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The Abundance of Fungi, Bacteria and Denitrification Genes during Insect Outbreaks in Scots Pine Forests

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Received: 20 June 2018; Accepted: 9 August 2018; Published: 14 August 2018

Abstract: Outbreaks of defoliating insects may affect microbial populations in forests and thereby mass balances and ecosystem functioning. Here, we investigated the microbial dynamics in Scots pine (*Pinus sylvestris* L.) forests during outbreaks of the nun moth (*Lymantria monacha* L.) and the pine-tree lappet (*Dendrolimus pini* L.). We used real-time PCR (polymerase chain reaction) to quantify genes that characterize bacterial and fungal abundance and the denitrification processes (*nirK*, *nirS*, *nosZ* clades I and II) in different forest compartments and we analyzed the C and N content of pine needles, insect feces, larvae, vegetation layers, organic layers, and mineral soil horizons. The infestation of the nun moth increased the bacterial abundance on pine needles, in the vegetation layer, and in the upper organic layer, while fungal populations were increased in the vegetation layer and upper organic layer during both outbreaks. In soil, the abundance of *nirK* increased after insect defoliation, while the C/N ratios decreased. *nosZ* clades I and II showed variable responses in different soil layers and to different defoliating insects. Our results illustrate changes in the microbial populations in pine forests that were infested by defoliating insects and changes in the chemical soil properties that foster these populations, indicating a genetic potential for increased soil N_2O emissions during the defoliation peak of insect outbreak events.

Keywords: insect outbreak; Scots pine forest; soil; phyllosphere; bacterial 16S; fungal 18S; *nirK*; *nirS*; *nosZ*; real-time PCR

1. Introduction

Organic input into the soil increases with outbreaks of tree-canopy defoliating insects, through insect feces, cadavers, litter and other plant material [1–3]. This results in high amounts of labile C and extractable N [4–6], which can increase mineralization rates [7,8]. Because soil microorganisms can respond quickly to natural disturbances [9], increased soil microbial respiration rates are commonly observed following the input of organic material during forest pest outbreaks [4,10]. Despite this phenomenon, little is known about the changes in the abundance of decomposer microbes during such biotic disturbances.

Fungal and bacterial populations in forest soils may respond differentially to organic inputs depending on the substrate composition (amount of labile C and N) and soil properties (prior C and N statuses) [11–13]. While bacterial growth decreases following N fertilization or N deposition owing to

C limitation (e.g., References [14,15]), the input of labile C compounds increases bacterial and fungal populations [6,16]. For example, oak (*Quercus robur* L.) defoliation by the gypsy moth (*Lymantria dispar* L.) led to increased populations of fungi and N_2-fixing bacteria in soil [17]. Increased populations of culturable fungi and Actinobacteria were found in pine forests (*Pinus sylvestris* L.) defoliated by the pine beauty moth (*Panolis flammea* D. et S.) and the pine-tree lappet (*Dendrolimus pini* L.) [6]. In contrast, a bark beetle (*Ips typographus* L.) induced tree dieback in a spruce (*Picea abies* L.) forest led to a decrease in the fungal biomass while the bacterial biomass was either unaffected or increased [18,19]. Overall, the effects of the organic input derived by phytophagous insects affecting fungal and bacterial gene abundance are poorly understood. However, bacteria are known to arrange a rapid turnover of easily decomposable compounds derived from litter, while fungi dominate a turnover of more complex organic compounds [20,21]. In acidic soils of pine forests, denitrification is expected to be an important driver for N turnover as nitrification rates in these "non-nitrifying" ecosystems are often negligibly low [6,22] and the nitrate reduction potential and nitrogen loss via denitrification are shown to be increased in these soils [23]. Given that the intensity and dispersal of insect outbreaks might increase as a result of climate change, alterations of nutrient availability and microbial communities caused by outbreaks gain importance in forest protection and C and N modeling [24].

In this study, we quantified the fungal, bacterial, and denitrification genes in different compartments of Scots pine forests (vegetation layer, organic layer, mineral soil, litter feces, dead larvae, and needles) suffering from insect outbreaks. We analyzed the abundance of fungal (18S) and bacterial (16S) rRNA genes, as well as genes related to denitrification in Scots pine forests infested by the nun moth (*Lymantria monacha* L.) and the pine-tree lappet using real-time PCR (qPCR, quantitative polymerase chain reaction). The functional genes involved in the NO_2-reduction were the Cu-nitrite reductase (*nirK*), the cd_1-nitrite reductase (*nirS*) and the nitrous oxide reductase (*nosZ* clade I and II).

We expected that abundance of bacteria and fungi during moments of high feces and needle litter input will increase. We further expected that not only the higher total organic input but also the different chemical and physical composition of insect feces compared to needle litter will lead to a shift in the abundance of denitrifying microorganisms. Due to the insect secretions and detritus on the needle surface, we assumed that the phyllosphere bacteria and fungi would be positively affected by phytophagous insects.

2. Materials and Methods

2.1. Study Site

In 2014, a 65-years old Scots pine forest (52°8′38″ N, 13°45′14″ E, 42 m a.s.l. (above sea level)) in the second year of an outbreak of the nun moth (80% defoliated canopy) was investigated. In 2015, we investigated a 92-years old Scots pine forest (52°17′42″ N, 12°19′32″ E, 47 m a.s.l.) within the first year of infestation by the pine-tree lappet (50% defoliated canopy). In each year, an adjacent non-infested site served as a control site (2014: 52°9′29″ N, 13°36′47″ E, 35 m a.s.l.; 2015: 52°17′19″ N, 12°20′46″ E, 50 m a.s.l.). All the investigated forests were located in the federal state of Brandenburg, Germany. The detailed site and soil properties were described earlier [25,26]. The soil types at the study sites in both years were classified as podzols (Food and Agriculture Organization of the United Nations (FAO) classification) derived from glacial and aeolian medium sand deposits. The pH range, as well as the C/N ratio, were measured at every sampling date for each soil layer in both years (Figure 1 and Table A1). In a pre-survey, the ammonia-oxidizing bacteria and archaea *amoA* gene copy numbers determined by qPCR were below the detection limit at both study sites (Tables A2 and A3). The annual mean temperature was 10.8 °C and 10.6 °C and the total average annual precipitation was 474 mm and 496 mm (DWD: German Federal Meteorological Service (DWD) and Climate Data Center (CDC), Weather Station Lindenberg (Station ID: 3015), 16.02.2017) at the sites in 2014 and 2015, respectively.

