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Changes of Scots Pine Phyllosphere and Soil Fungal Communities during Outbreaks of Defoliating Insects

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Abstract: Outbreaks of forest pests increase with climate change, and thereby may affect microbial communities and ecosystem functioning. We investigated the structure of phyllosphere and soil microbial communities during defoliation by the nun moth (*Lymantria monacha* L.) (80% defoliation) and the pine tree lappet (*Dendrolimus pini* L.) (50% defoliation) in Scots pine forests (*Pinus sylvestris* L.) in Germany. Ribosomal RNA genes of fungi and bacteria were amplified by polymerase chain reaction (PCR), separated by denaturing gradient gel electrophoresis (DGGE), and subsequently sequenced for taxonomic assignments. Defoliation by both pests changed the structure of the dominant fungal (but not bacterial) taxa of the phyllosphere and the soil. The highly abundant ectomycorrhizal fungal taxon (*Russula* sp.) in soils declined, which may be attributed to insufficient carbohydrate supply by the host trees and increased root mortality. In contrast, potentially pathogenic fungal taxa in the phyllosphere increased during pest outbreaks. Our results suggest that defoliation of pines by insect pest, change the structure of fungal communities, and thereby indirectly may be contributing to aggravation of tree health.

Keywords: insect outbreaks; Scots pine; phyllosphere; soil; microbial community; PCR-DGGE

1. Introduction

Over the last decades, biotic disturbances like insect outbreaks increased dramatically worldwide [1]. There is evidence that increasing intensity, frequency, duration, and extent of insect outbreaks are closely related to climate and global change [1,2]. Pest insects like the nun moth (*Lymantria monacha* L.) can evidently benefit from these changing environmental conditions [3]. For example, in Brandenburg, eastern Germany, about 5800 ha of forest were infested with nun moth, including 366 ha of completely defoliated area in 2013, and more than 11,000 ha of forest had to be treated with insecticides [4]. Besides substantial economic losses and expenditure for forest protection, outbreaks exhibit tremendous impact on forest ecosystem functions such as carbon sink potential and net ecosystem production [5–7].

The impacts of forest pest outbreaks on microbial community are not well understood. In recent years, our knowledge of the composition, diversity, and function of phyllosphere microbial communities expanded by the use of culture-independent methods like denaturing gradient gel electrophoresis (DGGE) [8]. Next generation sequencing (NGS) technologies offer the highest sampling depth, allowing the detection of low-abundant taxa [9], but the costs for the analysis of large numbers of sample sets are prohibitive. DGGE enables the monitoring of dominant taxa in a large number of

samples at a fraction of the costs of NGS. The technique has been successfully used to characterize microbial communities of various environments (e.g., [10–15]). In a pioneering study by Yang et al. [15], DGGE revealed that microbial populations inhabiting the phyllosphere are much more complex than previously assumed. Studies on honeydew-excreting aphids suggest that C-rich insect excreta promote the growth of phyllosphere bacteria, filamentous fungi, and yeasts [16,17]. Beside excreta, the growth of microorganisms inhabiting the phyllosphere is enhanced by nutrient leaching from leaves damaged by folivorous insects [18–21].

During an outbreak of pine tree lappet, C and N canopy-to-soil fluxes as well as the C/N ratio in the throughfall solution in Scots pine forests in northern Germany increased significantly [22]. Total net C and N canopy-to-soil fluxes of sessile oak (*Quercus petraea* L.) forests during infestation with winter moth (*Operophtera brumata* L.) and mottled umber (*Erannis defoliaria* L.) were 30- and 18-fold higher compared to uninfested forests [23]. Some studies show stimulation of the soil microbial activity accompanied by alterations in soil nutrient cycling during insect outbreaks [22,23]. For example, increased input of easily biodegradable organic matter with a narrow C/N ratio during pine beauty (*Panolis flammea* D.) and pine tree lappet infestations led to changes in the soil bacterial community of a Scots pine forest in south-western Poland [24]. In addition to matter input, canopy damage by insect defoliation can affect belowground microbial communities, particularly microorganisms that form symbiosis with damaged trees. Soil fungal communities are generally expected to decline in abundance and richness during outbreak events, particularly those of tree symbiotic ectomycorrhizal (EM) fungi [25,26], while saprophytic and endophytic fungi species may benefit [26].

The effects of outbreaks of single pests either on phyllosphere or soil microbial communities have been studied already (e.g., [21,24,27–35]), but these data did not simultaneously compare the effect of pest outbreaks on phyllosphere and soil microbial communities and did not differentiate between the effects of different pest insects in the same forest ecosystem. Here, we analyzed the effect of outbreaks of two defoliating insects, nun moth or pine tree lappet, in Scots pine stands in eastern Germany on the bacterial and fungal community structure in the soil and the phyllosphere using DGGE. We hypothesize that defoliation of pine trees by pest insects would reduce the abundance of tree symbiotic microorganisms (e.g., rhizobacteria and mycorrhizal fungi), and that bacterial and fungal communities of soils and the phyllosphere would respond differentially to defoliation.

