM; *P. massoniana* mature plantation, PM; *P. massoniana* overmature plantation, PO; *P. massoniana* + local broadleaved trees, PB; secondary forests, SF.

Table 1. Results of Kruskal–Wallis H test showing the difference of main fungal phyla in different forests.

Phylum	H Statistic		*p*-Value	
	Topsoil	Subsoil	Topsoil	Subsoil
Basidiomycota	12.43	1.61	*	ns
Ascomycota	28.12	10.08	***	*
Mortierellomycota	33.57	16.02	***	**
Mucoromycotina	6.05	5.49	ns	ns
Rozellomycota	28.37	35.54	***	***

*** $p < 0.001$; ** $p < 0.01$; * $p < 0.05$; ns $p > 0.05$.

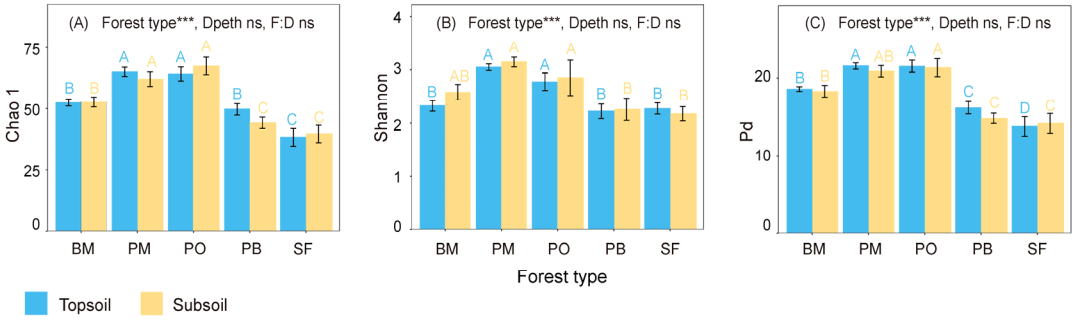

Figure 3. Multiple comparisons showing different alpha diversity of soil fungi in different forest types. (**A**) Chao 1; (**B**) Shannon; (**C**) Phylogenetic diversity. Significant differences ($p < 0.05$) in different forest types are indicated by letters in different colours. Broadleaf-oriented transformation, BM; *P. massoniana* mature plantation, PM; *P. massoniana* overmature plantation, PO; *P. massoniana* + local broadleaved trees, PB; secondary forests, SF. *** $p < 0.001$; ns $p > 0.05$.

3.2. Soil Fungal Guilds

The relative abundance of saprotrophs and symbiotrophs was significantly influenced by the forest type rather than soil depth (Table A3). The dominant fungal guild was saprotrophs and symbiotrophs with a relative abundance ranging from 32.65% to 85.95% and from 4.04% to 61.08% in plantations and the secondary forest, respectively (Figure 5A,B). The saprotrophs had the highest relative abundance in PB and the lowest abundance in SF. On the contrary, symbiotrophs demonstrated an opposite pattern. Additionally, the relative abundance of saprotrophs increased, but symbiotrophs decreased with increasing age in *P. massoniana* stand (Figure 5A,B). Ectomycorrhizal fungi, the dominant symbiotroph,

governed in the plantations (3.93%–60.74%), its relative abundance decreased with increasing age of *P. massoniana* stand and was minimized in PB (Figure 5C,D). In the subsoil, animal pathogens had the highest abundance in PB (Figure 5F). In addition, the relative abundance of soil saprotroph, the dominant saprotroph, increased with the increasing age of *P. massoniana* stands, and was minimized in PB at both depths (Figure 5G,H).