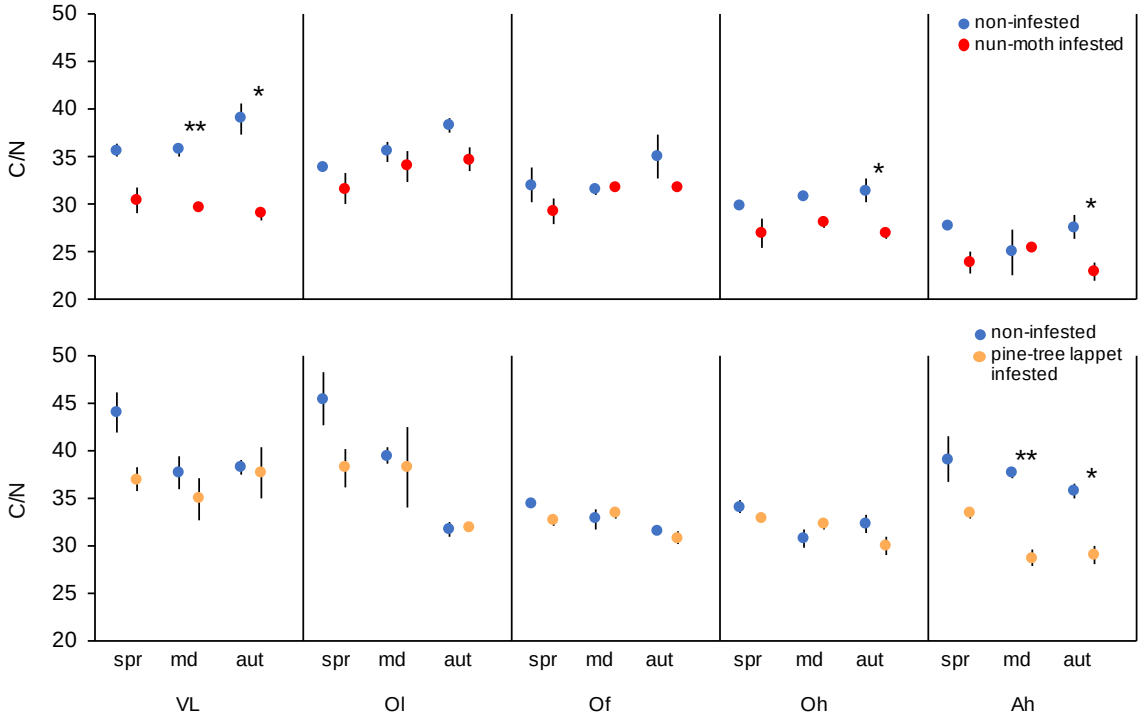

Figure 1. The mean ± standard error of the C/N of the vegetation layers, organic layers, and the mineral soil of the nun moth (*Lymantria monacha* L.) and pine tree lappet (*Dendrolimus pini* L.) infested and non-infested Scots pine (*Pinus sylvestris* L.) forest site. VL = vegetation layer, Ol = litter organic, Of = fibric organic, Oh = humic organic, Ah = humic mineral, spr = spring, md = main defoliation, aut = autumn, $n = 9$, the asterisks indicate significant differences between the infested and non-infested sites at same date and layer based on pairwise Student's t-test, ** $p < 0.010$, * $p < 0.050$.

2.2. Sampling

Sampling in both years was conducted before the outbreak in late March ('spring'), during the main defoliation in late May 2014 and June 2015 ('main defoliation') and after the outbreak in September ('autumn'). Three plots of 300–350 m^2 were established on the infested and non-infested site, respectively. Approximately 200 g of material from the moss vegetation including the incorporated fresh litter (vegetation layer = VL), the organic layers (litter organic = Ol, fibric organic = Of and humic organic = Oh) as well as the mineral soil horizon (humic mineral = Ah), were sampled at four randomly chosen sampling points per plot, dried at 40 °C for 24 h and ground.

Approximately 100 g of mixed needle samples were obtained from five individual branches of the south exposed lower tree crown (~11 m) of three individual trees per plot. Litter, larval feces, and larval cadavers at the infested site were collected in three nylon nets with a varying net size of 6–10 m^2. The needle litter, feces, and dead larvae were sampled after no longer than 48 h from the nets, subsequently separated, frozen on dry ice for preservation and transportation and freeze-dried for 72 h.

2.3. DNA Extraction

The DNA was extracted as described previously (cf. Reference [26]). Briefly, the DNA from 50 mg of finely ground soil, needle, feces, and litter was extracted following a CTAB protocol with two chloroform/isoamylalcohol steps with an intermediate phenol purification [27] and subsequent polyethylene glycol precipitation [28]. The DNA quality and quantity were checked on 0.8% (w/v) agarose gels stained with ethidium bromide. The inhibitory effects on PCR performance were eliminated by diluting the samples with a ratio of 1:100 in ddH_2O. The samples were stored at −20 °C until use.

2.4. Standard Curves

The nirK standard originated from environmental clones, *nirS* from *Pseudomonas fluorescens* (strain C7R12), *nosZ*I from *Bradyrhizobium japonicum* (strain USDA 110), *nosZ*II from *Gemmatimonas aurantiaca* (strain T-27), and bacterial 16S rRNA from *Pseudomonas aeruginosa* (strain Pa01). The standards were provided by D. Bru, French National Institute for Agricultural Research, Dijon, France. All target genes were cloned in plasmid pGEM-T (Promega, Mannheim, Germany) and multiplied in *Escherichia coli* JM109 grown in an LB medium [26] with ampicillin (50 µg/mL). For plasmid linearization, the restriction enzyme SalI was used. Following digestion, the cultures were streaked on agar plates with LB and ampicillin (5 mg/mL) and incubated at 37 °C overnight. Single colonies were transferred to a 3 mL LB medium with ampicillin (5 mg/mL) and incubated at 37 °C overnight under continuous rotation. The plasmid DNA was extracted using the alkaline lysis method [27]. For fungal 18S rRNA genes, the genomic DNA of *Verticillium longisporum* 43—provided by A. von Tiedemann, University of Goettingen, Germany—dissolved in 25 µL TE was used as a standard. The standard curves for qPCR were obtained with seven serial dilutions in 0.5× TE buffer with 1:3 ratio. The PCR conditions were optimized for each primer pair to achieve optimal amplification (Table A2).

2.5. Real-time PCR

Primers, primer sequences, and the expected amplicon lengths are listed in Table A3. Amplification of target genes was conducted in a CFX 384 Thermocycler (Biorad, Rüdigheim, Germany) in 384 well microplates using SYBR Green (Invitrogen, Karlsruhe, Germany) for detection in a total reaction volume of 4 µL. The reactions contained 3 µL of mastermix (10 mM Tris-HCl, 10 mM KCl, 0.1%, pH 8.3 at 25 °C), 100 µM of each dNTP (Bioline, Luckenwalde, Germany), varying final concentrations of $MgCl_2$, varying primer concentrations (Table A2), 0.1× SYBR Green, 1 mg/mL bovine serum albumin, 0.03 u hot start Taq DNA polymerase (New England Biolabs, Beverly, MA, USA), and 1 µL of template DNA or ddH_2O for negative controls. The annealing and extension time and temperature for each primer pair are listed in Table A2. Following amplification, the samples were heated to 95 °C for 60 s and cooled to 55 °C for 60 s followed by a temperature increase from 55 °C to 95 °C by 0.5 °C per cycle with continuous fluorescence measurement to obtain specific melting curves of the PCR products. PCR products exhibiting melting curves that differed from the ones of the standards were checked on 1.7% (*w*/*v*) agarose gels.

