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Effects of Naphthalene Application on Soil Fungal Community Structure in a Poplar Plantation in Northern Jiangsu, China

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Abstract: The soil food web is essential for the functioning of terrestrial ecosystems. The application of naphthalene is a commonly employed experimental treatment for expelling soil fauna to examine faunal effects on litter decomposition processes, for which is it assumed that naphthalene has negligible effects on soil microbial communities. An experiment was conducted to examine the potential soil-fauna-repellent effect of naphthalene application (100 g/m^2/month, TR) on a soil fungal community during litter decomposition. The results showed that TR greatly suppressed the abundance and taxonomic richness of soil fauna by 83.7 ± 14.2% and 48.1 ± 17.2%, respectively, and reduced the rates of poplar leaf litter decomposition compared to the control (CK, without naphthalene treatment). Among the fungal communities, the abundance of Thelephorales in the TR soil was suppressed, while the abundance of Capnodiales was stimulated, although TR did not significantly alter the carbon and nitrogen content in the soil microbial biomass nor the diversity of soil fungal communities and the most abundant fungal phylum. Thus, both the suppressed soil arthropod abundance and altered soil fungal community might contribute to the observed slowdown in litter decomposition. These results suggest that naphthalene, as a soil fauna repellent, can alter the abundance of specific taxa in a soil fungal community, thereby impeding the effort to elucidate the contribution of soil fauna to ecosystem functioning (e.g., with respect to litter decomposition).

Keywords: litter decomposition; fungal community; naphthalene; soil fauna; Thelephorales; poplar plantation

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1. Introduction

The interactions and feedback between aboveground and belowground communities play fundamental roles in controlling terrestrial ecosystem processes [1]. Aboveground communities can be both directly and indirectly affected by soil food web organisms, and, in turn, these plant community changes influence soil biota [2,3]. Studies regarding litter decomposition have been conducted for decades. Recently, a greater number of scientists have focused on how this process is driven by complex soil food web interactions that alter soil biogeochemical cycles affected by climate change [4–6]. Exploration of the interrelationships between aboveground community and belowground decomposers can enhance our understanding of biogeochemical cycles in terrestrial ecosystems [1,7,8].

Soil fauna, an important group of belowground decomposers, plays a critical role in the litter decomposition process [9], and the interactions between soil fauna and fungal communities have received considerable attention worldwide [6,10,11]. Researchers

often employ physical (litterbags) and/or chemical (repellents) techniques to exclude soil fauna in order to elucidate the roles of soil fauna in decomposition experiments for decades [12–16]. Naphthalene, a commonly used soil fauna repellent, is either administered to the soil surface adjacent to litterbags or added into litterbags to the reduce the number of soil arthropods [15–18]. Naphthalene application has proven to be easy to implement and effective for repelling soil arthropods [16,19–21]. As the simplest polyaromatic hydrocarbon and a decomposable organic compound with two fused benzene rings, naphthalene may provide exogenous carbon for soil microbes [19], thereby directly effecting soil microbial communities.

Research conducted in a tallgrass prairie concluded that the application of naphthalene was effective in reducing soil micro-arthropods and had negligible direct effects on soil microbial abundance and C dynamics [20]. However, studies from an alpine forest ecosystem located on the eastern Tibetan Plateau of China implied that naphthalene treatment might have affected active bacterial community abundance but not soil bacterial community structure [22]. Moreover, other researchers have demonstrated that naphthalene treatments not only significantly reduce the density and species richness of soil arthropods but also indirectly influence soil C-, N-, and P-degrading enzymes [16,23]. The effects of naphthalene application on fungal communities and the related belowground ecological processes have not been thoroughly investigated.

Furthermore, both graded mesh size litterbags and chemical treatments presented positive impacts of soil fauna on the litter decomposition process, for which the extent of the impacts varied. Some researchers have concluded that the effect of insecticide experiments (naphthalene) tends to be double that of the mean graded mesh effect (2.45 versus 1.19) on litter decomposition rate [24], and a similar result was also found in a recent meta-analysis [25]. This discrepancy between the two methods can result from either (1) the incomplete exclusion of animals when using the graded mesh bags or (2) the presence of enhanced microbial activity when using naphthalene with organic carbon additives [20,22].