2. Materials and Methods

2.1. Study Design and Site Characteristics

The bacterial and fungal community structure of phyllosphere and soil was investigated on infested and adjacent, uninfested control forest sites in 2014 and 2015. All sites were located in the federal state of Brandenburg, Germany. In 2014, a nun moth infested site (80% defoliation) and control site were located in a 65-year-old Scots pine stand located 3 km north of Märkisch Buchholz (52°8'38" N, 13°45'14" E, 42 m a.s.l.), Germany, and 10 km west of Teupitz (52°9'29" N, 13°36'47" E, 35 m a.s.l.), Germany, respectively. In 2015, the infested (52°17'42" N, 12°19'32" E, 47 m a.s.l.) and control (52°17'19" N, 12°20'46" E, 50 m a.s.l.) sites were located in a 92-year-old scot pine stand in Ziesar. The infested site showed abundant infestation (50% defoliation) with pine tree lappet. All soil types at all sites in both years were classified as podzols with a pH (1:10 in H₂O) ranging from 3.2 to 3.9 in the Ah horizon and a fine to medium sand of glacial origin as parent material. The annual mean temperature was 10.8 and 10.6 °C and the total average annual precipitation was 474 and 496 mm (DWD: German Federal Meteorological Service (DWD) and Climate Data Center (CDC), Weather Station Lindenberg (ID:3015), 16.02.2017) for the sites in 2014 and 2015, respectively.

Three plots with comparable site conditions were selected for sampling, designated plot I–III for the infested and plot IV–VI for the control site. To represent the phyllosphere bacterial and fungal community in the canopy, samples of one to three-year-old pine needles were taken from five individual branches with a minimum length of 20 cm in the south exposed tree crown at 11 m height

(average tree height was 13 m with the tree crown starting at approximately 9 m) of three individual trees per site plot. We collected all needles of sampled branches to achieve a representative sample. On infested sites, particularly damaged branches were sampled, whereas on the control sites branches without visible insect damage were sampled. Needle samples of each site plot were pooled after DNA extraction to obtain one mixed sample per site plot. Two replicate soil samples of 200 g fresh weight were taken from each of four soil organic layers (L Ol, Of, Oh) as well as the mineral soil horizon (Ah). After DNA extraction the replicates were pooled to obtain one mixed sample per layer and horizon at each site plot. Since the forest floor vegetation was exclusively characterized by mosses and lichens, soil samples were not sieved and tree roots were not removed so as to include tree root associated microorganisms. Further, soil samples were taken at similar distances from the trees to ensure a similar proportion of root biomass in all samples. Immediately after sampling, soil samples were dried at 40 °C for 24 h and ground; needle samples were freeze-dried for 72 h and ground.

Related to the developmental stages of the insect pests, samples were taken in early May (pre-defoliation), late May (main defoliation), and early October (post-defoliation and tree recovery period), whereas needle samples in 2014 were only available for late May and early October.

2.2. DNA Extraction from Soil and Needles

Total DNA extraction was performed using a cetyltrimethyl ammonium bromide (CTAB) extraction protocol with polyethylene glycol precipitation [36] downscaled to 50 mg material followed by phenol extraction [37]. Finely ground soil or needles were suspended in 1 mL of CTAB buffer (20 mM Na₂EDTA, 10 mM Tris, 0.13 M sorbitol, 0.03 M N-laurylsarcosine, 0.02 M hexadecyltrimethylammonium bromide, 0.8 M NaCl, 1% (v/v) polyvinylpolypyrrolidone, adjusted to pH 8.0 with NaOH), 1 µL 2-Mercaptoethanol, and 1 µL of Proteinase K solution (20 mg/mL) were added. After gentle vortexing, samples were incubated at 42 °C for 10 min and subsequently at 65 °C for 10 min, inverting the tubes frequently. Following adding 800 µL of chloroform/isoamylalcohol (24:1 (v/v)), the mixture was shaken, incubated on ice for 10 min, and centrifuged at 7380× g for 10 min. We mixed 700 µL of the supernatant thoroughly with 700 µL phenol and centrifuged at 7380× g for 10 min. The supernatant (650 µL) was extracted with the same amount of chloroform/isoamylalcohol (24:1 (v/v)) in the same manner. Following this, 600 µL of the liquid phase were transferred to a new Eppendorf tube containing 200 µL of polyethylene glycol (PEG) 6000 (30% (w/v)) and 100 µL of NaCl (5 M). After mixing the content thoroughly, the tubes were incubated at room temperature for 20 min and centrifuged at 16,000× g for 15 min to obtain DNA pellets. The supernatant was discarded and the pellet was washed with 500 µL of 70% (v/v) ethanol twice. The remaining ethanol in the tubes was evaporated under vacuum, the pellets were resuspended in 50 µL TE buffer (10 mM Tris, 1 mM Na₂EDTA, adjusted to pH 8.0 with HCl) and incubated at 42 °C for 2 h to facilitate dissolving the DNA.

The concentration and quality of DNA was assessed by agarose gel electrophoresis. A volume of 5 µL DNA solution were mixed with 2 µL loading buffer (100 mM EDTA, 50% (v/v) glycerol, 0.025% (v/v) bromophenol blue) and loaded into a 0.8% agarose gel in 1× TAE buffer (40 mM Tris, 20 mM sodium acetate, 1 mM Na₂EDTA, adjusted to pH 7.6)). Gel electrophoresis was carried out at 4.6 V/cm for 60 min, gels were stained with ethidium bromide solution (1 mg/L (w/v)) for 12 min and rinsed with double distilled water for 12 min before visualizing DNA by fluorescence in UV light. Replicate samples from each site plot were pooled for each sampling date and stored at −20 °C until analysis.