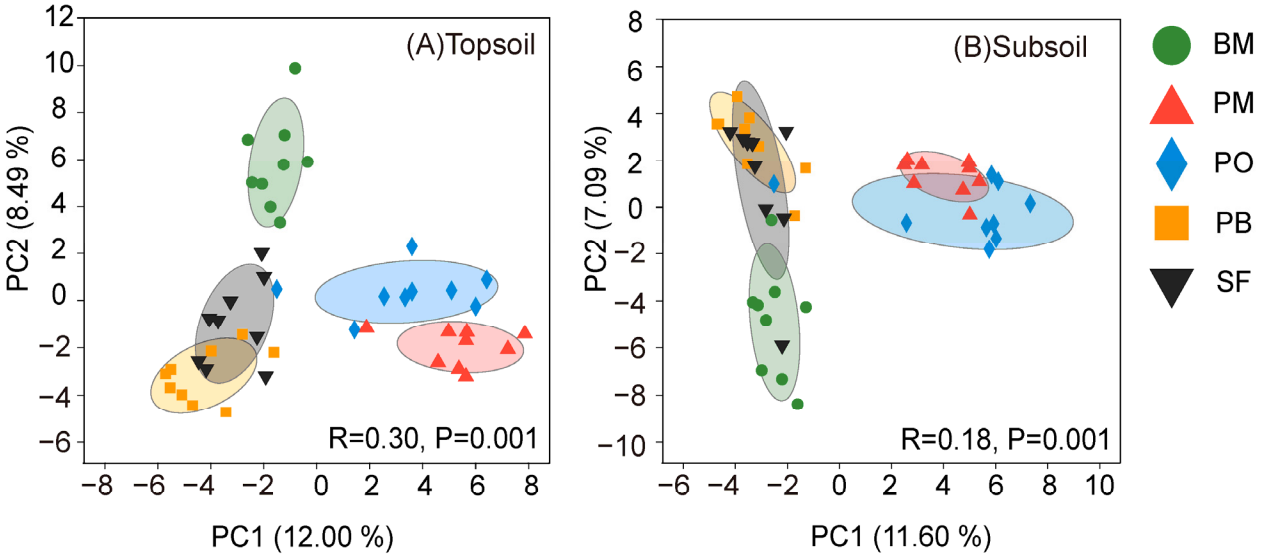

Figure 4. Principal component analysis of fungal communities in different forest types. Broadleaf-oriented transformation, BM; *P. massoniana* mature plantation, PM; *P. massoniana* overmature plantation, PO; *P. massoniana* + local broadleaved trees, PB; secondary forests, SF.

3.3. Relationship between Environmental Predictors and Fungal Communities

The relative abundance of Ascomycota and Rozellomycota was significantly and negatively correlated with pH and MBN content (Figure 6A,B; $p < 0.05$); Mortierellomycota was significantly and positively correlated with NH_4^+-N and MBN contents ($p < 0.05$) (Figure 6A,B). Further, the five most important predictors for the major phyla were identified by random forest models. These correlations were consistent with the results from random forest models that pH and soil nitrogen contents strongly affected these dominant phyla (Figure 6).

Similarly, Pearson correlation showed that diversity indices were mainly correlated to pH, NH_4^+-N, AP and MBN contents in the topsoil, and correlated with TN, NH_4^+-N and MBN contents in the subsoil (Table 2; $p < 0.05$). Similarly, the random forest model showed that pH and NO_3^--N content were the most important factors for the variation in Shannon index in the topsoil and subsoil, respectively (Figure 6C,D).

Table 2. Correlations between fungal diversity indices and litter and edaphic properties in topsoil and subsoil.

		$L_{c:n}$	Moisture	pH	Clay	TC	TN	NH_4^+-N	NO_3^--N	AP	MBC	MBN
Topsoil	Chao 1	0.26	−0.24	−0.69 ***	−0.10	−0.06	−0.27	−0.30 *	0.00	0.48 ***	−0.10	−0.45 **
	Shannon	0.07	−0.25	−0.50 ***	−0.08	−0.15	−0.08	−0.21	−0.14	0.27	0.19	−0.34 *
	Pd	0.29	−0.18	−0.64 ***	−0.09	−0.04	−0.27	−0.39 **	−0.15	0.42 **	−0.11	−0.49 ***
Subsoil	Chao 1	0.23	−0.11	−0.03	0.20	−0.11	−0.31 *	−0.25	−0.03	0.23	−0.03	−0.26
	Shannon	0.06	−0.22	0.07	0.04	−0.22	−0.30 *	−0.45 **	−0.14	0.04	−0.04	−0.34 *
	Pd	0.24	−0.08	−0.06	0.20	−0.15	−0.32 *	−0.35 *	−0.10	0.14	−0.02	−0.34 *

Pd, phylogenetic diversity; Litter C:N ratio, $L_{c:n}$; total carbon content, TC; total nitrogen content, TN; ammonium nitrogen, NH_4^+-N; nitrate nitrogen, NO_3^--N; available phosphorus, AP; microbial biomass carbon, MBC; microbial biomass nitrogen, MBN. *** $p < 0.001$; ** $p < 0.01$; * $p < 0.05$.