2.6. Soil, Feces, Needle and Dead Larvaea Chemical Analyses

The soil pH was measured in dH_2O (with a ratio of 1:10 for the organic layers and 1:2.5 for the Ah) with samples dried at 60 °C for 48 h. For the total C and N concentration analysis, soil, feces, needle litter, and dead larvae were dried at 105 °C for 24 h and finely ground prior to analysis in a total organic carbon (TOC) analyzer multi C/N (Analytik Jena, Jena, Germany).

2.7. Statistical Analyses

The qPCR data were tested for distribution of normality and homogeneity of variances by applying the Shapiro-Wilk test and the Levene's test, respectively. For C/N data, the Fligner-Killeen test of homogeneity of variances was used followed by Student's *t*-test to test for differences between the infested and non-infested site. The statistical analyses of soil, needle, litter, feces, and larval cadaver data were performed by employing the 'FSA' package version 0.8.16 [29]. The Kruskal-Wallis test, with subsequent post-hoc test (Dunn's test) and Benjamini-Hochberg corrected *p*-values for multiple pairwise comparisons, was used to detect differences between the infested and non-infested sites, soil layers, and years. All statistical analyses were executed in R, version 3.3.3 [30].

3. Results

3.1. Fungal 18S and Bacterial 16S rRNA Gene Abundance in Soil

Detectable target genes in the percentage of all samples and positive sample sizes during both outbreaks are listed in Tables A4 and A5. During the nun moth main defoliation, the fungal 18S rRNA gene abundance in the VL layer was four times higher compared to the non-infested site and the bacterial 16S rRNA was seven times higher ($p \leq 0.004$) and two times higher in the Ol layer ($p = 0.010$) (Figure 2). During the pine-tree lappet main defoliation, the 18S and 16S rRNA gene abundances showed a similar trend, but the variability was high and did not differ significantly between infested and non-infested sites. In autumn, during the nun moth outbreak, the same trend was detectable in the Ol layer ($p = 0.025$). In spring, during the pine-tree lappet outbreak, the 18S rRNA gene abundance was 5 times higher in the VL layer ($p = 0.010$) and in the Ol layer ($p = 0.025$) of the infested site compared to the non-infested site. No differences in the 18S/16S rRNA gene copy ratio in any layer for either outbreak were observed.

3.2. Denitrification Gene Abundance in Soil—*nirK*, *nirS*, *nosZ*I, *nosZ*II

The abundances of the denitrification genes in the soil are summarized in Table 1. During the nun moth's main defoliation, the VL and the Ol layers of the infested site exhibited ten and five times the gene copy numbers of *nirK* compared to the non-infested site, respectively ($p = 0.006$ and $p = 0.016$, respectively) while the *nosZ*II abundance in the Of layer was lower at the infested site compared to the non-infested site ($p = 0.047$). No differences were found for *nosZ*I during the nun moth defoliation.

During the pine-tree lappet defoliation, the *nosZ*I abundance was higher in the Ah during the main defoliation, the Of during autumn, and the *nosZ*II gene abundance in the VL was higher in the main defoliation, respectively, at the infested site ($p = 0.034$, $p = 0.049$ and $p = 0.180$). In contrast, during the pine-tree lappet defoliation, the *nirK* abundance in the Ol during the main defoliation and *nosZ*II abundance the VL in spring was lower at the infested site ($p = 0.006$ and $p = 0.021$).

No differences were detectable for *nirS* between the infested and non-infested sites for both the pest insects at any sampling date. Overall, the mean *nirK* gene abundance was four and five times higher than the *nirS* gene abundance during the nun moth and the pine-tree lappet outbreak, respectively.

3.3. Soil Chemical Properties

The C/N ratio of the mineral soil (Ah) decreased at both infested sites ($p = 0.049$ and $p < 0.001$), while the C/N ratio in the VL and Oh decreased only during the nun moth defoliation ($p < 0.001$ for both, Figure 1). The soil pH did not differ between the infested and non-infested sites but decreased from the top to mineral soil layers (Table A5).

3.4. C and N in Dead Larvae, Feces and Needle Litter

The C and N contents of dead larvae were similar for nun moth and pine-tree lappet with 57.3% and 62.1% N and 9.3% and 9.0% N, respectively. The needle litter derived from the nun moth contained 59.6% C and 1.6% N. Contents of 61.0% C and 0.9% N were obtained for the needle litter of the pine-tree lappet. Feces of the nun moth contained 58.3% of C and 0.8% of N; the feces of the pine-tree lappet are 60.6% for C and 0.8% for N.

3.5. Fungal 18S rRNA and Bacterial 16S rRNA Gene Abundance in Needles

Bacterial 16S rRNA genes in infested needles were higher during the nun moth outbreak in autumn, as well as during the pine-tree lappet outbreak at the main defoliation and in autumn compared to non-infested needles ($p \leq 0.010$) (Figure 3). No difference in the 18S rRNA gene abundance between the infested and non-infested site nor between the two pests was detectable.

