

**Effects of insect mass outbreaks on the C and N balance
in forest ecosystems**

Dissertation

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Summary

Intensive defoliation by phytophagous insects in forests is accompanied by high amounts of organic litter deposition (e.g., feces, needle litter, cadaver, cocoons) which affects biogeochemical processes in soil, thereby changing nutrient recycling and release as well as the soil microbial community. Besides its effects on soil, defoliation has negative impacts on tree health (e.g., reduced photosynthesis and growth rates, altered water balance), thus changing the trees' metabolic processes and altering tree-soil interactions. In consequence, one of the key forest functions – the uptake and storage of carbon – can be affected by insect outbreaks.

Single fluxes and process changes in insect infested forests have been previously investigated and quantified for different forest types. However, most studies cover only a limited subset of parameters, which provides just fragmentary insights on ecosystem turnover processes. This is the first multidimensional and holistic study combining aspects of tree physiology, microbiology, and biogeochemical C and N flux and pool changes within forest ecosystems infested by phytophagous insects.

One of the main objectives of this thesis was to retrace and quantify effects of insect derived organic inputs on soil properties and processes with a special focus on the forests' overall C and N cycling. Further this led to the question whether a change in soil nutrient cycling has feedback links on tree nutrition, tree health and the microbiome in different forest compartments.

To answer these questions, mass outbreaks of the nun moth (*Lymantria monacha* L.) and the pine-tree lappet (*Dendrolimus pini* L.) in two Scots pine forests (*Pinus sylvestris* L.) and two non-infested control forests (endemic insect abundances) in the federal state of Brandenburg, Germany, were investigated. The C and N inputs (solid and liquid) and the outputs (soil solution and gaseous soil emissions) during the outbreak years were quantified. Effects of soil C and N pools on microbial populations were quantitatively and qualitatively characterized by DNA-based methods (qPCR and DGGE followed by DNA sequencing) in the organic layers, the mineral soil, and other forest compartments. An additional microcosm incubation experiment with different organic matter treatments examined the effects of insect feces compared to needle litter on soil CO₂ and N₂O fluxes. Consequences of insect induced defoliation for tree nutrition and tree health were detected by a ¹⁵N uptake experiment of fine roots and the quantification of different N metabolites in needles and fine roots of infested and non-infested Scots pines. Further, effects on the trees phyllosphere and the understorey vegetation were detected by the above-mentioned DNA-based methods.

During insect outbreaks the total fluxes of N via insect feces, litter, and throughfall were on average increased by 1.8 times in relation to the biomass loss caused by the insects (study 1). Increased bacterial growth in the phyllosphere triggered by the insect outbreak may have contributed to the 4.5 times increased input fluxes via throughfall (study 3). Even though the additional N input to the soil resulted in increased soil N availability and decreased C/N (study 1 and 3), trees did not respond with increased N acquisition but were decreased in their net N uptake capacity for inorganic N and glutamine N by 30% to 65% (study 1). One contributor to the decrease of the net N uptake capacity is the impaired symbiosis with ectomycorrhizal fungi (study 2) which is probably a consequence of insufficient carbohydrate supply and increased fine root mortality. Moreover, infested trees accumulate total soluble protein-N, total amino acid-N, and structural N from tree internal sources in their fine roots and needles (study 1). Further, the soil N input accelerates fungal and bacterial abundance in the organic layers and changes the soil fungal community structure (study 2 and 3). High amounts of the additional C and N are lost via nitrate leaching (59% increased fluxes on average, study 1) and CO₂ emissions (32% increased emissions on average, study 4). The incubation experiment revealed that besides CO₂-C also N₂O-N emissions can be triggered by insect feces additions (study 4), which is reaffirmed in 10.4 times increased *nirK* gene abundance (Cu-nitrite reductase, a functional gene involved in the NO₂ reduction and thereby N₂O production) detected in forest soils during the main defoliation (study 3). These findings demonstrate that C and N pools, fluxes and turnover processes in the tree-soil-atmosphere continuum are heavily affected by insect mass outbreaks. Increased organic input triggered a change in microbial abundance and resulted in increased losses of C and N. At the same time, tree nutrition, phyllospheric colonization by microorganisms and mycorrhizal symbiosis is altered by the insect outbreaks. These results illustrate the complexity of process and flux modifications by insects and reveal the importance to evaluate changes differentiated temporally and spatially in multiple forest compartments to assess feedback links at ecosystem scale.