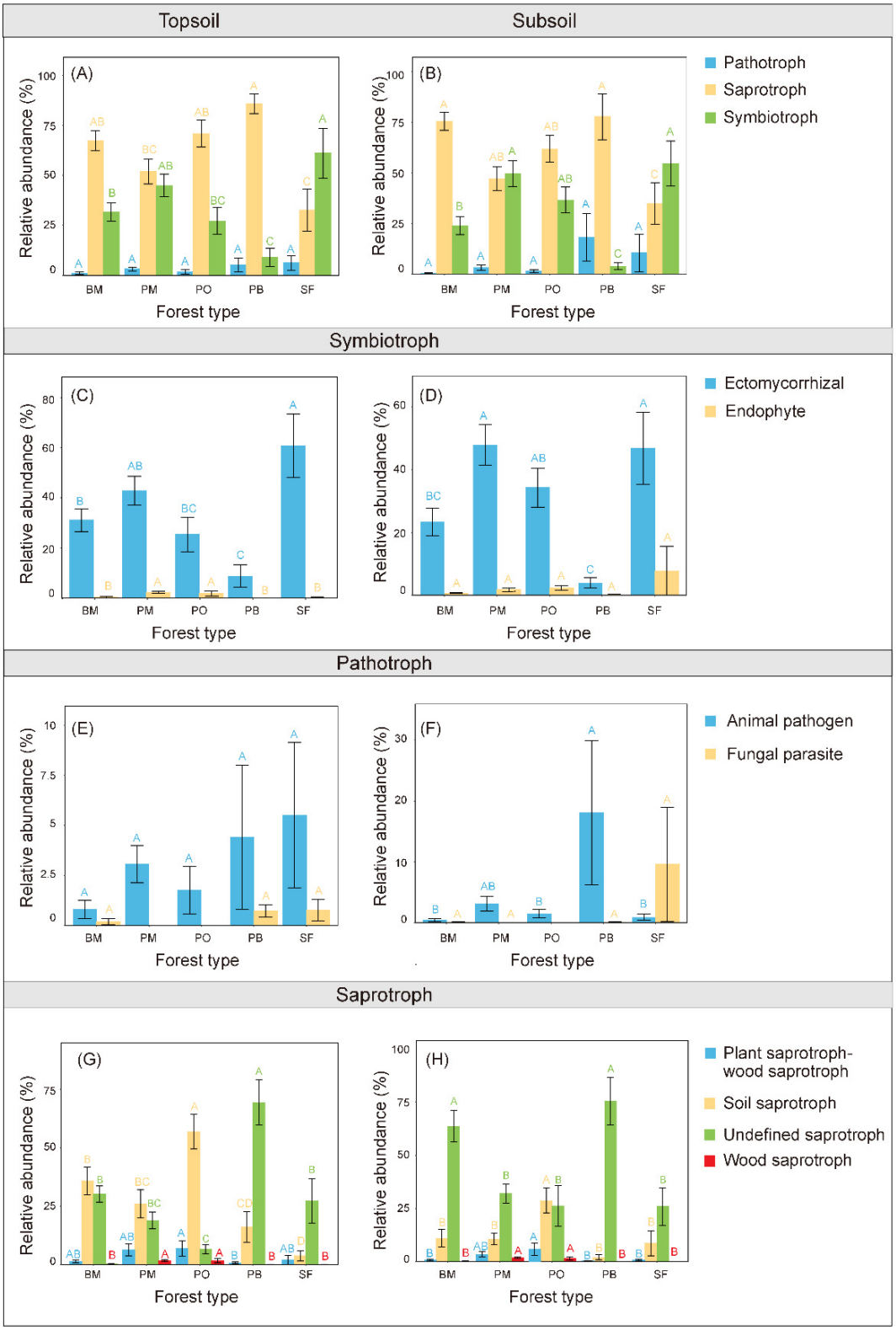

Figure 5. Relative abundance of fungal guilds among different forest types. (**A**,**C**,**E**,**G**) topsoil; (**B**,**D**,**F**,**H**) subsoil; Significant differences ($p < 0.05$) in different forest types are indicated by letters in different colours. Broadleaf-oriented transformation, BM; *P. massoniana* mature plantation, PM; *P. massoniana* overmature plantation, PO; *P. massoniana* + local broadleaved trees, PB; secondary forests, SF.