Thus, we do not have a quantitative and clear understanding of the effects of the application of naphthalene to the soil surface on soil fungal community composition. In this study, a field experiment was conducted to examine the effect of naphthalene on the soil fungal community in a poplar plantation, which represents the most widely distributed type of afforestation in Northern China owing to the tress' rapid growth, strong adaptability, and high yields [26,27]. As a deciduous tree species, the decomposition of poplar leaf litter plays an important role in the carbon-cycling process [28,29]. Previous studies implied that naphthalene effectively expelled soil fauna in poplar plantations and decreased litter decomposition and soil nitrogen mineralization rates [30–32]. Nevertheless, the effects of naphthalene application on the soil fungal communities in poplar plantations remained elusive.

The aims of this study were (1) to quantify the effects of naphthalene application on soil fungal diversity and (2) assess if naphthalene can be utilized by soil fungi and thus affect its community structure. We hypothesized that naphthalene application would (1) elevate fungal diversity and (2) increase the abundance of specific taxa of soil fungal communities because the addition of naphthalene and the reduction in detritivores would provide extra carbon sources for the growth of soil fungi [33,34] and the reduced number of fungivores would promote the fungal growth of the predated taxa [35–37].

2. Materials and Methods

2.1. Site Description and Experimental Design

This study was conducted at the Sihong Forest Farm (33°19′20″ N, 118°18′30″ E, approximately 16 m a.s.l.) in Northern Jiangsu, China. The study site is in a poplar plantation dominated by a hybrid of *Populus deltoides* Bartr × *P. euramericana* [Dode] Guineir. The climate is influenced by a strong subtropical monsoon with a mean annual temperature of 14.4 °C and a mean annual precipitation value of 972.5 mm. The poplar plantation was es-

tablished in March 2007 with 1-year-old seedlings planted at a density of 400 stem/ha. The total area of the experimental site was estimated to be 2.5 ha. A randomized block design was employed, which consisted of three 20 m × 20 m replicate plots that were randomly arranged at the same topographic position and elevation. In addition, six randomly located 1 m × 1 m subplots were selected in each plot. The average diameter at breast height and tree height were 24.4 cm and 23.9 m, respectively, in 2018. The soil is a clayloam Gleysols according to WRB [38], which contains approximately 13.73 mg/g of total organic carbon and 1.12 mg/g of total nitrogen in the top 10 cm of soil. Additional detailed information on soil properties can be found in Feng et al. (2019) [29].

Three subplots were treated with solid naphthalene balls (TR: 100 g/m^2/month from January 2019) [23,39,40] on the surface of the ground in each plot, while the remaining three subplots received no naphthalene (CK). In May 2019, three soil samples (0–5 cm depth) were collected using soil corers (2.5 cm in diameter) following the removal of herbs and surface litter in each subplot for microbial and chemical analysis. To conduct fungal community analysis, soil samples from the same subplots were then mixed and placed in a thermotank (−30 °C) for transport. For soil arthropod extraction, three additional soil cores (4 cm in diameter) were collected up to a depth of 5 cm in each subplot, stored at room temperature, and transported to the laboratory within 24 h.

Seventy-two 4 mm mesh size litterbags (15 cm × 20 cm) were each filled with 10 g of air-dried poplar leaf litter. Of the 24 bags at each plot, 4 were randomly placed on the soil surface in either the CK or TR subplots. The litterbags were collected after 4, 9, 16, and 24 months to examine poplar leaf litter decomposition and nutrient release process (unpublished data). This study focuses on the retrieval of the first 18 litterbags.

2.2. Chemical Analysis and Soil Arthropod Extraction

After 4 months of decomposition in the field, 18 litterbags (9 from each treatment) were collected, placed into separate plastic bags to minimize any loss of small litter fragments and animals, and immediately returned to the laboratory for weighting and chemical analysis. Soil microbial biomass carbon (MBC) and nitrogen (MBN) fractions were determined using the chloroform fumigation extraction method [41,42], wherein MBC can be estimated by determining the difference between organic C extracted with 0.5 M K_2SO_4 from $CHCl_3$-fumigated and non-fumigated soil and MBN can be measured similarly. Soil macrofauna was extracted by hand, and meso- and micro-fauna were extracted from each soil sample using modified Tullgren extractors over a period of 48 h (Tullgren Funnel Unit, BURKARD, Rickmansworth, UK) [43]. All extracted faunal samples, including both imago and larvae, were preserved in 75% ethanol and then sorted under a microscope (Carl Zeiss Microscopy GmbH Konigsallee 9-21, Gottingen, Germany) into taxonomic orders (e.g., Acari, Oribatid, Mesotigmatid, Collembola, Hymenoptera, Coleoptera, and Araneae) [44].