Zusammenfassung

Massenvermehrungen phytophager Insekten reduzieren die Blattbiomasse eines Waldes, was mit einem vermehrten Eintrag organischer Streu (z.B. Raupenkot, Nadeln, Kadaver, Kokons) in den Boden einhergeht. Dies beeinflusst die biogeochemischen Bodenprozesse, wodurch die Mineralisierung, die Nährstofffreisetzung sowie das Mikrobiom verändert werden können. Der Baum wiederum reagiert auf den Blattmasseverlust häufig mit einer reduzierten Photosyntheserate und einem gestörten Wasserhaushalt, was wiederum weitere interne Stoffwechselprozesse sowie die Zuwachsrates negativ beeinflussen kann. Infolgedessen ist eine der maßgeblichen Waldfunktionen – die Aufnahme und Speicherung von Kohlenstoff – durch Insektenmassenvermehrungen gefährdet. Diese Auswirkungen auf den Boden sowie durch den Befall veränderte physiologische Prozesse innerhalb des Baumes beeinträchtigen die Wechselbeziehungen zwischen Baum, Boden und Atmosphäre. In früheren Studien zu Insektenmassenvermehrungen in Wäldern wurden meist einzelne Parameter oder Prozesse erfasst, wodurch nur ein unvollständiges Verständnis der ökosystemaren Auswirkungen ermöglicht wurde. Diese Arbeit ist die erste multidimensionale und holistische Studie, die Aspekte der Baumphysiologie, Mikrobiologie sowie der biogeochemischen C- und N-Flüsse und -Speicher innerhalb mehrerer Waldkompartimente eines von Schadinsekten befallenen Waldökosystems berücksichtigt.

Ein Hauptziel dieser Arbeit ist es, die Auswirkungen vermehrten organischen Inputs durch Insektenbefall auf Bodeneigenschaften und -prozesse nachzuvollziehen und zu quantifizieren, wobei ein besonderer Schwerpunkt auf die veränderten C- und N-Kreisläufe innerhalb der Waldökosysteme gelegt wird. Daraus ergibt sich die Frage, ob eine Änderung der Umsetzungsprozesse im Boden Rückkopplungen für den Ernährungs- und Gesundheitszustand der Bäume sowie für das Waldmikrobiom impliziert. Um dies zu beantworten, wurden Massenausbrüche der Nonne (*Lymantria monacha* L.) und des Kiefernspinners (*Dendrolimus pini* L.) in zwei Kiefernwäldern (*Pinus sylvestris* L.) sowie entsprechende Wälder mit latenten Populationsdichten von Schadinsekten als Kontrollflächen in Brandenburg untersucht. Der C- und N-Eintrag via Kronentraufe und Streufall sowie der Austrag durch die Bodenlösung und Bodenatmung während der Ausbruchsjahre der Schädlinge wurden ermittelt. Der Einfluss von erhöhten organischen Einträgen auf mikrobielle Gemeinschaften im Boden wurde quantitativ und qualitativ durch DNA-basierte Methoden (qPCR und DGGE, gefolgt von DNA-Sequenzierung) in den organischen Auflagehorizonten, im Mineralboden und in anderen Waldkompartimenten charakterisiert. In einem Inkubationsversuch wurden die Auswirkungen von Insektenkot im Vergleich zu Nadelstreu auf die CO₂- und

N₂O-Emissionen des Bodens untersucht. Die Folgen von insektenverursachtem Blattmasseverlust für die Baumernährung und -gesundheit wurden durch ein ¹⁵N-Aufnahmeexperiment an Feinwurzeln sowie durch die Quantifizierung von verschiedenen N-Metaboliten in Nadeln und Feinwurzeln überprüft. Die Auswirkungen auf die Bodenvegetation und die Phyllosphäre der Bäume wurden ebenfalls mittels der DNA-Analysen untersucht.