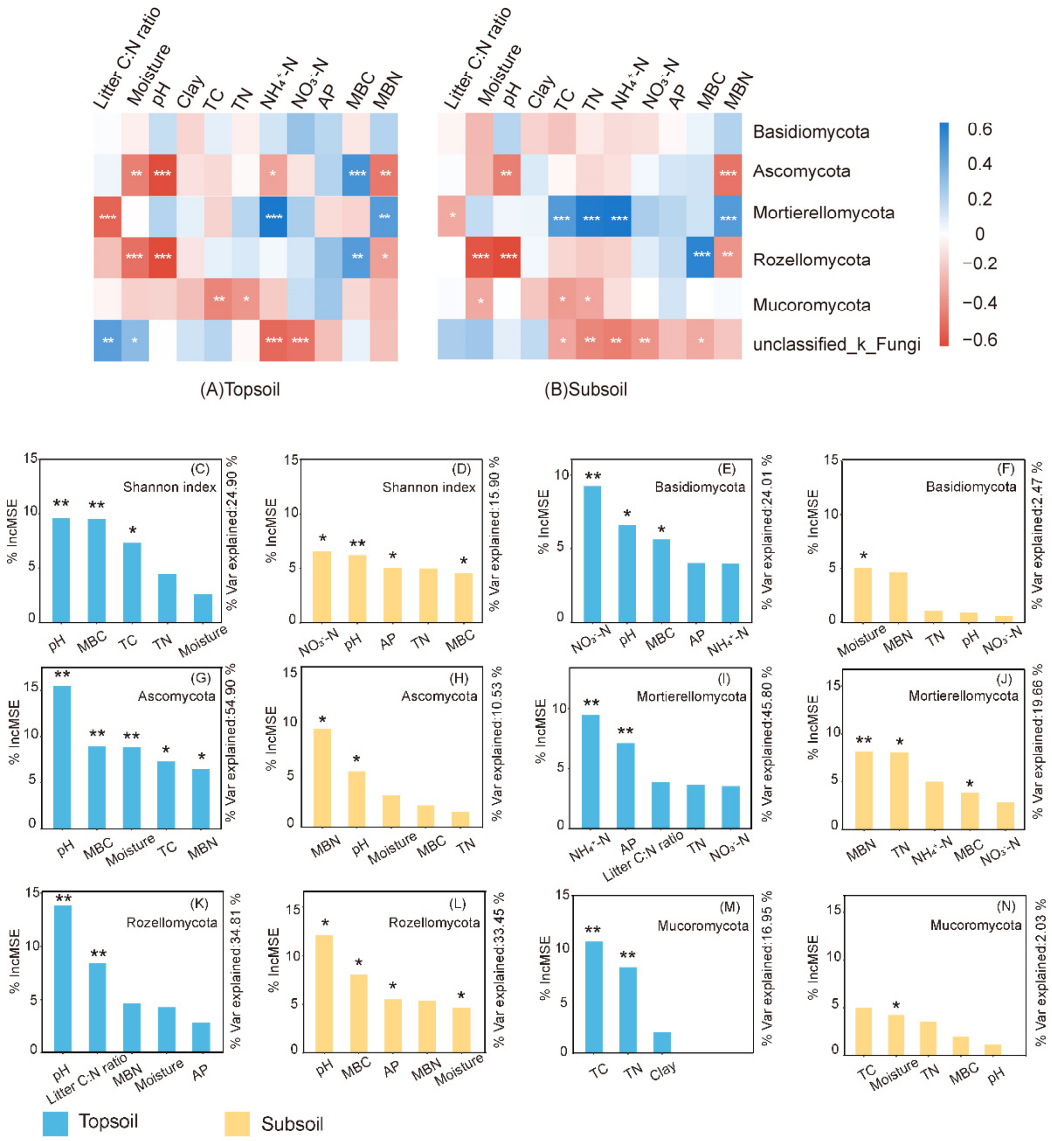

Figure 6. Environmental predictors of the fungal diversity and dominant phyla in different forest types based on Random Forest analysis and Correlation analysis. (**A**,**C**,**E**,**G**,**I**,**K**,**M**) topsoil; (**B**,**D**,**F**,**H**,**J**,**L**,**N**) subsoil. *** $p < 0.001$; ** $p < 0.01$; * $p < 0.05$.