2.3. PCR-DGGE

Polymerase chain reaction (PCR) was performed in a pEqSTAR 96 universal gradient thermocycler (PEQLAB, Erlangen, Germany) using 1:100 dilutions of the pooled DNA samples in a total reaction volume of 15 µL.

Bacterial 16S rDNA gene fragments were amplified using the primer set F968-GC and R1401-1a that included the hypervariable regions V6–V8 [14]. PCR mixtures were composed of 16 mM (NH₄)₂SO₄, 67 mM Tris-HCl, 0.01% Tween-20, pH 8.8 (NH₄ reaction buffer, Bioline, Luckenwalde,

Germany), 3 mM MgCl₂, 200 µM of each deoxyribonucleoside triphosphate, 0.8 µM of each primer, 1 mg/mL bovine serum albumin (BSA), 0.25 u Biotaq DNA-polymerase (Bioline, Luckenwalde, Germany) and 1 µL template DNA solution. PCR conditions were: initial denaturation for 5 min at 95 °C; 10 touchdown cycles of 60 s denaturation at 95 °C, 60 s annealing beginning at 60 °C with a decrease of annealing temperature by 0.5 °C per cycle, and 2 min extension at 72 °C; 30 cycles consisting of 60 s at 95 °C, 60 s at 55 °C, and 2 min at 72 °C; and final extension for 30 min at 72 °C.

Fungal 18S rDNA gene fragments were amplified using the primer set FR1-GC and FF390 that included hypervariable regions V7 and V8 [38]. The composition of PCR mixture was identical with PCR conditions for 16S rDNA except for 1.5 mM MgCl₂, 0.5 µM of each primer, and 0.05 u Biotaq DNA-polymerase (Bioline, Luckenwalde, Germany). The thermocycle program consisted of an initial denaturation for 3 min at 95 °C; 10 touchdown cycles of 30 s at 95 °C, 45 s annealing beginning at 55 °C with a decrease by 0.5 °C per cycle, and 2 min at 72 °C; 30 cycles of 30 s at 95 °C, 45 s at 50 °C, and 2 min at 72 °C; and final extension for 10 min at 72 °C.

Amplified bacterial 16S rDNA and fungal 18S rDNA gene fragments were separated on a 7.5% (w/v) acrylamide/bisacrylamide gel (37:5:1) gel with a 30–70% denaturing gradient (100% denaturant correspond to 7 M urea, 40% (v/v) formamide) in TAE running buffer (40 mM Tris, 20 mM sodium acetate, 1 mM Na₂EDTA, adjusted to pH 7.4 with glacial acetic acid). Gel electrophoresis was carried out using an INGENY phorU-2 system (Ingeny, International BV, Goes, The Netherlands) at 70 V (2.6 V/cm) for 16 h at 58 °C and 60 °C for fungal 18S rDNA and bacterial 16S rDNA gene fragments, respectively. PCR products were inspected on 1.7% (w/v) agarose electrophoresis gels.

2.4. Silver Staining and Gel Drying

A slightly modified protocol of the ProMega (Madison, USA) SILVER SEQUENCE™ DNA silver staining protocol [39] was used. All of the following steps were performed under continuous gentle shaking in plastic trays and all solutions were prepared with double distilled water at 4–10 °C. The use of deionized (or demineralized) water instead of doubly-distilled water for staining DGGE gels led to in-gel precipitation and a high background, which was most likely caused by organic traces leaking from the ion exchanger. To avoid these effects, double distilled water was used for all staining steps. Glass plates were removed, gels were fixed in 10% (v/v) glacial acetic acid for 20 min, rinsed two times with double distilled water for 2 min each and stained using a 0.1% (w/v) silver nitrate, 0.06% (v/v) formaldehyde solution for 35 min. Immediately after staining, the gels were rinsed with double distilled water for 30 s and transferred to a developer solution (3% (w/v) sodium carbonate, 0.06% (v/v) formaldehyde, 0.0002% (w/v) sodium thiosulfate) and the development of the image was monitored. Once DNA bands became visible, the reaction was stopped by adding 2 volumes of 10% (v/v) glacial acetic acid. The gels were kept in the solution for 4 min and rinsed with double distilled water two times for 4 min each, soaked in 3 mL of a 3% (v/v) glycerol solution and dried in a frame between two hydrated cellophane foils at room temperature for 48 h.

2.5. Selection of Soil DGGE Gels and Profiles for Sequencing

Fungal DGGE profiles of infested and uninfested plots differed distinctly for samples from late May (main defoliation) but not for samples from early May (pre-defoliation) and October (post-defoliation). Several reasons are conceivable: high population density of pests and high defoliation rates during the main defoliation might have enhanced the effect of defoliators on the fungal communities in these samples. Therefore, we further analyzed only samples taken during main defoliation in late May. Additionally, exclusively DGGE gels of the upper soil organic layers (L and OL) were selected for DNA sequencing since ordination analysis revealed that separation between clusters of infested and uninfested control plots decreased with the position of soil organic layers and mineral soil. Decreased fungal abundance and diversity in lower soil horizons is mainly caused by the decrease of oxygen level with soil depth, which may explain why the differences between fungal populations of infested and uninfested plots declined with soil depth [40]. DNA bands exhibiting largest differences

in DGGE profiles from infested pines and uninfested controls during main defoliation in late May were selected for sequencing. When sequencing did not enable discrimination of taxa on family level, taxa were assigned to the next higher taxonomic level (order).