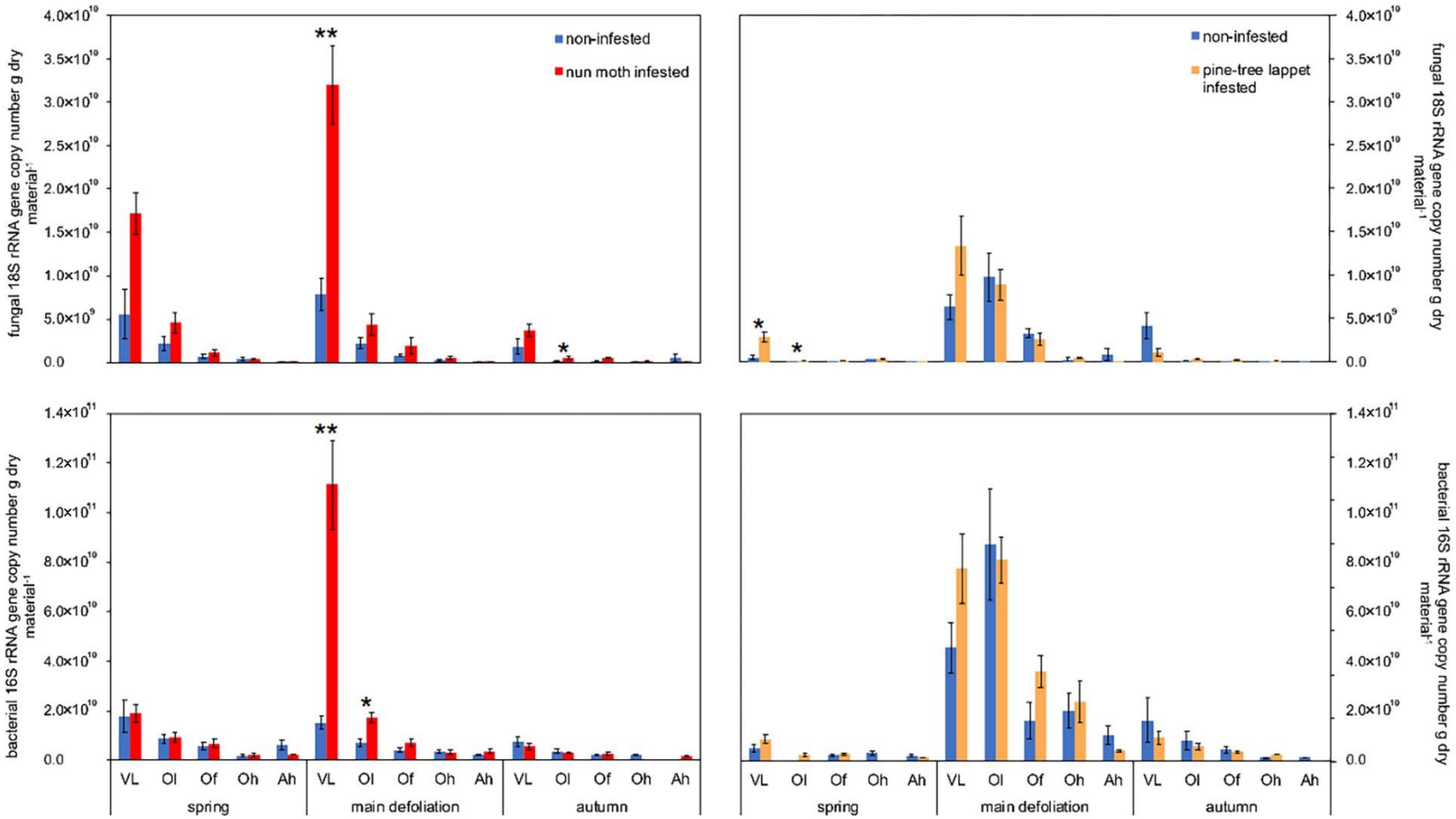

Figure 2. The mean fungal 18S rRNA and bacterial 16S rRNA gene copy numbers per gram of dry soil/plant material of the vegetation layers, organic layers, and the mineral soils of the nun moth (*Lymantria monacha* L.) and pine-tree lappet (*Dendrolimus pini* L.) infested and non-infested Scots pine (*Pinus sylvestris* L.) forest sites. VL = vegetation layer, Ol = litter organic, Of = fibric organic, Oh = humic organic, Ah = humic mineral. The error bars represent the standard error of the mean with varying sample sizes (see Table A5); an asterisk indicates significant differences between the infested and non-infested site on the same sampling date and layer based on the Kruskal-Wallis test, ** $p < 0.010$, * $p < 0.050$).

Table 1. The mean ± standard error of the *nirK*, *nirS* and *nosZ*I and *nosZ*II gene copy numbers per of gram dry material of the vegetation layer, organic layer, and the mineral soil of the nun moth (*Lymantria monacha* L.) and pine-tree lappet (*Dendrolimus pini* L.) infested and non-infested Scots pine (*Pinus sylvestris* L.) forest sites. VL = vegetation layer, Ol = litter organic, Of = fibric organic, Oh = humic organic, Ah = humic mineral. (varying sample size, see Table A5, n.d. = not detectable.); the asterisks indicate significant differences between infested and non-infested sites at the same date and layer based on a pairwise Kruskal-Wallis test, ** $p < 0.010$, * $p < 0.050$).

Target Gene	Site	Nun Moth Infested					Pine-Tree Lappet Infested				
		VL	Ol	Of	Oh	Ah	VL	Ol	Of	Oh	Ah
nirK	non-infested	$2.04 \times 10^8 \pm 1.07 \times 10^8$	$1.36 \times 10^8 \pm 5.32 \times 10^7$	$6.95 \times 10^7 \pm 2.15 \times 10^7$	$4.09 \times 10^7 \pm 1.43 \times 10^7$	3.87×10^7 -	$5.58 \times 10^8 \pm 3.17 \times 10^8$	$1.89 \times 10^9 \pm 5.91 \times 10^8$	$4.32 \times 10^8 \pm 1.89 \times 10^8$	$3.61 \times 10^8 \pm 1.42 \times 10^8$	$4.17 \times 10^7 \pm 2.06 \times 10^7$
	infested	1.53×10^9 ** $\pm 2.16 \times 10^8$	4.73×10^8 * $\pm 2.20 \times 10^8$	$1.64 \times 10^8 \pm 5.95 \times 10^7$	$2.84 \times 10^8 \pm 1.90 \times 10^8$	$1.40 \times 10^8 \pm 2.51 \times 10^7$	$8.52 \times 10^8 \pm 1.81 \times 10^8$	1.39×10^9 * $\pm 8.25 \times 10^8$	$3.70 \times 10^8 \pm 1.19 \times 10^8$	$1.47 \times 10^8 \pm 1.01 \times 10^8$	$1.96 \times 10^8 \pm 1.28 \times 10^8$
nirS	non-infested	$9.93 \times 10^7 \pm 3.66 \times 10^7$	n.d. -	8.37×10^7 -	n.d. -	n.d. -	1.66×10^8 -	$1.50 \times 10^8 \pm 4.36 \times 10^5$	$1.22 \times 10^8 \pm 1.29 \times 10^7$	$8.42 \times 10^7 \pm 7.29 \times 10^6$	n.d. -
	infested	$1.06 \times 10^8 \pm 1.73 \times 10^7$	$7.47 \times 10^7 \pm 1.24 \times 10^7$	n.d. -	n.d. -	n.d. -	1.66×10^8 -	$2.25 \times 10^8 \pm 3.68 \times 10^7$	$8.25 \times 10^7 \pm 6.75 \times 10^6$	$6.59 \times 10^7 \pm 2.84 \times 10^7$	6.05×10^7 -
nosZI	non-infested	$8.53 \times 10^5 \pm 4.92 \times 10^5$	$3.83 \times 10^5 \pm 9.62 \times 10^4$	$2.16 \times 10^5 \pm 2.39 \times 10^4$	$2.43 \times 10^5 \pm 3.73 \times 10^4$	$5.33 \times 10^5 \pm 2.88 \times 10^5$	$3.59 \times 10^6 \pm 3.22 \times 10^6$	$8.58 \times 10^5 \pm 4.49 \times 10^4$	$1.40 \times 10^6 \pm 5.13 \times 10^5$	$9.74 \times 10^5 \pm 2.43 \times 10^5$	$1.59 \times 10^5 \pm 4.06 \times 10^4$
	infested	$8.02 \times 10^5 \pm 1.32 \times 10^6$	$6.05 \times 10^5 \pm 7.86 \times 10^4$	$5.44 \times 10^5 \pm 2.04 \times 10^5$	$4.73 \times 10^5 \pm 1.01 \times 10^5$	$4.60 \times 10^5 \pm 2.06 \times 10^5$	$1.87 \times 10^6 \pm 1.31 \times 10^5$	$3.21 \times 10^5 \pm 9.76 \times 10^4$	$1.29 \times 10^6 \pm 5.55 \times 10^5$	$6.04 \times 10^5 \pm 1.97 \times 10^5$	5.89×10^5 * $\pm 1.48 \times 10^5$
nosZII	non-infested	$2.53 \times 10^7 \pm 1.68 \times 10^7$	$2.38 \times 10^7 \pm 1.04 \times 10^7$	$9.37 \times 10^6 \pm 3.79 \times 10^6$	$4.17 \times 10^6 \pm 2.69 \times 10^6$	$2.62 \times 10^6 \pm 1.70 \times 10^6$	$1.96 \times 10^8 \pm 8.85 \times 10^7$	$4.75 \times 10^7 \pm 1.97 \times 10^7$	$3.62 \times 10^7 \pm 2.47 \times 10^7$	$1.51 \times 10^6 \pm 6.85 \times 10^5$	$4.45 \times 10^8 \pm 5.77 \times 10^8$
	infested	$3.26 \times 10^7 \pm 1.88 \times 10^7$	$1.93 \times 10^7 \pm 9.95 \times 10^6$	$1.13 \times 10^7 \pm 5.96 \times 10^6$	$6.58 \times 10^6 \pm 3.22 \times 10^6$	$1.32 \times 10^6 \pm 8.69 \times 10^5$	$2.32 \times 10^8 \pm 1.99 \times 10^8$	$3.75 \times 10^8 \pm 3.05 \times 10^8$	$7.97 \times 10^6 \pm 4.40 \times 10^6$	$2.94 \times 10^6 \pm 1.89 \times 10^6$	2.04×10^5 -