2.3. Extraction of Fungal Genome

DNA was then extracted from three TR and three CK soil samples using an E.Z.N.A. Soil DNA Kit (Omega Bio-tek, Norcross, GA, USA). We employed a 20 μL reaction system of TaKaRa rTaq DNA Polymerase to conduct Polymerase Chain Reaction (PCR) tests. The primer sets of ITS1F (5′-CTTGGTCATTTAGAGGAAGTAA-3′) and ITS2R (5′-GCTGCGTTCTTCATCGATGC-3′) were designed to amplify the ITS rRNA genes of soil fungi. The PCR reaction system was designed as follows: 10× buffer (2.0 μL), 2.5 Mm dNTPs (2.0 μL), 5 μM forward and reverse primers (0.8 μL), r Taq polymerase (0.2 μL), BSA (0.2 μL), and template DNA (10.0 ng), whose volumes were supplementarily filled to 20 μL with sterilized ultrapure water. The amplification procedure was as follows: 3 min of denaturation at 95 °C and 35 cycles consisting of 30 s at 95 °C, 30 s for annealing at 55 °C, 45 s for elongation at 72 °C, and 10 min for a final extension at 72 °C. There were three replicates for each sample, and we combined the amplicons prior to sequencing. Furthermore, 2% agarose gel electrophoresis was employed to detect the quality of the

PCR products. The PCR products were purified using an AxyPrep DNA Gel Extraction Kit (Axygen Biosciences, Union City, CA, USA).

2.4. Illumina MiSeq Sequencing and Processing

Purified amplicons were equimolarly pooled and paired-end-sequenced (2 × 300) using an Illumina Miseq platform following the standard protocols of Majorbio Bio-Pharm Technology Co. Ltd. (Majorbio, Shanghai, China). The sequencing data were spliced and quality-controlled, and low-quality sequences were removed. Illumina Miseq sequencing yielded 353,063 medium- to high-quality sequences.

QIIME (Quantitative Insights into Microbial Ecology, version 1.9.1, http://qiime.org) was employed to cluster the operational taxonomic units (OTUs), for which more than 97% of the similarity between sequences was classified into one OTU. The taxonomy of each ITS rRNA sequence was analyzed using the Ribosomal Database Project (RDP) Classifier algorithm (version 2.11, http://rdp.cme.msu.edu/). Species data were compared to identify fungal communities, and the grouping data for each OTU sequence at the boundary, phylum, class, order, family, genus, and species levels were obtained using the UNITE database (version 8.0, https://unite.ut.ee/).

2.5. Data Analysis

Shannon index, Simpson index, Chao1 index, and Coverage were employed in this study, which were indexed to evaluate the alpha diversity, abundance, and coverage of fungal communities. They were calculated as follows:

$$Shannon\ index = -\sum_{i=1}^{S_{obs}} \frac{n_i}{N} ln \frac{n_i}{N}, \quad (1)$$

where S_{obs} is the actual observed number of OTUs, n_i refers to the sequences included in the ith OTU, and N is the total number of sequences.

$$Simpson\ index = \frac{\sum_{i=1}^{S_{obs}} n_i(n_i - 1)}{N(N-1)}, \quad (2)$$

In the above equation, the abbreviations are the same as those listed for Equation (1).

$$Chao1\ index = S_{obs} + \frac{n_1(n_1 - 1)}{2n_2 + 1}, \quad (3)$$

In the above equation, n_1 is the number of OTUs with only one sequence, n_2 is the number of OTUs with only two sequences, and the other variables are the same as those given above.

$$Coverage = 1 - \frac{n_1}{N}, \quad (4)$$

In this equation, the abbreviations are the same as those given above.