2.6. Band Excision, Reamplification, and Sequencing

Based on the NMDS, bands were excised from dried gels using a razor blade and placed in double distilled water for 20 s. Cellophane was removed and imbibed polyacrylamide was ground by sterile plastic cones in 50 µL double distilled water and incubated at 4 °C over night. Polyacrylamide was removed by centrifugation and supernatants were used to reamplify DNA fragments using primers identical to the primers used to generate DGGE samples but without GC-clamps; the same PCR conditions without the touchdown phase was used. PCR products were inspected on 1.7% (*w/v*) agarose gels; DNA was precipitated with isopropanol in a final concentration of 70% (*v/v*) at room temperature for 10 min and centrifuged at $16,160\times g$ for 10 min. The pellet was washed with 70% (*v/v*) ethanol and dissolved in 12 µL double distilled water and send to Macrogen Europe (Macrogen Europe, Amsterdam, The Netherlands) for sequencing. To verify the identity of bands occurring at the same position on a DGGE gel but within different samples, at least two bands occurring at the same position were sequenced. The sequences were assigned to fungal and bacterial taxa as follows. Published sequences with $\geq 99\%$ identity were retrieved from the GeneBank database at the National Center for Biotechnology Information (NCBI) using the software BLAST [41] with default settings. The origin of the strains from which these sequences originated and their taxonomic characterization were checked with the help of metadata attached to the accessions and sequences without reliable taxonomic assignment were discarded.

2.7. Data Analysis of DGGE Profiles

DGGE gels were placed on a light-table and photographed for visual band identification. Although the test design prevented inaccuracies in comparing profiles between two or more gels [37], gel images were first adjusted for exposure and contrast and then converted into black and white to achieve the maximum number of identifiable bands and minimize the bias of the staining intensity.

A matrix based on relative intensities of DGGE bands (0 = absent, 1 = least intense, 4 = most intense) was constructed for each gel for semiquantitative analysis [42,43]. Ordination analysis of the data was performed using non-metric multidimensional scaling ordination (NMDS) of Euclidean distance matrices. The analysis was executed using the R (Version 3.3.1 GUI 1.68 Mavericks build, R Foundation for Statistical Computing, Vienna, Austria, 2016) package *vegan* v.2.0-4 [44].

3. Results

3.1. Bacterial Communities

Ordination analysis as well as manual reviewing of DGGE profiles revealed no significant differences between infested and control plots in samples under insect outbreaks at any sampling date. Thus, fragments of bacterial DNA separated by DGGE were not sequenced.

3.2. Fungal Communities

3.2.1. Phyllosphere Fungi

The fungal community of the phyllosphere showed well separated clustering between infested and uninfested control forest plots during the main period of defoliation in late May compared to early May and October 2014 and 2015 (Figure 1). Taxonomic assignments based on the sequences of the phyllosphere community in both years revealed that all fungi were assigned to the phylum Ascomycota (Figure 2, Tables 1 and 2).

Products assigned to Hypocreales (family: Nectriaceae) and Dothideales (family: Dothideaceae) were present in both sample types from pine trees in 2014 but they differed in their relative intensities: the taxon assigned to Nectriaceae and one of the two taxa belonging to Dothideaceae were more abundant in uninfested control samples while the other taxon of Dothideaceae was more abundant in infested pine tree samples. One amplicon, assigned to Xylariales (family: Amphispheariaceae), occurred in both years in both sample types but was more abundant in the samples from infested pine trees. The majority of taxa were assigned to Capnodiales, occurring in both years with different relative intensities: in 2014 these taxa occurred either only in infested (1 taxon) or uninfested (4 taxa) phyllosphere samples; in samples taken in 2015 taxa assigned to Capnodiales (family: Mycosphaerellaceae) were either only detected in infested (2 taxa) or uninfested control samples (1 taxon) but also exhibited different abundance between infested and uninfested samples (5 taxa). Both PCR products assigned to Pleosporales (family: Pleosporaceae) occurred only in the samples of infested phyllosphere from 2015.