Our RDA explained 57.35% and 35.56% of the variation in fungal guilds in topsoil and subsoil, respectively (Figure 7). In the topsoil, the first two axes separated BM and SF from other forests, suggesting a significant shift in the functional composition in broadleaved forests (Figure 7A). Ectomycorrhizal fungi influenced the functional composition of BM most, which was driven by soil MBC and NO_3^--N contents. By comparison, the functional composition of other forests was mainly affected by saprotrophs, which were driven by soil pH and nitrogen contents (Figure 7A). In the subsoil, the first axis significantly separated BM and PB from other forests, and the pattern was driven by soil nitrogen and clay contents (Figure 7B; $p < 0.05$). The SF, PM and PO were arranged along the second axis, which was mainly affected by saprotrophs and strongly driven by pH (Figure 7).

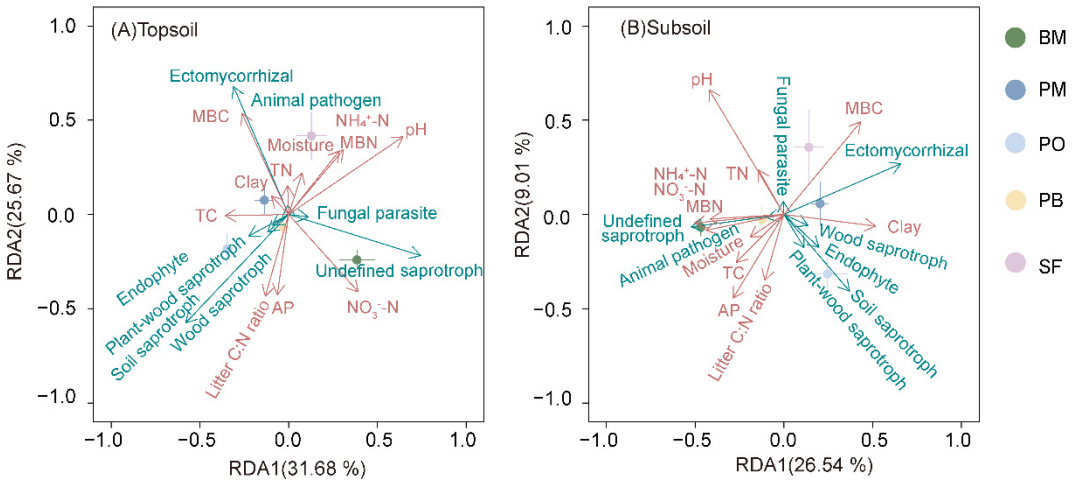

Figure 7. The redundancy analysis of soil guilds with litter and soil physicochemical factors in different forest types. Broadleaf-oriented transformation, BM; *P. massoniana* mature plantation, PM; *P. massoniana* overmature plantation, PO; *P. massoniana* + local broadleaved trees, PB; secondary forests, SF. Total carbon content, TC; total nitrogen content, TN; ammonium nitrogen, NH_4^+-N; nitrate nitrogen, NO_3^--N; available phosphorus, AP; microbial biomass carbon, MBC; microbial biomass nitrogen, MBN.

4. Discussion

Soil fungi respond sensitively to global change and vegetation shift, which strongly influence the soil food webs and carbon turnover [14,34]. Compared with previous studies on mixture planting, we assessed the consequence of two different multispecies restoration modes compared to two stand-age monocultures in both topsoil and subsoil to make our findings more convincing. Specifically, we compared the fungal community and functionality in multispecies restoration to that of the secondary forest to illustrate whether the soil heath could recover to a level of natural forest to an extent. Herein, our findings supported our first hypothesis that multispecies restoration significantly altered the fungal taxonomic composition in the topsoil and subsoil of *P. massoniana* plantations, and there was similar to the secondary forests in subtropical areas (Figure 4). However, contrasting with the hypothesis, we found fungal diversity was lower in the two modes of multispecies restoration (Figure 3). In addition, the two restoration modes also reduced the relative abundance of ectomycorrhizal fungi and wood saprotrophs in the Pine monocultures (Figure 5), which supported our second hypothesis. Furthermore, we found these changes in fungal composition were regulated by the soil pH and nitrogen contents, which supported our last hypothesis.