Während der Massenvermehrung der Nonne waren die N-Einträge durch Insektenkot, Nadelstreu und Kronentraufe in Abhängigkeit von der Fraßintensität durchschnittlich 1,8-fach erhöht (Studie 1). Die Bakterienabundanz in der Phyllosphäre nahm durch den Schädlingsbefall zu und trug damit zu dem 4,5-fachen Anstieg der N-Flüsse in der Kronentraufe bei (Studie 3). Obwohl der zusätzliche Eintrag in den Boden zu einer erhöhten N-Verfügbarkeit und einem engeren C/N Verhältnis führte (Studie 1 und 3), zeigten die Kiefern keine vermehrte N-Allokation, sondern waren in ihrer N-Aufnahmekapazität für anorganisches N und Glutamin-N um 30% bis 65% reduziert (Studie 1). Zu dieser Reduktion könnten die Ektomykorrhiza beigetragen haben (Studie 2), welche sich wahrscheinlich als Folge der unausgewogenen Versorgung durch den Symbiosepartner sowie erhöhter Feinwurzel mortalität während des Insektenbefalls dezimiert haben. Einhergehend mit der verschlechterten N-Versorgung durch die Wurzelaufnahme akkumulieren befallene Bäume Protein-N, Aminosäure-N und strukturelles N in ihrer Feinwurzel- und Nadelbiomasse, welches primär aus bauminternen Speichern stammt (Studie 1). Darüber hinaus erhöht der N-Eintrag nicht nur insgesamt die Pilz- und Bakterienabundanz sowie die Struktur der pilzlichen Gemeinschaft in den organischen Auflagehorizonten (Studie 2 und 3), sondern führt zu vermehrter Nitratauswaschung (durchschnittlich 59% erhöhte Nitratgehalte in der Bodenlösung, Studie 1) und CO₂-Emissionen (durchschnittlicher Emissionsanstieg um 32%, Studie 4). Das Inkubationsexperiment verdeutlichte, dass neben CO₂ auch die im Rahmen von Insektenmassenausbrüchen bisher wenig beachteten N₂O-Emissionen durch den vermehrten Eintrag von Insektenkot zunehmen können (Studie 4). Dies spiegelt sich auch in einem 10,4-fach erhöhten Auftreten des *nirK*-Gens (Cu-Nitrit-Reduktase, ein an der NO₂-Reduktion und damit indirekt an der N₂O-Produktion beteiligtes funktionelles Gen) in Waldböden während eines Massenausbruchs von Schädlingen wieder (Studie 3).

Diese Ergebnisse zeigen, dass C- und N-Speicher, -Flüsse sowie -Umsatzprozesse im Baum-Boden-Atmosphäre-Kontinuum durch Massenvermehrungen von Schadinsekten direkt beeinträchtigt werden. Ein vermehrter organischer Input löst eine Veränderung der mikrobiellen Abundanz und damit der Mineralisierungsprozesse aus und führt zu erhöhten Austrägen von C und N. Gleichzeitig werden die N-Versorgung der Bäume, die

Besiedlung der Phyllosphäre durch Mikroorganismen sowie die Mykorrhizasymbiose durch die Insektenausbrüche beeinträchtigt. Diese Ergebnisse verdeutlichen die Komplexität von Prozess- und Flussmodifikationen durch Insekten und zeigen wie wichtig es ist die Veränderungen in jedem Waldkompartiment zeitlich und räumlich differenziert zu betrachten, um mögliche Rückkopplungsinterferenzen auf ökosystemarer Ebene beurteilen zu können.

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Abbreviations

a.s.l.	–	above sea level
Ah	–	humic mineral layer
Aut	–	autumn
C	–	carbon
CTAB	–	cetyltrimethyl ammonium bromide
C _{tot}	–	total carbon
DBH	–	diameter breast height
ddH ₂ O	–	double distilled H ₂ O
DGGE	–	denaturing gradient gel electrophoresis
DN	–	dissolved nitrogen
DNA	–	deoxyribonucleic acid
dNTP	–	deoxyribose nucleoside triphosphates
DON	–	dissolved organic nitrogen
dw	–	dry weight
ECD	–	electron capture detector
EDTA	–	ethylenediaminetetraacetic acid
EM	–	ectomycorrhizal fungi
FAO	–	Food and Agriculture Organization of the United Nations
fw	–	fresh weight
GC	–	gas chromatographic system
GHG	–	greenhouse gas
GIS	–	geographic information system
ICP-OES	–	inductively coupled plasma optical emission spectrophotometer
IRMS	–	isotope-ratio mass spectrometry
LB	–	lysogeny broth
MD	–	main defoliation
N	–	nitrogen
n.s.	–	not significant
NCBI	–	national center for biotechnology information
NGS	–	next generation sequencing
NMDS	–	non-metric multidimensional scaling
N _{tot}	–	total nitrogen
Of	–	fibric organic layer
Oh	–	humic organic layer
OI	–	litter organic layer
PCR	–	polymerase chain reaction

PEG	–	polyethylene glycol
PVC	–	polyvinyl chloride
PVC-U	–	polyvinyl chloride unplasticized
PVP	–	polyvinylpyrrolidone
qPCR	–	quantitative (real-time) polymerase chain reaction
rDNA	–	ribosomal deoxyribonucleic acid
rRNA	–	ribosomal ribonucleic acid
Spr	–	spring
Sum	–	summer
t	–	time
TAA-N	–	total soluble amino acid nitrogen
TAE	–	Tris acetic acid EDTA buffer
TE	–	Tris plus EDTA buffer
TOC	–	total organic carbon
TSP-N	–	total soluble protein nitrogen
UV	–	ultraviolet
v/v	–	volume per volume
VL	–	vegetation layer
w/v	–	weight per volume

Extended Summary

1. Introduction

Insects are an integral component of forest ecosystems but can adversely change ecosystem functioning during a mass progradation (FAO 2009). There are growing evidences that climate change will increase forest disturbances by insects due to direct changes in the insects' development and survival as well as impacts on trophic interactions and abiotic environmental conditions (Ayres and Lombardero 2000; Seidl et al. 2017). For example, in 2015 85,518 million ha of forest were affected by insect pests, which is 2.14% of the total forested area worldwide and values are believed to increase (van Lierop et al. 2015; Keenan et al. 2015).