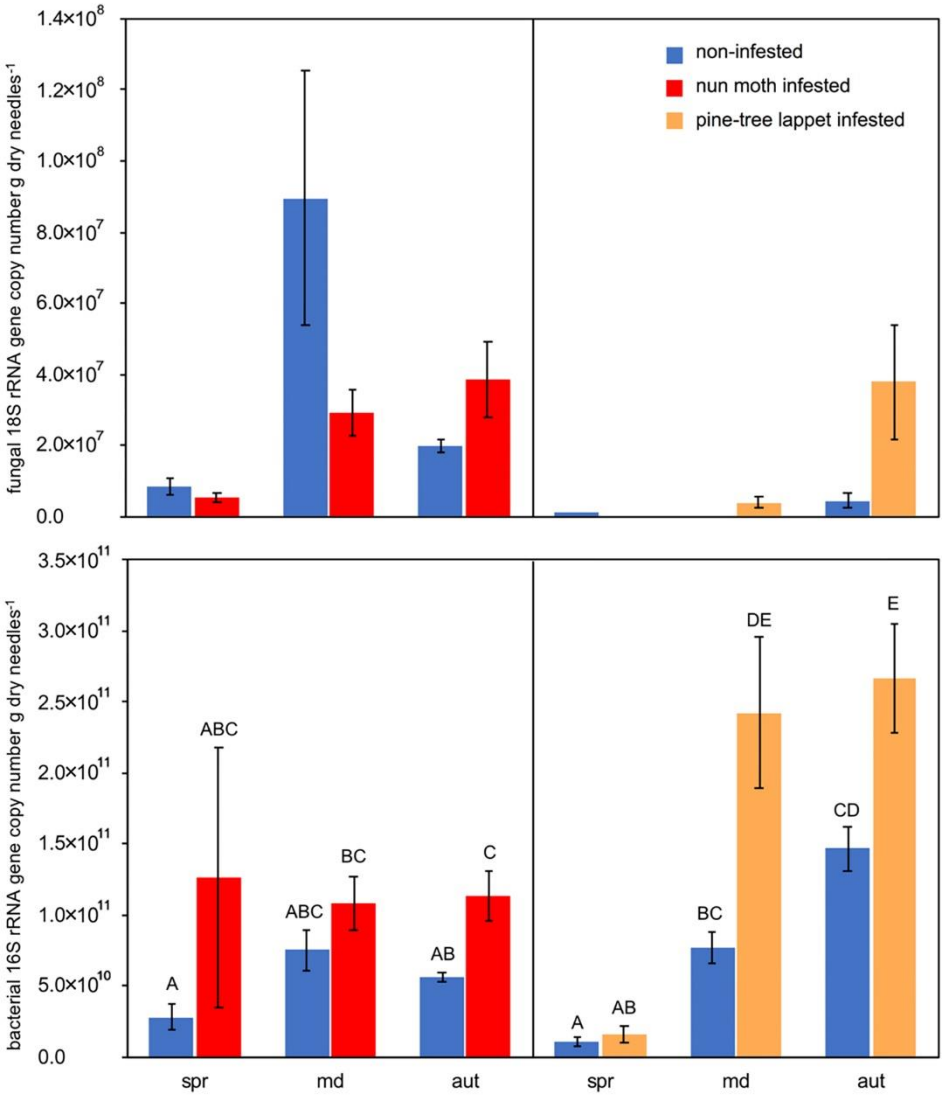

Figure 3. The mean fungal 18S rRNA and bacterial 16S rRNA gene copy numbers per gram of dry needles of the nun moth (*Lymantria monacha* L.) and pine tree lappet (*Dendrolimus pini* L.) infested and non-infested Scots pine (*Pinus sylvestris* L.) forest site (the error bars represent standard error of the mean, spr = spring, md = main defoliation, aut = autumn. Varying sample sizes, see Table A6, different letters indicate statistically significant differences between the infested and non-infested needles based on the Kruskal-Wallis test with the subsequent Dunn's test, $p < 0.050$). For fungal 18S rRNA, no statistical differences were found between the infested and non-infested needles.

3.6. The Abundance of Fungal 18S and Bacterial 16S rRNA Genes in Feces, Needle Litter, and Larvae

The fungal 18S rRNA gene abundance at the nun moth infested site was higher for all three sample types ($p \leq 0.050$) and the bacterial 16S rRNA gene abundance was higher for feces, and litter ($p \leq 0.050$) compared to the pine-tree lappet infested site (Table 2). Larvae cadavers generally showed higher bacterial 16S rRNA gene abundance than litter, while the pine-tree lappet cadavers additionally had a higher bacterial 16S rRNA gene abundance than the feces of the corresponding year ($p < 0.010$ for all).

Table 2. The mean ± standard error of the fungal 18S rRNA and the bacterial 16S rRNA gene copy number per gram of dry material of the organic input (litter, insect feces and larval cadavers) of the nun moth (*Lymantria monacha* L.) and pine tree lappet (*Dendrolimus pini* L.) infested Scots pine (*Pinus sylvestris* L.) forest sites.

Type of Organic Input	Fungal 18S rRNA Gene Abundance		Bacterial 16S rRNA Gene Abundance	
	Nun Moth	Pine-Tree Lappet	Nun Moth	Pine-Tree Lappet
Litter	4.15×10^9 * $\pm 1.56 \times 10^9$	1.42×10^8 $\pm 7.95 \times 10^6$	2.88×10^{11} * $\pm 1.22 \times 10^{10}$	4.13×10^{10} $\pm 6.14 \times 10^9$
Feces	5.36×10^9 * $\pm 4.38 \times 10^8$	1.58×10^9 $\pm 4.92 \times 10^8$	3.04×10^{11} * $\pm 2.96 \times 10^{10}$	1.57×10^{11} $\pm 3.46 \times 10^{10}$
Cadaver	1.44×10^{10} * $\pm 4.74 \times 10^9$	1.66×10^9 $\pm 8.37 \times 10^7$	1.23×10^{12} ♦ -	7.34×10^{11} $\pm 5.77 \times 10^9$

Samples were collected after no longer than 48 h ($n = 3$, ♦$n = 1$, asterisks indicate significant differences between the nun moth and pine-tree lappet infested site based on the Kruskal-Wallis test, * $p < 0.050$).