4.1. Multispecies Restoration Altered the Fungal Taxonomic Composition

Consistent with the previous studies, phyla of Basidiomycetes, Ascomycota and Mortierellomycetes favoured the fungal communities in our sites [17,35] and differed among different forests (Table 1). In support of our first hypothesis, our findings demonstrated that the dominance of topsoil fungal communities shifted from Ascomycota at the two *P. massoniana* plantations to Basidiomycota at modes of multispecies restoration and natural secondary forests. By contrast, Ascomycota and Rozellomycota had comparatively higher relative abundance in the two *P. massoniana* plantations; Mortierellomycota was most dominant in the PB and SF (Figure 2). It is reported that oligotrophic Ascomycetes are prevalent in carbon- or nutrient-poor environments or under drought pressure [36]. This was also supported by the negative correlations between the relative abundance of Ascomycotes with moisture, NH_4^+-N, and MBN contents, indicating their high resistance to environmental stress [37]. On the other hand, Basidiomycetes including ectomycorrhizal and saprotrophic fungi, are traditionally known as the key decomposers for recalcitrant

lignocellulose and cutin in forest soils [38]. In the topsoil, the relative abundance of Basidiomycete was highest in PB, which was caused by the "priming effect" via liable litter mixing into the pine needles that were rich in lignin [39]. However, this effect could not act in the subsoil. Similarly, a previous study reported that most ectomycorrhizal fungi, as members of Basidiomycota, play critical roles in nitrogen mining and water availability in coniferous soils [40].

These findings indicated that multispecies restoration effectively altered the fungal taxonomic composition in both topsoil and subsoil according to the clustering of PO and PM, and further highlighted that multispecies restoration altered the soil trophic status in our subtropical areas [36]. Additionally, the overlap of SF and PB indicated this restoration mod was an effective close-to-nature strategy and that the fungal taxonomic composition was expected to recover to the level of secondary forests even in the subsoil.

4.2. Multispecies Restoration Decreased the Fungal Diversity

Fungal diversity reflects soil health and stability [6]. To test our hypothesis, we presented the fungal taxonomic diversity by three widely used indices of Chao 1, Shannon and PD taking the richness, diversity and evolution into consideration. In agreement with the previous studies, forest type had a significant impact on fungal taxonomic diversity [7,10]. However, in contrast with our hypothesis that multispecies restoration would increase the fungal diversity, the three indices were consistently highest in the two *P. massoniana* monocultures, followed by the two modes of multispecies restoration compared with the secondary forests (Figure 3). Huang et al., (2022) found mixing with Pines increased the fungal diversity of *S. superba* plantation as a result of reduced soil quality [35]. Many studies defined fungi as oligotrophs that prefer acidic soils with low nutrient availability [5,35]. In line with the previous study, this habitat preference explained the pattern of fungal diversity in our study, which was further supported by the significant and negative correlation between these indices and soil pH and nitrogen contents (Table 2) [35]. Remarkably, fungal diversity did not significantly differ between topsoil and subsoil, although topsoil contains higher microbial biomass (Table A2; Figure 3). This phenomenon was also reported in other research where fungal biomass decreased but the proportion of mycorrhizal fungi increased with depth [41]. We speculated that the unchanged pattern could be explained by the joint effects of decreased soil nutrients but increased pH along the depth.

4.3. Multispecies Restoration Altered the Soil Fungal Guilds

Supporting our hypothesis, fungal guilds significantly differed among forests independent of the soil depth, and saprotroph- and symbiotroph-dominated communities were favoured in plantations and secondary forests, respectively (Table A3; Figure 5). This finding agreed with the previous report that tree species affected the respective rhizosphere microenvironments and underground food webs within one year of their establishment [42]. In addition, fungal guilds converting ectomycorrhizal fungi to saprotrophs were also observed in Canadian forests after forest disturbances [7].