During a massive outbreak situation, insects do not only affect tree health but can act as regulators for ecosystem processes and functions due to herbivory mediated alterations of carbon (C) and nitrogen (N) cycling (Schowalter 2016; Hunter 2001). Effects vary in velocity, duration, intensity, magnitude, and are highly variable depending on tree species, insect species, climate, and ecosystem properties (Pinkard et al. 2011; Hicke et al. 2012). Besides the limited ability to predict future outbreak events significant knowledge gaps still exist concerning the impacts on biogeochemical cycles in the forest ecosystems (Hicke et al. 2012). It becomes increasingly important to clarify and quantify the consequences of insect outbreaks for forests C and N cycling and thereby ecosystem functioning regarding the prospect of growing endangerment of forests by herbivory disturbances.

1.1. Effects of defoliation on the tree

Aboveground

Insect pests cause foliar damage up to complete defoliation of the tree, which reduces the photosynthetic active area. Consequently, either an increased photosynthesis rate of the remaining foliage as a compensatory reaction or a reduced photosynthesis rate were observed (Hoogesteger and Karlsson 1992; Lovelock et al. 1999; Vanderklein and Reich 2000). Further, allocation of N rich compounds in the foliage as compensatory reaction were discovered (Millard and Grelet 2010; Fang et al. 2016). Changes in the microbial community size and structure colonizing the phyllosphere were reported during insect infestations and were either qualitatively or quantitatively characterized in previous studies (Lilley et al. 1997; Müller et al. 2003; Menkis et al. 2015). Studies combining both approaches in one forest ecosystem during insect infestations apparently have not been conducted so far.

Phyllosphere microorganisms can play an important role for C and N transport from the canopy to the soil (Müller et al. 2006). On the one hand, the phyllosphere inhabiting microorganisms can alter throughfall chemistry and vertical nutrient transport and on the other hand they make the tree more susceptible toward secondary infestations by pathogens (Stadler and Müller 2000; Menkis et al. 2015). Further, the loss of biomass and transpiration area causes an imbalance between root water uptake and leaf transpiration which can adversely affect the trees water and nutrient balance (Bréda et al. 2006; Aroca et al. 2012).

Belowground

Trees show frequently altered patterns of biomass allocation during and/or after an infestation by phyllophagous insects: while fine root mortality increases (Britton 1988), an increased aboveground biomass growth is a possible reaction which further results in a decreased above:belowground biomass ratio (Cannell 1985; Russel 2004). Influences on root dynamics (e.g., decreased rhizodeposition and root growth) and altered root-soil-microorganism interactions are often observed to be a consequence of defoliation (Saravesi et al. 2015; Kosola et al. 2001; Pineda et al. 2017). These changes can lead to modified uptake of inorganic N by defoliated trees. However, studies exhibit divergent results: increased N uptake (e.g., Stoelken et al. 2010; Li et al. 2015) as well as reduced N uptake (Kosola et al. 2001) or no effect of defoliation (Lovett and Tobiessen 1993; Rubino et al. 2015) were reported. So far, the organic N acquisition by trees in context of insect outbreaks has never been investigated, although organic N sources are believed to play a major role in tree N nutrition (Ohlund and Näsholm 2004). The links between root nutrition, root N uptake capacity and root-colonizing ectomycorrhiza during biotic stress are still unclear and will therefore be included in this thesis (study 1 and 2).

1.2. Effects of defoliation on soils

Input change

In addition to the direct effects on the tree, outbreaks of defoliating insects in forests strikingly increase the organic input into the soil by the deposition of feces, cadavers, litter, and other plant material, usually during the insect's larval stage (Stadler et al. 2005; Müller et al. 2006; le Mellec and Michalzik 2008). Besides the solid inputs, washouts of damaged needles as well as wash offs of insect excretions and microorganisms from the needle and twig surface with high concentrations of N reach the soil within the throughfall solution (le Mellec et al. 2009; Stadler and Michalzik 1998). In addition to the changed timing and quantity of the input, the quality of the C and N compounds is easily available