Differences in soil fungal community structure between samples were visualized using Bray–Curtis and principal co-ordinates analysis (PCoA). Soil fauna abundance and taxonomic richness data were first natural-log-transformed. Differences between the compositions of soil fungal communities and diversity indices, litter mass remaining, MBC, MBN, and soil fauna abundance and taxonomic richness between the TR and CK were analyzed using t-tests. All the sequence-related data were analyzed using the Majorbio cloud platform (www.majorbio.com). Statistical tests were considered significant at $p < 0.05$. The raw sequence data for this study are available in the Sequence Read Achieve (SRA) database of the National Centre for Biotechnology Information (NCBI) under accession number PRJNA720294.

3. Results

3.1. Soil Fauna Abundance and Litter Mass Remaining

Naphthalene treatment significantly suppressed soil fauna abundance (83.7 ± 14.2%, $p < 0.001$) and taxonomic richness (48.1 ± 17.2%, $p = 0.021$) in the poplar plantation, and poplar leaf litter decomposed slower in the TR compared to the CK treatments after four months (6.2 ± 1.9%, $p < 0.001$) (Figure 1a–c). There was no statistically significant difference in the carbon and nitrogen content of the soil microbial biomass between the CK and TR treatments (Figure 1d,e).

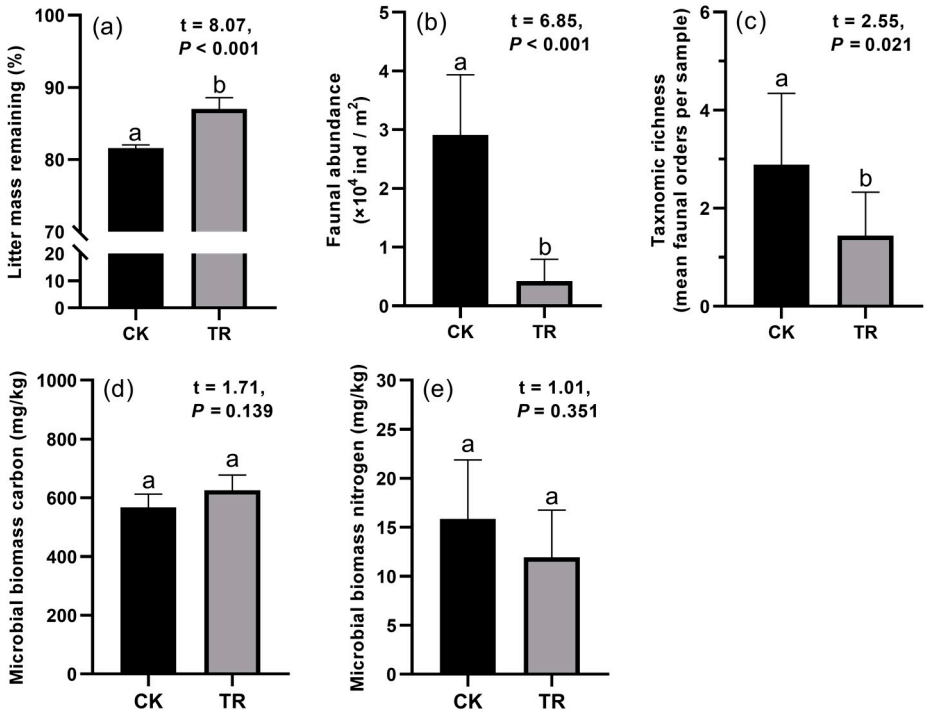

Figure 1. Effect of naphthalene treatment (TR) on (**a**) litter mass remaining, (**b**) soil faunal abundance, (**c**) taxonomic richness at the order level, (**d**) carbon content in soil microbial biomass, and (**e**) nitrogen content in soil microbial biomass compared with the control (CK) in a poplar plantation in northern Jiangsu, China. Bars (±standard error) with different letters indicate significant differences between CK and TR ($p < 0.05$).