4. Discussion

The analysis of bacterial and fungal gene abundances showed that the infestation of pine forests by herbivorous insects can stimulate bacterial populations in pine needles, the vegetation layer, and the upper organic layer, while fungal populations increased in the vegetation layer and upper organic layer. The *nirK* gene abundance in the soil increases with down-scaled C/N ratios that may stimulate the decomposition of organic matter. Our results show the enhancement of both fungal and bacterial rRNA gene abundance in infested forests and suggest increased rates of nutrient cycling and accelerated soil respiration in the course of pest outbreaks.

4.1. The Soil Fungal and Bacterial Population Size Responds to Insect Derived Litter

Fungi and bacteria are known to respond fast to increasing organic input [11,31]. During nun moth outbreaks in pine forests, the N input via throughfall was 24% higher and 300% higher via feces and needle litter on defoliated sites during the vegetation period [25]. Thus, the observed increase in the microbial population size during insect outbreaks was likely to be triggered by the defoliation events and the associated high organic inputs on the infested sites.

The increased quantity and quality of the organic inputs during insect outbreaks are important parameters affecting soil microbial communities [6]. The response of the microbial populations to additional N supply may depend on the form of N and the current soil N status, moisture status, organic C content, and pH [20,32,33]. For example, the bacterial community structure shifts in a bark beetle (Scolytidae spp.) infested forest site have been shown to correlate with soil the NH_4^+ concentrations and C/N ratio [34]. At our study sites, high amounts of dissolved organic C and N in throughfall, needle litter, larval cadavers, and particularly feces were introduced to the soil (cf. Reference [25]). Feces have a physiochemically easy biodegradable structure and most of the N in the feces is soluble and labile, thereby giving an impulse to the microbial growth and decomposition processes in the organic layer [4,5,20,35]. This may account for the increased bacterial community size during main defoliation. However, the N input can lead to a cascade of soil chemical reactions on which fungi and bacteria are distinctly adapted and differ in their reaction time [36]. Additionally, it has been observed that only moderate to high amounts of organic input lead to changes in soil microbial population growth [37]. The lower defoliation intensity of the pine-tree lappet (50%) compared to the nun moth outbreak (80%) may, therefore, explain the differences in the bacterial and fungal population sizes in our study. The limitation of the bacterial increase to the main defoliation sampling date implies a certain regenerative capacity of microbial populations. However, to see if our observations are accompanied by functional community changes, different methods (e.g., DNA-based amplicon sequencing) are necessary.

4.2. Insect Outbreaks Lower the Soil C/N Ratio

The vertical transport of C and N derived from insect feces in soils during insect outbreaks is accelerated by the high water solubility of most of these compounds [5,38]. Even though the changes of fungal and bacterial population sizes were limited to the VL and Ol layers during the nun moth outbreak, alterations of the C/N were still detected in the Ah layer at the main defoliation and autumn. Our observed vertical differences in the C and N contents agree with a study of lodgepole pine (*Pinus contorta* Dougl. ex Loud.) forest infested with bark beetles (Scolytidae spp.) [34,39]. Therein, the biogeochemical response to the outbreak was most prominent in the mineral soil and dependent on the percentage of dead trees [34,39]. Even though the trees at our study site were not killed by the outbreaks, the higher intensity of the nun moth outbreak compared to the pine-tree lappet outbreak can be one explanation for the observed differences between the sites. A decreased C/N in soil was previously observed in a nutrient release experiment of insect feces under laboratory conditions [5] and during *Operophtera brumata* L. and *Erannis defoliaria* L. outbreaks in the organic layers of an oak forest [40]. The lower C/N in fresh needle biomass which enters the soil surface in contrast to the senescent needles may also contribute to the decreased C/N during outbreaks [41]. In contrast to the observed short-term changes in soil microbial population size, the soil C and N contents in mineral soil appear to be spatially and temporally more sensitive to insect outbreaks.

4.3. The Varying Effects on Denitrification Genes

N additions in various forms have previously been shown to change N cycling-related gene abundance in soils [15,42]. However, the response of the denitrification genes remains controversial since denitrification is determined by many interacting environmental and soil biological factors [33,42]. While *nirK* was positively affected by defoliation of the nun moth, no effect on the *nirS* gene abundance was detected in our study. In contrast, N fertilization in Chinese fir (*Cunninghamia lanceolata* L.) forests showed negative effects on the *nirS* and *nirK* gene abundances [15]. Long-term N deposition in a Norway spruce (*Picea abies* L.) forest showed no effect on the *nirK* gene abundance, while *nosZ* abundance was positively affected [43]. In our study, the *nosZ*I and *nosZ*II gene abundances were either positively affected, negatively affected, or not affected by pest infestation. Thus, it appears that it might be difficult to directly compare the abundances of denitrification genes among the study sites, and this may be related to the great importance of the form of N and the site conditions prior to insect outbreaks (cf. References [14,33]).

Our findings are consistent with the assumption that denitrifying microorganisms do not show coherent responses to environmental changes [44,45]. For example, *nirK* proved to be more responsive to changes such as land use practices [46], vegetation cover [47], and water source quality [48], compared to *nirS*. Furthermore, *nirK* has a higher sensitivity towards nutrient additions than *nirS* [23,49]. Further, it appears that there may be a certain resilience of the denitrifying community to environmental changes. Additionally, their ability to recover fast following disturbances [43,50,51] might account for the non-existing (*nirS*) and inconsistent (both *nosZ* clades) changes between the infested and non-infested sites.

Moreover, the active part of the microbial biomass often accounts for only a fractional amount of the total microbial biomass [52]. Conventional DNA-based methods allow the assessment of the potential activity of genes by quantification, but are unable to differentiate between DNA derived from active, dormant or dead microorganisms [34]. Abundance and activity patterns of denitrifying microorganisms often do not respond congruently to environmental changes [50]. Even within DNA-based methods, the differences in DNA extraction methods, DNA yield, the choice of primer sets, as well as qPCR efficiencies, limit the transferability of the results to other studied ecosystems [53]. Nevertheless, both *nir* genes are known to correlate with soil net N_2O fluxes [33,54–56]. *nirS*-possessing organisms usually are comprised of *nosZ* genes which enable complete denitrification, while *nirK* organisms mostly lack *nosZ* [57]. This implies that soil N_2O emissions are predominantly determined by *nirK*-, rather than by *nirS*-type microorganisms. Thus, the *nirS*/*nirK* ratio may be negatively

correlated with the soil N_2O sink capacity [58,59]. Therefore, the increased *nirK* gene abundance in combination with the consistent or decreased amounts of *nirS* and *nosZ* genes can be seen as a predictor of enhanced potential for N_2O emissions from infested forest sites. However, to date, there is no long-term field study on the effects of insect outbreaks on N_2O emissions from forest soils to assess the impact of biotic disturbance on the emissions from denitrification.