for microbial turnover compared to more recalcitrant senescent leaf litter during non-infestation conditions of the tree. Insects primarily consume the fresh foliage before trees resorb N during leaf senescence (Lovett et al. 2002). The passage of fresh plant material through the insect gut alters the quality of C and N compounds in distinct ways (Lovett and Ruesink 1995). Insect feces N content consists of about 9% inorganic N (NH_4^+ and NO_3^-) and 91% organic forms (e.g., uric acid) (Lovett and Ruesink 1995). Insects need the N-rich plant proteins for the production of structural molecules (such as chitin) as well as for maintaining their internal enzymatic activities (Lind and Barbosa 2012). While insects feeding on Scots pine can utilize carbohydrates (fructose, glucose, and sucrose) to up to 100%, the utilization of N is rather low with <20% of the original needle N content (Jensen 1991; Lovett et al. 2002). As comparison, the tree retranslocation of N during needle senescence is 67 – 74%, resulting in lower N contents and wider C/N ratio of needle litter compared to insect feces (Nieminen and Helmisaari 1996). Thus, the high C and N content of feces and a lower C/N ratio compared to needle litter in combination with the high input rates can change C and N contents in the soil (Lovett et al. 2002; Madritch et al. 2007; le Mellec et al. 2009).

Soil processes

The input change in form of high amounts of labile C and extractable N in insect feces can facilitate the nutrient access in soils during insect infestations (Lovett and Ruesink 1995; Zimmer and Topp 2002; Stremińska et al. 2006). This nutrient input by the feces modifies important soil processes in C and N cycling, for example increase of microbial activity and mineralization rates, especially in N-limited ecosystems (Belovsky and Slade 2000; Chapman et al. 2003; le Mellec and Michalzik 2008). Increased populations of soil fungi and bacteria were observed following insect defoliation in forests (Stremińska et al. 2006; de Graaff et al. 2010; Oneț et al. 2016). However, both bacteria and fungi show a fast response toward organic input changes but perform partially distinct tasks in decomposition processes: bacteria are specialized in the rapid turnover of easily decomposable compounds while fungi dominate in the turnover of more complex organic compounds (Wardle et al. 2002; Poll et al. 2008). Therefore, the quality of the input may be a critically important variable for the possible changes of soil microbial community structure and abundance. For example, the NH_4^+ concentrations and C/N ratio in soil has been shown to correlate with the bacterial community structure in bark beetle (Scolytidae spp.) infested forests (Mikkelsen et al. 2017).

Besides accelerating effects on the nutrient cycling, decelerating effects are possible (Ritchie et al. 1998). With the feces input from Scots pines, large amounts of recalcitrant compounds in form of secondary metabolites such as terpenes, rosin acid and phenols

reach the forest soil (Kainulainen and Holopainen 2002; Kagata and Ohgushi 2013). The decomposition of these compounds can negatively affect microbial degradation processes for several years (Kainulainen and Holopainen 2002). A N retention due to microbial immobilization or creation of recalcitrant complexes in soil is another possible pathway of the additional N (Lovett and Ruesink 1995; Madritch et al. 2007; Madritch and Lindroth 2015). Microbial bound N is temporarily inaccessible for trees. Therefore, the time scale (fast reactions versus long-term changes) is an important but still not sufficiently investigated parameter to consider when evaluating microbial responses to insect outbreaks and, thus, is particularly discussed in this work. Further, the link between microbial population size and composition and changes in soil functions during insect outbreaks are still unclear (Nannipieri et al. 2003) and therefore study 2 and 3 are dedicated to that topic.

Losses

Labile C and N from feces is rapidly incorporated in the microbial biomass or dissolved in the soil solution from where different pathways are possible: N can be taken up by trees or the understorey vegetation, can be reused by microorganisms or absorbed to the soil matrix and thus contribute to the soils internal C and N pools (accumulation and stabilization) or it can be lost via leaching or gaseous emissions. Increased heterotrophic respiration (CO₂ emissions) from forest soils during and following insect infestations are commonly observed (le Mellec et al. 2011; Kurz et al. 2008; Lovett and Ruesink 1995; Michalzik and Stadler 2000; Oren et al. 2001). In contrast, little attention is given to possible alterations of soil N₂O fluxes during insect outbreaks, probably because of its high spatial and temporal variability in forests and coherent difficulties in generalizing flux quantification over a larger area (Groffman et al. 2009; Butterbach-Bahl et al. 2013). However, a correlation between N deposition and gaseous N emissions in N poor ecosystems is assumed (Kitzler et al. 2006). Next to N₂O, other gaseous N emissions such as NO or N₂ as a reaction to organic input are believed to be of minor magnitude and importance (Butterbach-Bahl et al. 2013). Leaching losses via soil solution, especially of nitrate, are another consequence of insect derived input (Swank et al. 1981; Pitman et al. 2010; le Mellec et al. 2011). N leaching losses of insect feces are higher and happen rapidly compared to N leaching from leaf litter (Hollinger 1986; le Mellec et al. 2011). A comprehensive comparison of different N-loss pathways within the same forest ecosystem during insect defoliation did not exist so far and is therefore one of the aims of the present thesis.