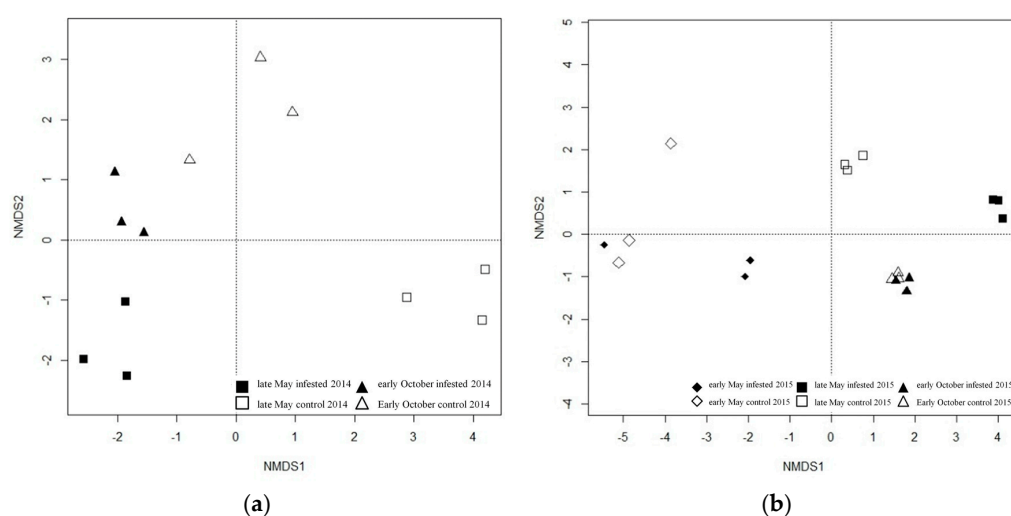


Figure 1. Non-metric multidimensional scaling ordination (NMDS) of Euclidean distance matrices of 18S rDNA denaturing gradient gel electrophoresis (DGGE) profiles of the phyllosphere fungal community in Scots pine (*Pinus sylvestris* L.) forests in 2014 (a) and 2015 (b).

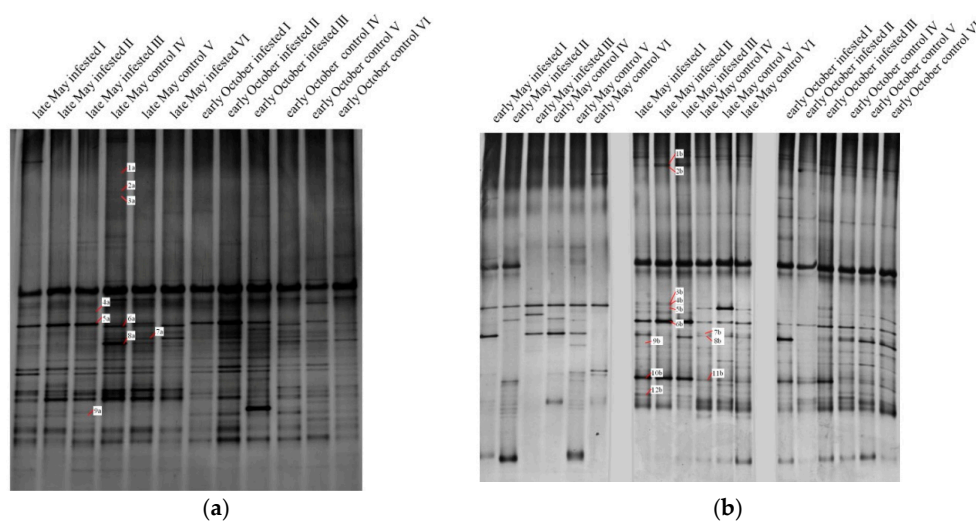


Figure 2. Denaturing gradient gel electrophoresis (DGGE) profiles of 18S rDNA gene fragments of the phyllosphere fungal community in Scots pine (*Pinus sylvestris* L.) forests in 2014 (a) and 2015 (b). Labelled bands were excised for sequencing.

Table 1. Taxonomic assignment of fungal DNA sequences obtained from the needles of Scots pine (*Pinus sylvestris* L.) in late May 2014. Phylum and order of the taxa are listed. Positions of the corresponding denaturing gradient gel electrophoresis (DGGE) bands are shown in Figure 2a.

Band	Phylum	Order	Infested Plots			Control Plots		
			I	II	III	IV	V	VI
1a	Ascomycota	Capnodiales	—	—	—	+	+	+
2a	Ascomycota	Capnodiales	—	—	—	+	+	+
3a	Ascomycota	Capnodiales	—	—	—	+	+	+
4a	Ascomycota	Dothideales	—	+	+	—	—	—
5a	Ascomycota	Xylariales	+++	+++	+++	+	++	—
6a	Ascomycota	Capnodiales	—	—	—	+++	++	+++
7a	Ascomycota	Dothideales	—	—	+	++	++	+++
8a	Ascomycota	Hypocreales	+	+	—	+++	++	+
9a	Ascomycota	Capnodiales	+	+	+	—	—	—

+, ++ and +++ indicate increasing relative band intensity; — indicates absence of bands.

Table 2. Taxonomic assignment of fungal DNA sequences obtained from the needles of Scots pine (*Pinus sylvestris* L.) in late May 2015. Phylum and order of the taxa are listed. Positions of the corresponding denaturing gradient gel electrophoresis (DGGE) bands are shown in Figure 2b.

Band	Phylum	Order	Infested Plots			Control Plots		
			I	II	III	IV	V	VI
1b	Ascomycota	Capnodiales	+	++	++	+	+	+
2b	Ascomycota	Capnodiales	+	++	++	+	+	+
3b	Ascomycota	Capnodiales	+	+	+	—	—	—
4b	Ascomycota	Capnodiales	+	+	+	—	—	—
5b	Ascomycota	Capnodiales	+	+	+	++	+++	++
6b	Ascomycota	Xylariales	+++	+++	+++	++	++	++
7b	Ascomycota	Capnodiales	+	+	+	+	+	+
8b	Ascomycota	Capnodiales	++	—	—	+	++	+
9b	Ascomycota	Pleosporales	+	+	+	—	—	—
10b	Ascomycota	Pleosporales	+++	+++	+++	—	—	—
11b	Ascomycota	Capnodiales	—	—	—	+	+	+
12b	Ascomycota	Capnodiales	+	+	+	—	—	—

+, ++ and +++ indicate increasing relative band intensity; — indicates absence of bands.