4.4. Litter, Feces and Larval Cadavers as Habitat for Microorganisms and N Source

The higher N concentrations in the needle litter during the nun moth outbreak compared to the pine-tree lappet outbreak that we have found may have contributed to the increased fluxes of microorganisms from the phyllosphere to the forest floor (cf. References [59,60]). The relatively high N content on the nun moth infested litter may originate from the prolonged infestation, while the pine-tree lappet site was still in the first year of mass infestation during the sampling. High needle litter, feces, and larval cadaver inputs during insect outbreaks into the soil may serve as an additional source for N and may comprise large amounts of microorganisms which, in turn, have been shown to affect soil microbial population size [6].

4.5. Insect Outbreaks Increase Phyllosphere Bacteria Colonization

The recovered bacterial 16S and fungal 18S rRNA genes from the needle samples included both epiphytic and endophytic bacteria and fungi [61]. Bacteria numerically dominated over fungi in the phyllosphere at all sites and dates in our study. Further, we mainly found increased bacterial 16S (but not fungal 18S) rRNA gene abundance in needles from the infested site during insect outbreaks at the main defoliation until autumn. These findings are in accordance with previous studies [62]. The phyllosphere bacteria have comparatively rapid turnover rates and are influenced by both environmental conditions (e.g., temperature, UV exposure, moisture conditions) and tree characteristics and metabolism (e.g., resource availability, leaf cuticle properties, leaf age) [63,64]. Thus, seasonal increments may be triggered by increasing leaf age and changing environmental conditions throughout the vegetation period.

Defoliation caused by insect outbreaks can increase the production of nutritional substances in foliage and thereby improve the habitat conditions for microorganisms [64]. The leaching of substances from the destructed needle parts, as well as substances in insect saliva, can act as growth promoters of phyllospheric microorganisms [61,65]. For example, the infection with different powdery mildews (Erysiphaceae spp.) has been shown to stimulate the bacterial phyllosphere population of the Japanese spindle tree (*Euonymus japonicus* T.) [66]. Eastern hemlock (*Tsuga canadensis* L.) infested with Hemlock woolly adelgid (*Adelges tsugae* A.) showed an increased abundance of epiphytic microorganisms [1]. Bacteria can have parasitic, commensal and mutualistic interactions with trees [61]. Therefore, further studies on the function of the increased phyllosphere bacteria induced by insect outbreaks are needed to understand the interactions with their host and their environment.

5. Conclusions

Outbreaks of the nun moth and the pine-tree lappet in Scots pine forests foster bacterial and fungal populations differentially during the growing season, as well as in various forest compartments. Changes in microbial populations were of short duration and predominantly occurred in the vegetation layer and upper organic layer. The increase of the *nirK* gene abundance during the main defoliation of the nun moth in combination with the decreased C/N ratios in the soil suggests that alterations of the soil denitrifying populations are present, however, the duration and magnitude of these alterations remains largely unknown. Future studies may enlighten us on whether the observed increase in the genetic potential for soil N_2O emissions during pest outbreaks yields in increased soil N_2O emissions.

Author Contributions: Conceptualization and Supervision: A.I.-M.-A., P.K.; Investigation: M.M.G., L.B., S.M.; Formal analysis and Visualization: M.M.G.; Writing-Original Draft Preparation: M.M.G., L.B.; Writing-Review & Editing P.K. and A.I.-M.-A.

Funding: This study was funded by the Germany Research Foundation (DFG) (DFG Project No. ME 3861/2-1).

Acknowledgments: We would like to thank Annett Reinhardt, Carsten Beyer and Franziska Germeshausen for their support during the field work and Carsten Thies for helpful comments on the manuscript. We acknowledge support by the German Research Foundation and the Open Access Publication Funds of the Goettingen University.

Conflicts of Interest: The authors declare no conflict of interest.

Appendix A

Table A1. Mean [± standard error] of pH (soil:H_2O ratio 1:10 for the organic layers and 1:2.5 for the Ah) values of the vegetation layer, organic layer and the mineral soil of nun moth (*Lymantria monacha* L.) and pine tree lappet (*Dendrolimus pini* L.) infested and non-infested Scots pine (*Pinus sylvestris* L.) forest sites. VL = vegetation layer, Ol = litter organic, Of = fibric organic, Oh = humic organic, Ah = humic mineral.

Site	VL	Ol	Of	Oh	Ah
non-infested	4.33 ± 0.10	3.91 ± 0.07	3.51 ± 0.07	3.33 ± 0.05	3.42 ± 0.06
nun moth infested	4.57 ± 0.10	4.29 ± 0.07	3.94 ± 0.08	3.57 ± 0.06	3.57 ± 0.07
non-infested	4.27 ± 0.10	3.97 ± 0.06	3.61 ± 0.05	3.34 ± 0.05	3.47 ± 0.06
pine-tree lappet infested	4.21 ± 0.09	4.04 ± 0.04	3.74 ± 0.07	3.42 ± 0.04	3.58 ± 0.07

Table A2. QPCR conditions for the different target genes; B*amoA* standards were generated from genomic DNA of *Nitrosomonas europeae* (strain DSM 28437), A*amoa* standards were generated from environmental clones.

Target Gene	Final $MgCl_2$ Concentration (mM)	Primer Concentration (µM)	Initial Denaturation	Denaturation	Annealing	Extension
nirK	2.5	0.5	95 °C, 120 s	94 °C, 20 s	63 °C–1 °C/cycle for 6 cycles, 58 °C for 35 cycles 30 s	68 °C, 30 s
nirS	2.1	0.5	95 °C, 120 s	94 °C, 20 s	63 °C–1 °C/cycle for 6 cycles, 58 °C for 35 cycles 30 s	68 °C, 30 s
*nosZ*I	2.1	1	95 °C, 120 s	94 °C, 20 s	65 °C–1 °C/cycle for 6 cycles, 60 °C for 35 cycles 30 s	68 °C, 25 s
Target Gene	**Final $MgCl_2$ Concentration (mM)**	**Primer Concentration (µM)**	**1 cycle**	**35 cycles**		
			Initial Denaturation	**Denaturation**	**Annealing**	**Extension**
B*amoA*	2.0	0.3	95 °C, 120 s	94 °C, 20 s	62 °C, 30 s	68 °C, 35 s
A*amoA*	30	0.3	95 °C, 120 s	94 °C, 20 s	61 °C, 30 s	68 °C, 45 s
*nosZ*II	2.5	2.7	95 °C, 120 s	94 °C, 20 s	60 °C, 30 s	68 °C, 45 s
18S	2.6	0.3	95 °C, 120 s	94 °C, 20 s	59 °C, 30 s	68 °C, 25 s
16S	2.5	0.3	95 °C, 120 s	94 °C, 20 s	63 °C, 30 s	68 °C, 25 s

Table A3. Target genes, oligonucleotide primers and amplicon size.