1.3. Effects of defoliation on the ecosystem scale

Insect-caused defoliation can not only provoke distinct alterations of the tree health but additionally affects the whole forest ecosystem. Changes of tree vitality, growth, and an increased tree mortality have the potential to shift forest structure and composition (Stadler et al. 2005). For example, the understorey vegetation layer can shift toward plants with higher light and nutrient requirements (Wenk and Apel 2007). Furthermore, the susceptibility of the forest towards secondary pests is increased following an infestation with phytophagous insects, which further impairs the health of the forests (Annala et al. 1999; Oliva et al. 2016). Multiple consecutive years of severe defoliation increase tree mortality significantly (Van Asch and Visser 2007). Therefore, insect outbreaks can be a major perturbation on the forests' net primary production (Pinkard et al. 2011). In combination with decreasing C uptake by the biomass, greenhouse gas emissions from soil and plants and leaching losses following insect outbreaks the forest can turn from a C sink to a C source (Kurz et al. 2008; Clark et al. 2010; Dymond et al. 2010; Hadden and Grelle 2017). In addition to the ecological detriments, decreased growth, alterations of wood quality and tree dieback decrease the forest production function which causes economic losses for the timber industry and impairments of the protective and social forest functions (Straw et al. 2002; Lyytikäinen-Saarenmaa et al. 2002). However, almost all effects of insect outbreaks are strongly dependent on climate, site conditions, plant attributes and soil properties as well as the insect species and the outbreak characteristics (Pinkard et al. 2011). Therefore, our knowledge on insect outbreak consequences for C and N fluxes on the ecosystem scale remains incomplete. The comprehensive flux quantification during the insect infestations investigated in this thesis gives a first insight into the alterations of ecosystem balances with the purpose to improve the estimation of defoliation caused effects on ecosystem scale.

1.4. Objectives and aims

This thesis aims to detect and quantify changes in forests caused by infestations of phytophagous insects. Direct effects of the insects on the tree performance and nutrition and indirect effects on the soil by increased C and N inputs lead to a cascade of flux and process changes. Soil microbial populations are potentially quantitatively and qualitatively affected by the input change and thereby altering soil C and N turnover processes and ecosystem losses. At the same time, the defoliation and larval residues alter the phyllosphere with consequences for the phyllosphere inhabiting microorganisms and, thus, the tree health. Taken together, these alterations have the potential to alter ecosystem balances and functions. With this work I would like to answer the following questions:

- What is the fate of insect derived C and N inputs to the soil: Prevalence of losses or enhanced system internal recycling?
- What are the consequences of defoliation and insect derived inputs for tree N nutrition and tree health?
- Is the microbiota as indicator for turnover processes quantitatively and/or qualitatively affected by insect outbreaks?
- Does the increased C and N input enhance nitrification/denitrification processes in soil and consequently increase gaseous emissions?

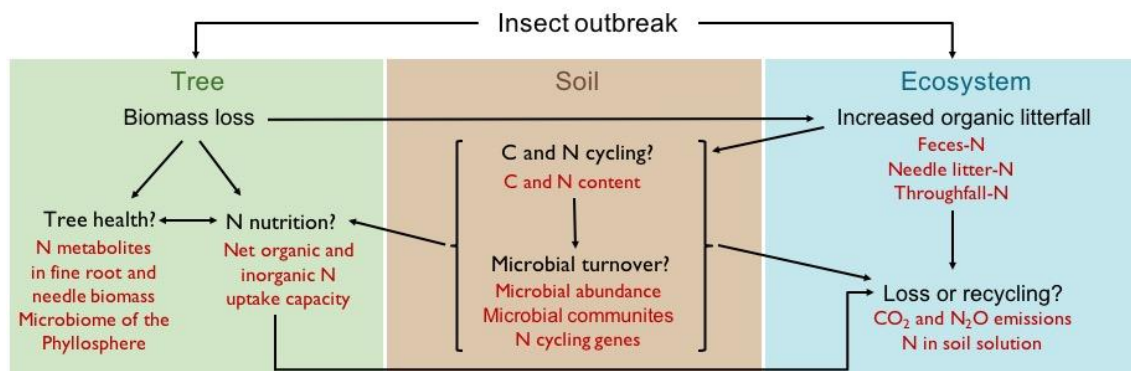


Figure 1: Links between major process changes and research questions (black) with corresponding parameters evaluated in this thesis (red).