3.2.2. Soil Fungal Community

In accordance with the fungal community of the phyllosphere, the soil fungal community showed separation between clusters of infested and uninfested forest plots in late May compared to early May and October 2014 and 2015 (Figure 3). Cluster separation between infested and uninfested plots during the main defoliation in late May decreased with increasing soil depth (data not shown). Therefore, only DNA bands exhibiting the largest differences in DGGE profiles of L and OI layers in late May were selected for sequencing (Figure 4); the L layer was analyzed using samples from 2014 and the OI layer using samples from 2015.

A taxon, assigned to Trichocomaceae, was only present in two of the three samples of the uninfested control plots in 2014 (Table 3). Taxa assignable to the genus *Rhizomucor* (family: Mucoraceae) accounted for the majority of sequenced bands in 2014 with varying relative intensities between infested and uninfested control samples: one of the three infested samples exhibited the same relative abundance pattern of *Rhizomucor* as the uninfested control samples which all show occurrences of these taxa; in the other two soil samples from the infested site only one of the four *Rhizomucor* taxa is present. One amplicon present in DGGE profiles of samples taken in 2015 was assigned to the genus

Russula (family: Russulaceae) (Table 4). This taxon was only detected in uninfested plots, despite a relatively low abundance in one of the three infested plots.

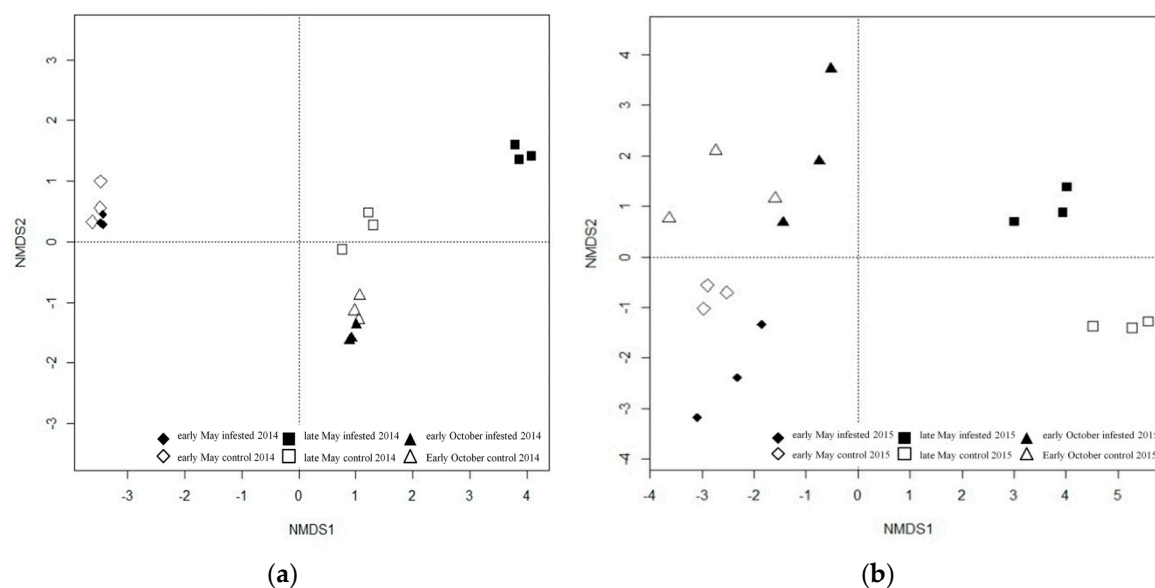


Figure 3. Non-metric multidimensional scaling ordination (NMDS) of Euclidean distance matrices of 18S rDNA denaturing gradient gel electrophoresis (DGGE) profiles of the phyllosphere fungal community of the L soil organic layer in 2014 (a) and Ol soil organic layer in 2015 (b).