Target Gene	Primer Name	Primer Sequence (5′–3′)	Amplicon Size (bp)	Reference
bacterial ammonia monooxygenase α subunit (B*amoA*)	amoA-1F amoA-2R	GGGGTTTCTACTGGTGGT CCCCTCKGSAAAGCCTTCTTC	491	Rotthauwe et al. (1997) [67]
archaeal ammonia monooxygenase α subunit (A*amoA*)	CrenamoA23f CrenamoA616r	ATGGTCTGGCTWAGACG GCCATACABCKRTANGTCCA	628	Tourna et al. (2008) [68]
Cu-nitrite reductase catalytic subunit (*nirK*)	nirK876F nirK1040R	ATYGGCGGVAYGGCGA GCCTCGATCAGRTTRTGGTT	165	Henry et al. (2004) [69]
Cd_1-nitrite reductase catalytic subunit (*nirS*)	cd3aF R3cd	GTSAACGTSAAGGARACSGG GASTTCGGRTGSGTCTTGA	410	Michotey et al. (2000) [70], Throbäck et al. (2004) [71]
nitrous oxide reductase catalytic subunit (*nosZ*I)	NosZ2F NosZ2R	CGCRACGGCAASAAGGTSMSSGT CAKRTGCAKSGCRTGGCAGAA	267	Henry et al. (2006) [72]
nitrous oxide reductase catalytic subunit (*nosZ*II)	1153 nosZ 8F 1888 nosZ 29R	CTIGGICCIYTKCAYAC GCIGARCARAAITCBGTR	698	Jones et al. (2013) [73]
fungal 18S rRNA (18S)	FR1 FF390	AICCATTCAATCGGTAIT CGATAACGAACGAGACCT	350	Vainio & Hantula (2000) [74]
bacterial 16S rRNA (16S)	341F 534R	CCTACGGGAGGCAGCAG ATTACCGCGGCTGCTGGCA	194	López-Gutiérrez et al. (2004) [75]

Table A4. Detectable target genes in % of all samples during the outbreak of the nun moth (*Lymantria monacha* L.) and pine tree lappet (*Dendrolimus pini* L.).

Target Gene	Soil		Needle		Litter		Feces		Cadaver	
	Nun Moth	Pine-Tree Lappet	Nun Moth	Pine-Tree Lappet	Nun Moth	Pine-Tree Lappet	Nun Moth	Pine-Tree Lappet	Nun Moth	Pine-Tree Lappet
18S	87.8%	76.7%	79.2%	22.9%	100%	100%	100%	100%	100%	100%
16S	65.6%	65.0%	93.8%	89.6%	100%	100%	100%	100%	33%	100%
nirK	59.4%	57.2%	NA	NA	NA	NA	NA	NA	NA	NA
nirS	13.9%	19.4%	NA	NA	NA	NA	NA	NA	NA	NA
*nosZ*I	32.8%	35.6%	NA	NA	NA	NA	NA	NA	NA	NA
*nosZ*II	72.2%	63.9%	NA	NA	NA	NA	NA	NA	NA	NA

Total sample number soil: $n = 180$, needle $n = 48$, litter, needle, feces: $n = 3$, NA = data not available.

Table A5. Samples above the detection limit for fungal 18S and bacterial 16S rRNA gene abundance, *nirK*, *nirS*, *nosZ*I and *nosZ*II for the different soil horizons and sampling dates in nun moth (*Lymantria monacha* L.) and pine tree lappet (*Dendrolimus pini* L.) infested and non-infested Scots pine (*Pinus sylvestris* L.) forest sites.

	Target Gene	VL						Ol						Of						Oh						Ah					
		spr		md		aut		spr		md		aut		spr		md		aut		spr		md		aut		spr		md		aut	
		inf	non-inf	inf	non-inf	inf	non-inf	inf	non-inf	inf	non-inf	inf	non-inf	inf	non-inf	inf	non-inf	inf	non-inf	inf	non-inf	inf	non-inf	inf	non-inf	inf	non-inf	inf	non-inf	inf	non-inf
Nun Moth Outbreak	18S [a]	5	5	6	6	6	6	6	6	6	6	6	6	5	5	6	6	6	6	5	4	6	6	4	3	5	6	5	5	2	3
	16S [b]	6	5	6	6	6	5	6	5	6	6	3	5	6	4	5	6	4	2	2	3	5	4	0	3	1	2	2	2	2	0
	*nosZ*I	2	4	2	3	0	1	1	3	3	1	2	2	2	1	2	2	2	2	3	3	1	2	0	1	2	2	2	3	2	3
	*nosZ*II	6	5	6	6	6	5	6	6	6	6	6	4	5	5	5	5	5	5	5	3	6	6	2	3	0	0	1	2	2	2
	nirK	6	5	6	5	6	5	6	4	6	6	3	5	6	4	5	6	4	2	2	2	4	3	0	2	1	0	2	1	0	0
	nirS	6	1	5	3	2	0	4	0	3	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Pine-Tree Lappet Outbreak	18S [a]	6	6	6	6	6	6	6	6	6	6	6	6	6	6	6	6	6	5	2	0	6	6	3	1	1	0	6	5	0	1
	16S [b]	6	6	6	6	6	6	5	0	6	6	5	6	3	4	5	4	3	4	0	3	5	6	1	2	1	2	5	4	0	1
	*nosZ*I	4	1	2	2	4	2	2	1	2	0	2	2	2	4	2	4	3	3	1	3	4	2	3	2	1	0	3	3	0	0
	*nosZ*II	4	4	6	5	3	5	4	5	5	5	6	6	6	4	6	6	4	4	3	2	5	6	2	1	0	2	1	4	0	1
	nirK	5	6	6	6	6	5	4	0	6	5	5	6	3	4	5	4	3	2	0	2	5	6	1	2	1	0	1	3	0	1
	nirS	0	0	5	4	0	0	2	4	0	4	0	0	0	0	6	5	0	0	0	0	2	3	0	0	0	0	0	0	0	0

VL = vegetation layer, Ol = litter organic, Of = fibric organic, Oh = humic organic, Ah = humic mineral, spr = spring, md = main defoliation, aut = autumn. [a] = fungal 18S rRNA genes, [b] = bacterial 16S rRNA genes.

Table A6. Needle samples above the detection limit for fungal 18S and bacterial 16S rRNA gene abundance for the sampling dates in nun moth (*Lymantria monacha* L.) and pine tree lappet (*Dendrolimus pini* L.) infested and non-infested Scots pine (*Pinus sylvestris* L.) forest sites.

Target Gene	Nun Moth Outbreak						Pine-Tree Lappet Outbreak					
	Spring		Main Defoliation		Autumn		Spring		Main Defoliation		Autumn	
	Infested	Non-infested	Infested	Non-infested	Infested	Non-infested	Infested	Non-infested	Infested	Non-infested	Infested	Non-infested
fungal 18S rRNA	6	4	8	5	7	8	0	1	2	0	6	2
bacterial 16S rRNA	8	6	8	7	8	8	5	6	8	8	8	8

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