The more specific aims of the single studies were as follows:

- to quantify input fluxes of N during insect outbreaks compared to non-outbreak conditions (study 1)
- to clarify the effects of insect outbreaks on inorganic N uptake capacity and particularly to identify possible alterations on organic N acquisition and possible preferences for N sources of the infested trees (study 1)

- to characterize potential dominant taxa changes in the bacterial and fungal community structure in the soil and the phyllosphere during insect outbreaks especially with regard to possible ecosystem consequences (study 2)
- to quantify microbial populations (fungi, bacteria, denitrification genes) in the phyllosphere, feces, needle litter, dead larvae, the vegetation layer, the organic layer, and the mineral soil during insect outbreaks with respect to turnover of the organic input and the pest insect's life cycle (study 3)
- to elucidate the soil internal N conversion processes (especially denitrification) during insect outbreaks and their implications for potential soil N losses (study 3) and consequently
- to identify and quantify possible loss pathways for C and N (soil CO₂ and N₂O fluxes, total N, NO₃-N, dissolved organic nitrogen (DON) in the soil solution) and to assess their relevance for ecosystem fluxes during outbreak situations (study 1 and 4)

2. Material and Methods

2.1. Study Site

Study site for the study 1 to 4 was a Scots pine (*Pinus sylvestris* L.) forest site infested with the nun moth (*Lymantria monacha* L.), 40 km south of Berlin, Germany. For study 2 and 3 additionally a forest infested with the pine-tree lappet (*Dendrolimus pini* L.), 90 km southwest of Berlin, Germany, (see Figure 2) was investigated. For both infested forest sites, corresponding forests with non-outbreak conditions of the insects, comparable site conditions, and within spatial proximity served as controls. The soil type at all sites was an acidic, calcium carbonate-free and nutrient-poor podzol on fine to medium sand planted with adult Scots pine as monoculture. Samplings and measurements were conducted between 2013 and 2015 and were aligned to the developmental stage of the outbreak, in most cases pre-defoliation, main defoliation, and post-defoliation/tree recovery period.