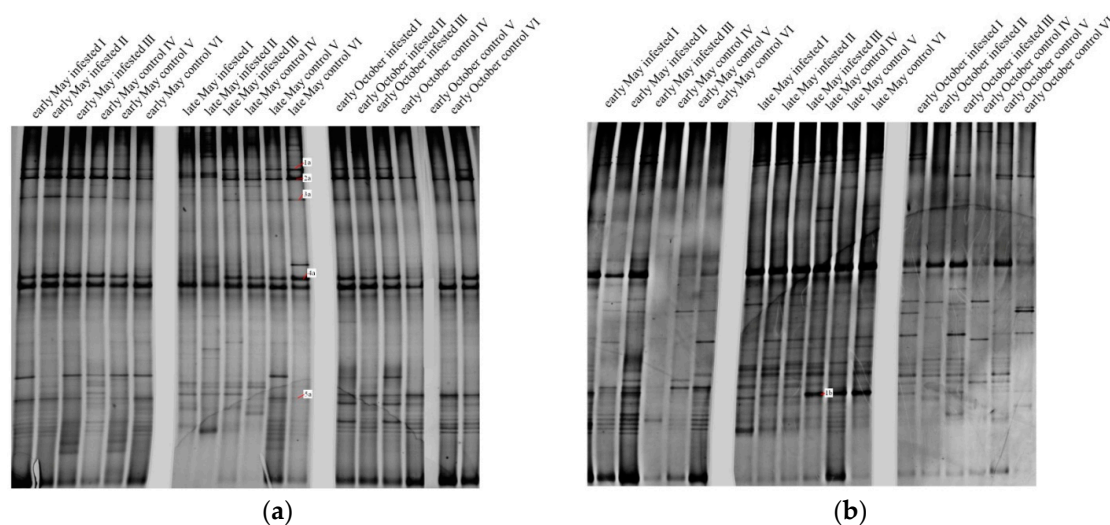


Figure 4. Denaturing gradient gel electrophoresis (DGGE) profiles of 18S rDNA gene fragments of the soil fungal community in Scots pine (*Pinus sylvestris* L.) forests of the L soil organic layer in 2014 (a) and the Ol soil organic layer in 2015 (b). Labelled bands were excised for sequencing.

Table 3. Taxonomic assignment of fungal DNA sequences obtained in the organic L layer of soil in Scots pine (*Pinus sylvestris* L.) forests in late May 2014. Phylum and family of the taxa are listed. Positions of the corresponding denaturing gradient gel electrophoresis (DGGE) bands are shown in Figure 4a.

Band	Phylum	Family	Infested Plots			Control Plots		
			I	II	III	IV	V	VI
1a	Zygomycota	Mucoraceae	—	—	++	++	++	+++
2a	Zygomycota	Mucoraceae	—	—	+++	+++	+++	+++
3a	Zygomycota	Mucoraceae	+	+	++	++	++	++
4a	Zygomycota	Mucoraceae	—	—	+++	+++	+++	+++
5a	Ascomycota	Trichocomaceae	—	—	—	—	++	+

+, ++ and +++ indicate increasing relative band intensity. — indicates absence of bands.

Table 4. Taxonomic assignment of fungal DNA sequences obtained in the L layer of soil in Scots pine (*Pinus sylvestris* L.) forests in late May 2015. Phylum and family of the taxa are listed. Positions of the corresponding denaturing gradient gel electrophoresis (DGGE) bands are shown in Figure 4b.

Band	Phylum	Genus	Infested Plots			Control Plots		
			I	II	III	IV	V	VI
1b	Basidiomycota	<i>Russula</i>	—	+	—	+++	+++	+++

+, indicates low relative band intensity and +++ high relative band intensity; — indicates absence of bands.

4. Discussion

The analyses of bacterial and fungal community of soils and phyllospheres during insect pest outbreaks in Scots pine forests showed that defoliation changed the structure of dominant fungal communities of the phyllosphere and the soil, while bacterial communities were not affected. Moreover, infestation of pine trees with pests in the present study stimulated the colonization of needles with potential parasites and pathogens. Our results indicate that insect damages are accompanied by alterations in dominating fungal species. However, it should be noted that our analysis provides a picture that is limited by observation time, site conditions, as well as tree and insect species.

4.1. Bacterial Community of Pine Needles and Soil

Infestation of pine trees with foliar feeding pests neither affected the bacterial communities of needles nor soils in our study. Nonetheless, results documented in the literature on effects of insect damage on bacteria colonizing the phyllosphere remain controversial. It is known that defoliating insects contribute to the dispersal of bacteria on the phyllosphere [45]. Studies on honeydew-excreting aphids showed that C-rich insect excreta significantly increased the growth of phyllosphere bacteria, filamentous fungi and yeasts [16,17]. For example, heavy infestations of eastern hemlock (*Tsuga canadensis* L.) with hemlock woolly adelgid (*Adelges tsugae* A.) strongly stimulated bacteria, filamentous fungi, and yeasts in the phyllosphere [46]. A study conducted by Müller et al. [21] investigated the effects of lepidopterous larvae infesting European beech (*Fagus sylvatica* L.) and sessile oak on culturable phyllosphere bacteria. Defoliation of sessile oak increased the proportion of bacteria utilizing mineral N [21]. Growth-promoting effects on culturable bacteria, filamentous fungi, and yeasts of the phyllosphere were also observed on European beech and sessile oak during moth infestation [20]. Thus, changes in bacterial community structures in the phyllosphere appear to be related to species-specific insect pest-tree interactions.

In accordance with other studies, soil bacterial community structure appeared to be relatively stable with no changes under insect infestation. Similarly, culture-independent pyrosequencing in a 5-year investigation of bark beetle (*Ips typographus* L.) induced mortality of limber pines (*Pinus flexilis* E.) did not reveal substantial changes in the bacterial community of soil during outbreaks [27]. In contrast, altered soil bacterial community structure was found to be coupled with altered soil edaphic properties

under heavily (85% tree mortality) bark beetle-impacted lodgepole pine (*Pinus contorta*) trees [33]. Further, outbreaks of pine beauty and pine tree lappet on Scots pine increased the abundance of culturable actinobacteria, which was attributed to litterfall of easily biodegradable organic matter with a narrow C/N ratio [24]. An increase of N content in needles as a compensatory response to defoliation was often observed [47–49]. Needles with a higher N content, which get into the soil via litterfall, stimulate decomposition later in the season and are introduced into soil in increased rates during pest outbreaks [50]. In addition, the extent of infestation was found to determine the biogeochemical responses as well as the soil bacterial communities [33,35]. Mikkelsen et al. [34] elegantly highlighted the importance of rare bacterial taxa on the community dynamics and presumably biogeochemical cycling under bark beetle-induced tree mortality. Because DGGE visualizes only amplicons originating from dominant species, the effect of defoliation on rare taxa might have remained undetected, although they might be critical in maintaining ecosystem functioning.