The relative abundance of saprotrophs was the highest in PB and increased with the age of *P. massoniana* stands (Figure 5A,B). In particular, the specific wood saprotroph was enriched in the *P. massoniana* plantations (Figure 5G,H), which was ascribed to plentiful inputs of recalcitrant and hydrophobic compounds that derived from needles and fine-root residues of Pine [23,43]. These findings together with the abundant Basidiomycete reflected a higher decomposing rate in the coniferous forests; on the other hand, the "priming effects" could be responsible for the increased proportion of saprotroph in PB via mixing the fresh liable substrate into the pine needles in mixed stands [44].

The abundance of ectomycorrhizal fungi observed in Pine soils was likely to be related to the symbiosis of host trees, especially in subsoils (Figure 5C,D) [12,41]. The associated ectomycorrhizal fungi improved the survivability and resistance of Pine to environmental stresses in both young and mature forests [45]. However, we found the relative abundance of symbiotrophs was lower in plantations, and minimized in the PB, compared with the

secondary forest (Figure 5). We speculated that the lower abundance of ectomycorrhizal fungi was reasonable due to greater disruption of fungal hyphae extensions and fine root biomass after multispecies planting [45,46]. The relative abundance of ectomycorrhizal fungi decreased with the stand age of Pine, following the previous study that revealed that tree density was the conceivable driver for the fungal associations in the managed forests [47]. Interestingly, we found fungal parasites were more favoured in the topsoil of multispecies restoration and secondary forests (Figure 5E,F). These results pointed out that multispecies restoration altered the defensive capacity and resistance to insect infestations and fungal infections, and the effects relied on the soil depth to an extent [7,48].

4.4. Linking Litter and Soil Biogeochemistry to Soil Fungal Communities

We found that soil pH and nitrogen contents were the most important drivers for the fungal taxonomic and functional composition, which was consistent with the previous studies [12,49,50]. In other words, the multispecies restoration altered the fungal communities via the indirect effects on the soil pH and nitrogen contents. Soil pH exerted the most remarkable effect on fungal communities, such as the phyla of Ascomycota, Rozellomycota, or the functional group of saprotrophs in accordance with previous studies (Figures 6 and 7) [49,51]. Pine planting usually leads to soil acidification through the leaching of organic acids during the decomposition process of coniferous needles [14], accordingly, the broadleaf restoration may suppress the growth of a certain fungal guild by changing the soil acidification status [12,14].

Soil nitrogen and phosphorus contents also affect the fungal communities, especially in poor habits [35]. Microbial activities are more limited by phosphorus than nitrogen in the subtropical forests [52], which reasonably explained the positive correlation between the fungal diversity indices and the soil AP content (Table 2). However, we noticed that the relative abundance of dominant phyla was more related to the soil nitrogen content rather than AP content (Figure 6). For instance, Mortierellomycota benefited from the elevated soil NO_4^+-N content in our study although they can dissolve mineral phosphorus and accelerate carbon and phosphorus cycling by secreting oxalic acid [53,54]. In addition, soil nitrogen (e.g., MBN, NH_4^+-N and TN) contents were negatively related to the dominant phyla of Ascomycota, Mucoromycota and Rozellomycota, which is consistent with the finding that nitrogen addition suppressing oxidase for decomposing the lignin and aromatic compounds [39,51]. Moreover, we speculated that these relationships were mediated by the mycorrhizal traits of trees [55,56]. When further dividing the fungi into functional groups, we found the soil NO_3^--N and AP contents strongly and negatively influenced the relative abundance of ectomycorrhizal fungi, while the other guilds were mainly controlled by the soil pH (Figure 7). In addition, the dominant ectomycorrhizal trees, such as the *P. massoniana* in our study, depleted the nitrogen from soil organic matter by producing enzymes, which aggravated the nitrogen limitation for free-living saprotrophs [40].