3.2. Soil Fungal Community Diversity

The total values of effective sequences from the TR and CK treatments were 63,091 and 57,293, respectively (Table 1). Fungal alpha diversity indices, including Shannon index, Simpson index, and the Chao1 index, presented no statistically significant differences between the CK and TR soils. The coverage of all samples was more than 99%, which demonstrated that the depth of the sequences was sufficient. PCoA revealed that soil fungal communities at the order level differed between the TR and CK treatments (Figure 2). The contributions of the first two selected principal components to the differences in fungal community compositions were 30.31% and 26.41%, respectively.

Table 1. Sequence numbers and alpha diversity indices of fungal communities for the control (CK) and naphthalene treatments (TR) in a poplar plantation in northern Jiangsu, China. The same letters indicate a lack of significant differences between the CK and TR treatments ($p > 0.05$).

Group	Sequence	Simpson Index	Shannon Index	Chao 1 Index	Coverage
CK	57,293 ± 12,997 a	0.0543 ± 0.030 a	4.0675 ± 0.294 a	767.45 ± 72.52 a	99.78%
TR	63,091 ± 9125 a	0.0473 ± 0.013 a	4.1957 ± 0.041 a	850.18 ± 87.88 a	99.76%

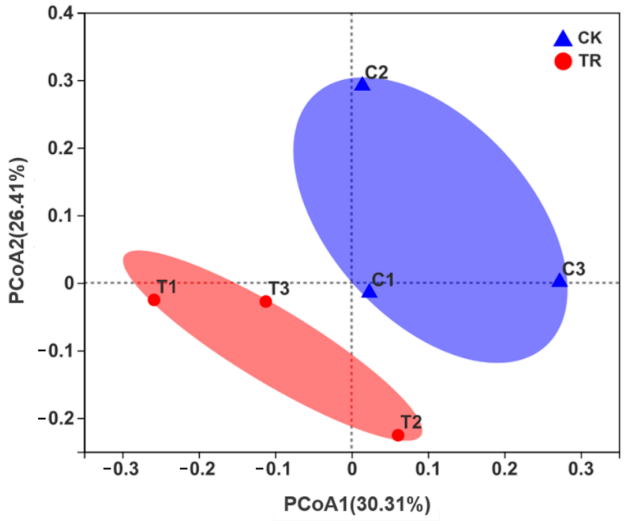

Figure 2. Principal coordinate analysis (PCoA) of fungal communities (based on Bray–Curtis distance and concerning differences at the order level) under control (CK) and naphthalene treatments (TR) in a poplar plantation in northern Jiangsu, China.

3.3. Soil Fungal Community Composition

The number of shared OTUs observed was 890, which accounted for 57.35% of all observed OTUs (2442; Figure 3). The number of unique OTUs for soil under the TR and CK treatments were 392 and 270, respectively. There was a total of 14 known phyla and 33 known classes of fungi across all the samples. The main fungal phyla included Ascomycota, Basidiomycota, Mortierellomycota, and Rozellomycota (Figure 4a). Cumulatively, the relative abundances of these phyla accounted for an average of 94.05% of all fungi. The main fungal classes encompassed Sordariomycetes, Dothideomycetes, Agaricomycetes, Eurotiomycetes, Mortierellomycetes, Tremellomycetes, Leotiomycetes, and Pezizomycetes (Figure 4b). Overall, the relative abundances of these classes accounted for an average of 82.57% of all fungi. Although the fungal communities at the phylum level were similar between the TR and CK soils, the relative abundance of several specific groups at the order level varied (Figure 5). Welch's *t*-test revealed that the abundance levels of Thelephorales ($p = 0.013$) and Capnodiales ($p = 0.01$) were significantly different between the TR and CK soil samples (Figure 5).