4.2. Fungal Community of Pine Needles

Pine needle fungi indicated considerable changes in community structure, with a high abundance of Ascomycota in the phyllosphere during outbreaks (cf. [30,31]). The phyllosphere of Norway spruce (*Picea abies* L.) stands following a spruce bud scale outbreak (*Physokermes piceae* S.) in Lithuania showed also high abundances of plant pathogenic fungi (*Rhizosphaera kalkhoffii*, *Exobasidium bisporum*, *Phialophora sessilis*) [30]. These are known to cause damage to plant tissues. Therefore, alteration of fungal communities during pest outbreaks, additionally may threat tree health [30]. In our study, a taxon assigned to the family Amphisphaeriaceae occurred in all plots with higher abundance in the infested plots. Many Amphisphaeriaceae are opportunistic pathogens colonizing damaged trees in temperate zones [51,52]. One taxon of the family Dothideaceae occurred only in infested plots, while another Dothideaceae taxon was predominantly found in uninfested plots. Dothideaceae are cosmopolitans that can be endophytic, biotrophic, necrotrophic or saprobic on various plants [53]. Some taxa (e.g., *Scirrhia pini* F. & P.) are known to cause economically relevant damage to conifers [54,55]. A taxon of the family Nectriaceae was less abundant during insect defoliation in 2014. Taxa belonging to the family Nectriaceae are cosmopolitans and show association with plant necromass or occur as pathogens of other fungi [56,57]. Two taxa assigned to the family Pleosporaceae were only detected in infested plots. The occurrence was related to outbreaks of the pine tree lappet. Pleosporaceae include necrotrophs and saprophytes [58,59], including economical important pathogens that can threaten tree health. Fungal taxa of the family Mycosphaerellaceae varied in response to insect infestation. Mycosphaerellaceae exhibit as diverse lifestyles as Dothideaceae [60], and may have contributed to the different abundances.

4.3. Soil Fungal Community

Soil fungal community structures responded significantly to insect outbreaks. The abundance of several taxa assigned to the genera *Rhizomucor* and the family Trichocomaceae was distinctly reduced in infested plots. *Rhizomucor* and Trichocomaceae are saprophytic and saprotrophic fungi, respectively, with global distribution in soils and decomposing vegetation [61–63]. The reduced abundance of saprophytic and saprotrophic fungi in soils from infested Scots pine plots stands is a new finding, and contrasts earlier studies (e.g., [26,28]) on Norway spruce and subarctic mountain birch (*Betula pubescens* ssp. *czerepanovii* H.-A.). Apparently, species-specific interactions and/or the biogeographical effects related to composition region appear to contribute to structuring of these fungal communities.

The abundance of an ectomycorrhizal fungus assigned to the genus *Russula* was strongly reduced in soil samples collected from infested trees during infestation in the L layer in 2015, supporting the hypothesis that defoliation would suppress the abundance of these fungi. This finding is in line with the results of an artificial defoliation experiment in Scots pine by Kuikka et al. [25] reporting reduced production of sporocarps as well as reduced diversity in ectomycorrhizal fungi under defoliated

trees (see also [28,29,32]). For example, in lodgepole pine (*Pinus contorta* Dougl. ex. Loud. var. *latifolia* Engelm.) pine beetle (*Dendroctonus ponderosae* Hopkins) outbreaks led to a decline in species richness [32] as well as in the abundance of ectomycorrhizal fungi [29]. Bark beetle outbreaks in Norway spruce stands also reduced ectomycorrhizal fungi, but increased saprophytic fungi [28]. Successive defoliation of subarctic mountain birch by autumnal (*Epirrita autumnata* B.) and winter moth (*Operophtera brumata* L.) larvae in northern Finland led to a decline of the abundance and richness of ectomycorrhizal fungi by 70–80%, but benefited saprophytic and endophytic fungi [26]. Ectomycorrhizal fungi depend on the supply of carbohydrates via host roots [64,65]. It is likely that high losses of needle biomass during defoliation decrease photosynthesis and consequently carbohydrate supply of this symbiotic association. Additionally, it is known that an impaired nutritional status can cause increased root mortality and depressed growth of fine roots [66]. Therefore, reduced root biomass in soil samples of infested sites may contribute to the observed lower abundance of ectomycorrhizal fungi.

5. Conclusions

The interactions of species in the course of biotic disturbances in forests need to be reconsidered. Defoliation of Scots pine by insects changed the structure of phyllosphere and soil fungal communities but not those of bacterial communities. In particular, the highly abundant symbiotic association of an ectomycorrhizal taxon (*Russula* sp.) declined during an outbreak of pine tree lappet, and thereby may affect ecosystem functioning. Since pest outbreaks are short term events (2–3 years) in the life span of a forest ecosystem, observed adverse changes of fungal communities appear to occur rather rapidly, emphasizing the importance of monitoring and management of forest pest.

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