5. Conclusions

By using high-throughput sequencing, we provided evidence for the effects of multispecies restoration on the fungal taxonomic and functional guild composition in both topsoil and subsoil. Our findings highlighted that multispecies restoration altered the soil trophic status, defensive capacity, and resistance in the subtropical areas studied. We found that fungal alpha diversity showed the highest value in the *P. massoniana* monocultures, and the majority of topsoil fungal communities converted from Ascomycota-dominated at *P. massoniana* plantations to Basidiomycota-dominated at other forests. We also observed the specific wood saprotroph was most abundant in the *P. massoniana* plantations. The restoration modes with broadleaf trees were effective close-to-nature strategies, and the fungal taxonomic composition was expected to recover to the level of secondary forests, particularly in the subsoils. However, the composition of fungal guilds were different among different forests and the recovery of fungal functionality should be further studied in the future. Moreover, we identified that soil pH and nitrogen availability strongly affect the dominant phyla and guilds. Soil NO_3^--N and AP contents strongly influenced the

ectomycorrhizal fungi, while the other fungal guilds were mainly controlled by soil pH. We proposed that fungal communities should be further linked to ecosystem functions such as the carbon mineralization process to explore the biological mechanisms for the "tree species–ecosystem function" relationship. Specifically, trees associated with arbuscular mycorrhizal and ectomycorrhizal fungi dominate in subtropical forests to acquire nutrients. It is important to consider the functional interactions between mycorrhizal fungi and free-living decomposers to evaluate carbon sequestration potential under different restoration modes of species mixing.

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Appendix A

Table A1. The number of major microbial groups at the level of phyla, orders, families, genera and species obtained by high-throughput sequencing.

	Depth	Phyla	Orders	Families	Genera	Species
BM	Topsoil	8	61	112	164	228
	Subsoil	10	77	138	204	273
PM	Topsoil	10	68	143	244	344
	Subsoil	10	85	164	277	375
PO	Topsoil	11	77	141	236	323
	Subsoil	12	83	178	313	441
PB	Topsoil	11	87	189	347	504
	Subsoil	11	89	185	314	457
SF	Topsoil	10	70	140	247	378
	Subsoil	11	78	163	273	415

Broadleaf-oriented transformation, BM; *P. massoniana* mature plantation, PM; *P. massoniana* overmature plantation, PO; *P. massoniana* + local broadleaved trees, PB; secondary forests, SF.

Table A2. Effect of forest type and soil depth on alpha diversity in the two-way ANOVA test.

Alpha Diversity	Forest Type			Depth			F:D		
	DF	F	*p*	DF	F	*p*	DF	F	*p*
Chao 1	4	33.60	***	1	0.17	ns	4	0.80	ns
Shannon	4	10.76	***	1	0.48	ns	4	0.30	ns
Phylogenetic diversity	4	0.80	***	1	0.60	ns	4	0.28	ns

*** $p < 0.001$; ns $p > 0.05$.

Table A3. Effect of forest type and soil depth on the relative abundance of fungal guilds in the two-way ANOVA test.

Fungal Trophic Type	Fungal Guilds	Forest Type			Depth			F:D		
		DF	F	*p*	DF	F	*p*	DF	F	*p*
Symbiotroph	Ectomycorrhizal	4	12.94	***	1	0.30	ns	4	0.85	ns
	Endophyte	4	0.75	ns	1	0.91	ns	4	0.89	ns

Table A3. *Cont.*

Fungal Trophic Type	Fungal Guilds	Forest Type			Depth			F:D		
		DF	F	*p*	DF	F	*p*	DF	F	*p*
	Total	4	13.85	***	1	0.00	ns	4	0.00	ns
Pathotroph	Animal pathogen	4	2.08	ns	1	0.42	ns	4	1.42	ns
	Fungal parasite	4	1.15	ns	1	0.76	ns	4	0.92	ns
	Total	4	1.74	ns	1	0.00	ns	4	0.00	ns
Saprotroph	Plant saprotroph-wood saprotroph	4	4.40	**	1	1.22	ns	4	0.18	ns
	Soil saprotroph	4	15.71	***	1	22.14	***	4	3.10	*
	Undefined saprotroph	4	17.36	***	1	8.69	**	4	1.54	ns
	Wood saprotroph	4	8.36	***	1	0.00	ns	4	0.04	ns
	Total	4	16.70	***	1	0.00	ns	4	0.00	ns

*** $p < 0.001$; ** $p < 0.01$; * $p < 0.05$; ns $p > 0.05$.

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