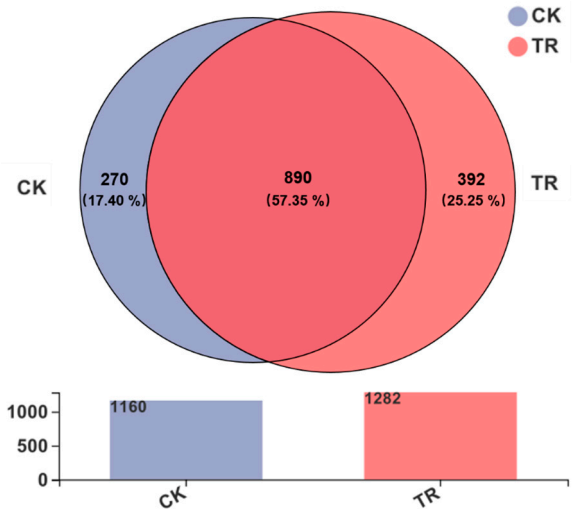

Figure 3. Venn graphs of the number of shared and unique operational taxonomic units (OTU) under the control (CK) and naphthalene treatments (TR) in a poplar plantation in northern Jiangsu, China. The overlapping section indicates shared OTUs; non-overlapping parts represent specific OTUs under the TR and CK treatments. The numbers on the graphs refer to the corresponding quantity of OTUs.

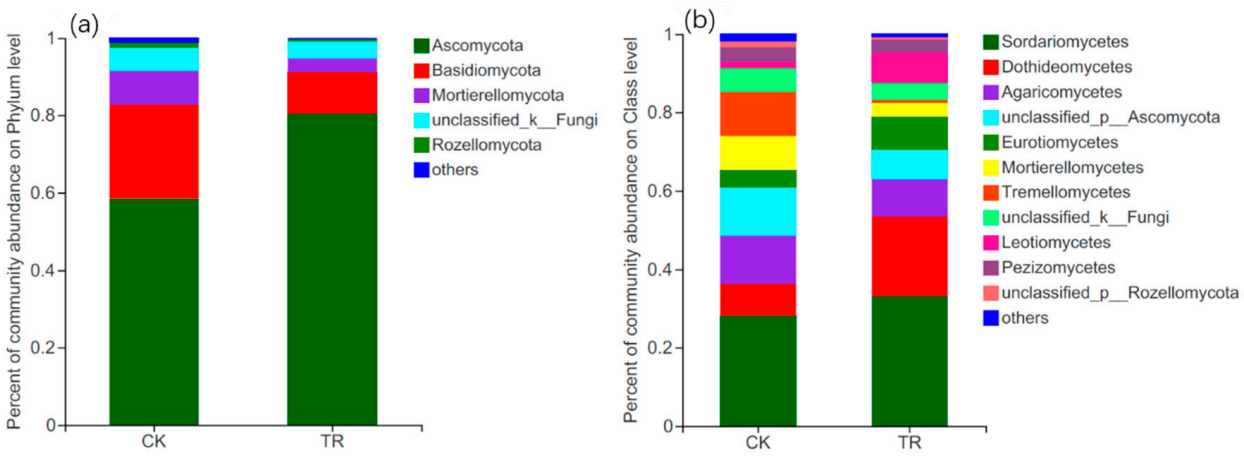

Figure 4. Relative abundance of soil fungal communities at the (**a**) phylum and (**b**) class level under the control (CK) and naphthalene (TR) treatments in a poplar plantation in northern Jiangsu, China.

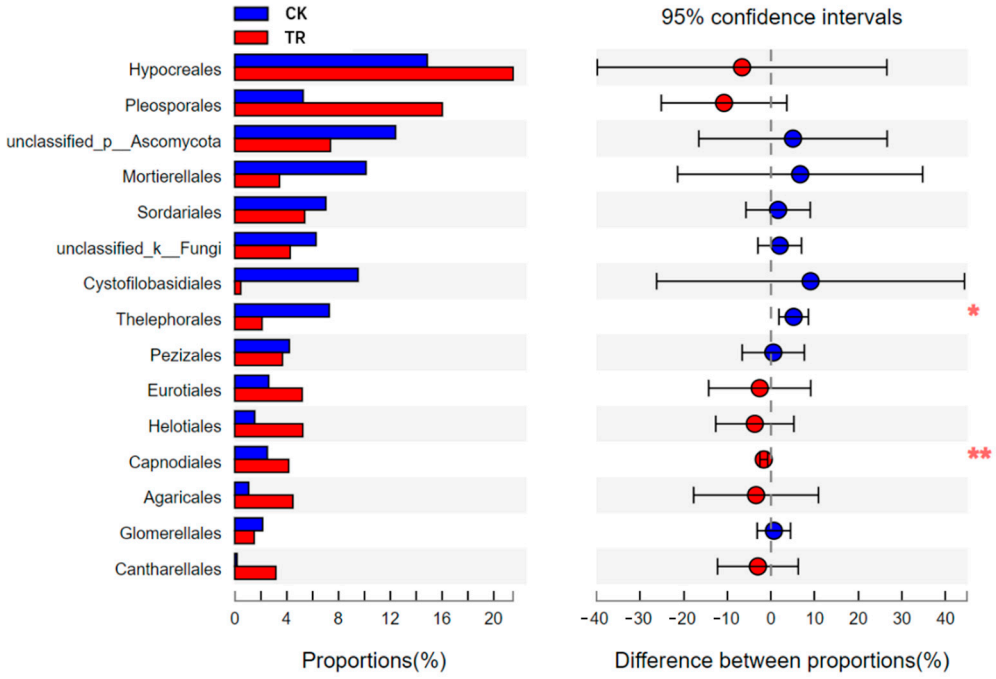

Figure 5. Comparison of relative abundances of soil fungal communities under control (CK) and naphthalene (TR) treatments at the order level in a poplar plantation in northern Jiangsu, China (Welch's *t*-test). * $0.01 < p \leq 0.05$; ** $0.001 < p \leq 0.01$.

4. Discussion

Naphthalene treatment is a commonly used method for suppressing soil fauna numbers when examining the contribution of soil fauna to litter decomposition; however, its effect on soil fungal community structure is often ignored. This study showed that, as expected, naphthalene treatment greatly suppressed soil arthropod numbers and reduced leaf decomposition rates [23,40]. Contrary to hypothesis one, fungal diversity was not increased under TR. However, naphthalene was found to exert significant effects on the abundance of specific taxa of soil fungi, but it did not significantly affect the carbon and nitrogen content of soil microbial biomass.

4.1. Naphthalene's Effects on Soil Fungal Communities and Litter Decomposition

The mechanisms by which naphthalene may affect soil fungal communities include the (1) direct addition of new organic carbon, (2) an indirectly elevated level of carbon

availability through reduced arthropod detritivores, (3) the direct inhibition of a fungal community, (4) an indirectly decreased level of predation by fungivores, and (5) the indirect alteration of substrates via the reduced production of arthropod feces and dead bodies [23,39,45]. In this study, microbial biomass carbon content and fungal diversity did not increase, suggesting that the improvement in carbon availability through mechanisms (1) and (2) did not benefit soil fungal community growth.

Both Ascomycota and Basidiomycota, which are saprophytic or symbiotic [46–48], were the predominant phyla in the TR and CK soils. However, they differed in terms of relative abundance, with higher Ascomycota numbers and lower Basidiomycota numbers resulting from the TR treatment compared to that of the CK treatment (Figure 4a). It was reported that Basidiomycota, particularly the saprotrophic, cord-forming basidiomycetes, gradually replaced Ascomycota during the course of litter decomposition [49,50]. As the most potent decomposers of cellulose, Basidiomycete fungi have often been demonstrated to be prominent decomposers during late stages of litter decomposition as their capacity to synthesize enzymes is required for the degradation of relative complex polymers [51,52]. The activity of extracellular enzymes produced by Basidiomycetes, such as β-glucosidase, cellobiohydrolase, N-acetylglucosaminidase, acid phosphatase, and phosphodiesterase, was proven to be most frequently affected by soil fauna activity (e.g., grazing), which can directly influence carbon, nitrogen, and phosphorus cycling [52]. The observed high relative abundance of Ascomycota and low relative abundance of Basidiomycota resulting from the TR treatment were likely a result of the unimproved substrate quality due to the suppressed level of soil fauna abundance and the reduced degree of litter decomposition under the TR treatment.

More specifically, on the order level, the abundance of soil-resident Thelephorales under the TR was significantly lower than that for the CK treatment, while Capnodiales presented the opposite outcome (Figure 5). Naphthalene application not only drives away soil arthropods but also reduces the amount of arthropod feces and the number of dead bodies, which can alter the carbon substrates (lower C:N ratios and higher dissolved organic carbon content) and thus induce fungal growth [53]. The decreased abundance of Thelephorales might have been due to both the direct inhibition of naphthalene and the lack of a suitable substrate for its growth. Thelephorales, belonging to Basidiomycota, constitutes one of the groups of ectomycorrhizal fungi (ECM) that plays a critical role in a number of key ecosystem functions, such as nutrient mobilization from soil organic matter, carbon cycling, and linkages between trees and soil through common mycorrhizal networks [54–56]. Similar results were found in a laboratory Petri plate study, wherein naphthalene reduced radial growth in eight out of nine Basidiomycete fungi [57], while another study concerning a secondary fir forest on the eastern Tibetan Plateau, China, showed different results, wherein naphthalene stimulated basidiomycete mycelia; this disparity might have been provoked by the vast environmental differences of the study sites [58]. The translocation of nutrients via mycelial connections at the soil–litter interface is a vital mechanism that controls the decomposition of plant residues [59,60]. Researchers observed that a greater release of N and P from roots was strongly correlated with an increased abundance of Thelephorales and Cantharellales, which suggested that ECM might be mining for nutrients from decaying roots (low C:N substrates) [60,61]. The lower abundance of Thelephorales under TR implied that the release of N and P from decaying litter may be decreased.

Higher abundance of Capnodiales was found under the TR treatment, thus partially confirming hypothesis two. Capnodiales, known as “sooty molds” due to their profuse superficial hyphal development, constitutes the second largest order in Dothideomycetes, which constitutes the largest class of Ascomycota [62–64]. Sooty molds often derive nutrients from the excretion of insects producing honeydew, but they can also survive without insects and absorb other nutrients [65,66]. Naphthalene may help reduce the numbers of predatory soil invertebrates, thus stimulating sooty mold growth [67]. Capnodiales exhibited saprobic traits [63,64], and saprotrophic fungi are important regulators of nutrient

cycling and SOM dynamics during the litter decomposition process [68,69]. However, there is almost no information on the interactions between litter decomposition, Capnodiales, and soil fauna; thus, further investigations are required.

4.2. Limitations and Future Work

The effect of naphthalene, coupled with the paucity of knowledge regarding its direct interfacial effects on soil fungal community structure, may translate to a misunderstanding of the interactions between soil fauna and the functions of soil fungi. The appearance of this stronger effect in the insecticide experiments (compared to the different mesh size litterbag experiments) examined in a meta-analysis [24,25] might have been partially due to the decreased number of specific taxa of the soil fungi decomposer community caused by naphthalene. This study demonstrated that naphthalene not only affected micro-arthropods but also altered specific soil fungal communities. This might cause an overestimation of the contribution of soil fauna in litter decomposition. This experiment discussed the potential mechanisms whereby soil fungal communities are affected by naphthalene used as a soil fauna repellent after four months of litter decomposition. However, further studies are needed to elucidate the direct effect of naphthalene, i.e., aside from the indirect faunal effect, on soil microbial communities and the litter decomposition process at different stages during a longer experiment.

5. Conclusions

Soil fauna and microbes constitute the complex soil food web, and their functions in belowground ecological processes have received considerable attention. This research concluded that the application of naphthalene greatly suppressed soil fauna numbers and decreased litter-decomposing rates. While the carbon and nitrogen content of soil microbial biomass were not significantly affected, naphthalene treatment altered specific soil fungal taxa, including by suppressing the abundance of Thelephorales and increasing the abundance of Capnodiales. This was the case despite the fact that Ascomycota and Basidiomycota remained the most abundant fungal phyla under both the naphthalene and control treatments. The decreased litter decomposition rate may be attributed to both the suppression of soil fauna abundance and the alteration of soil fungal taxa. The influence of naphthalene application on the specific taxa (Thelephorales and Capnodiales) of soil fungal communities should be taken into consideration when estimating the contribution of soil fauna to litter decomposition in future studies.

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Data Availability Statement: The dataset supporting the conclusions of this article is available at the NCBI Sequence Read Archive: https://www.ncbi.nlm.nih.gov/sra/PRJNA720294 (accessed on 31 December 2021). Final DNA sequence assembly was uploaded online. NCBI SRA accession: PRJNA720294. The data have been deposited in Dyrad at https://datadryad.org/stash/share/7Eg0BSnKiKvdXXvqZeYcixgobaMSMM_zqwkrMQB4l20 (accessed on 9 January 2023).

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