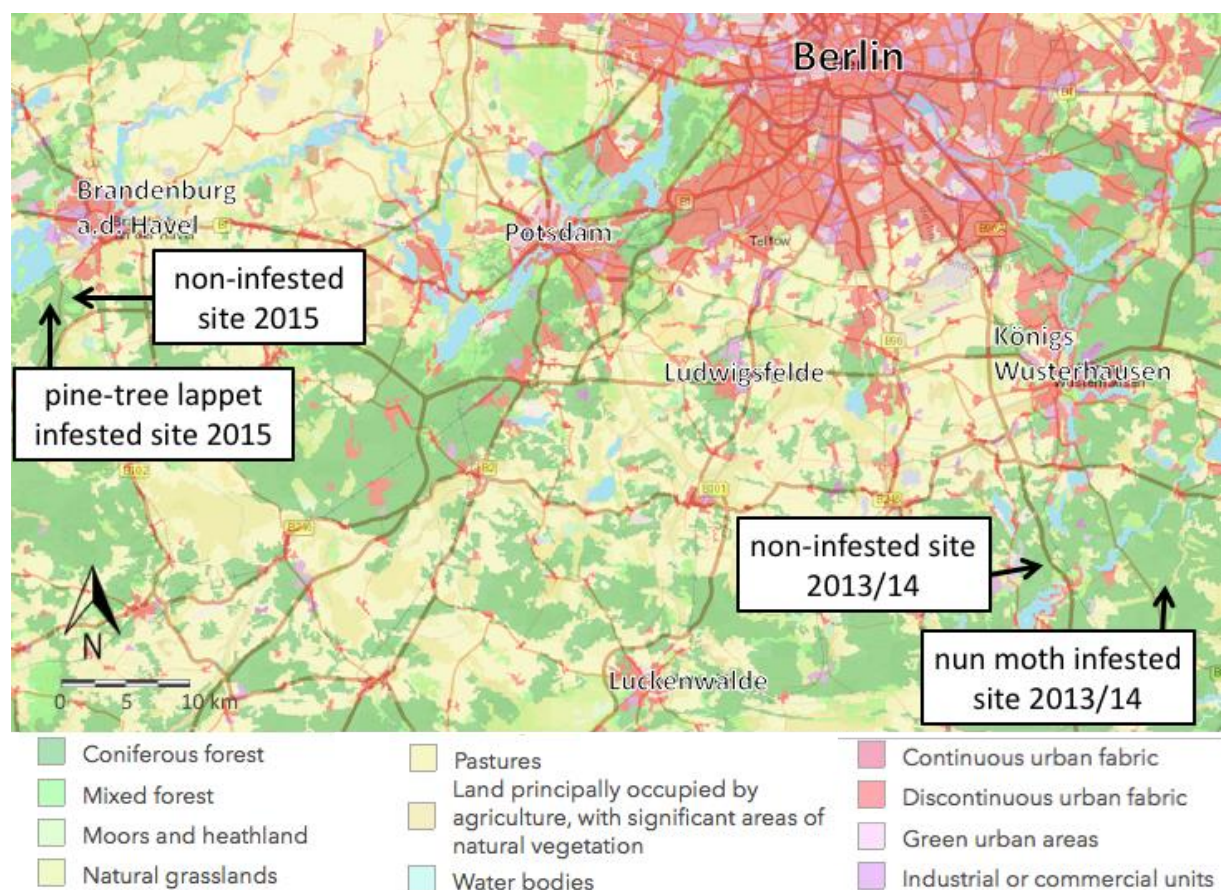


Figure 2: Geographical location and land use of the study sites in 2013/14 and 2015. Map source: European Environment Agency, 2013: *Corine Land Cover 2006*, seamless vector data (Version 17), Copenhagen, Denmark and Esri, HERE, Garmin, USGS et al.: *World Street Map*, Redlands, California, USA; <https://www.arcgis.com/home/webmap/viewer.html> (12.02.2019).

2.2. Sampling

On the nun moth infested and control sites, throughfall samplers, zero tension humus lysimeter (stationary underneath the organic layers) for collection of soil solution, and nylon nets for larval feces and needle litter sampling were permanently installed (Study 1). PVC frames were permanently inserted into the soil on the nun moth and pine-tree lappet infested and control sites to conduct soil gas sampling using the closed chamber approach in periodic intervals (study 4). Samples of the vegetation layer (VL), the organic layers (OI, Of, Oh), and the mineral soil (Ah) as well as fresh Scots pine needle and fine root biomass samples were taken for the determination of the C and N content and partly for the DNA extraction and determination of N metabolites in 2014 and in 2015. An experiment to quantify the net ^{15}N uptake capacity was conducted in 2014 on living Scots pine trees on four sampling dates. Further, a soil incubation experiment with needle litter, feces and needle litter plus feces treatment additions to measure soil gas fluxes (CO_2 , N_2O) was conducted in a climate chamber of the Büsgen institute, Georg-August University Göttingen.

2.3. Analytical Methods

- Total carbon (C_{tot}) and nitrogen (N_{tot}) content of the soil in study 1, 3 and 4, in fine roots and fresh needles in study 1, the vegetation-, and organic layers in study 3, and feces, and needle litter in study 4 were determined in dried and finely ground material by a TOC/TN analyzer.
- N content in the soil solution and the throughfall (N_{tot} , nitrate-N and DON) in study 1 was determined on filtered samples. N_{tot} was quantified using thermal oxidation, nitrate-N was determined by ion chromatography and DON was calculated as the difference between N_{tot} and nitrate-N.
- Net N uptake capacity of the fine roots in study 1 was determined after two hours of incubation in different artificial soil solutions, where for each N source the specific N was substituted by ^{15}N for NH_4^+ , NO_3^- as well as for the amino acids arginine and glutamine, respectively, while a non-labeled solution served as control. Fresh and dry weight of the incubated fine roots were determined, samples were finely ground and ^{15}N content was determined with an element analyzer coupled to an isotope ratio mass spectrometer.
- Total soluble protein-N, total amino acid-N, NH_4^+ -N, NO_3^- -N and structural N content in fine roots and needles in study 1 were determined in samples finely ground in liquid N_2 . For total soluble protein-N and total amino acid-N samples were extracted in

specific buffer solutions, respectively, before absorption measurement using a spectrometer. $\text{NH}_4^+\text{-N}$ and $\text{NO}_3^-\text{-N}$ were measured with an ion chromatograph after sample preparation in polyvinylpyrrolidone solution. Structural N was calculated by subtracting total soluble protein-N, and amino acid-N from total N.

- Total DNA used in study 2 and 3 was extracted from the dried and finely ground material following a cetyltrimethyl ammonium bromide (CTAB) protocol including two chloroform/isoamylalcohol steps with an intermediate phenol purification and subsequent polyethylene glycol precipitation.
- For the fingerprinting analyses of the phyllosphere and soil fungal and bacterial community DNA samples were pooled, bacterial 16S and fungal 18S rRNA genes were amplified in a polymerase chain reaction (PCR) using specific primer sets with a GC clamp and then separated on an acrylamide/bisacrylamide gel containing a denaturing urea and formamide gradient (denaturing gradient gel electrophoresis (DGGE)). Gels were fixed by acetic acid, stained in silver nitrate and formaldehyde solution before transferring to a developer solution and subsequent drying. Bands of interest were cut out, DNA was reamplified with a PCR, precipitated and sent for sequencing for taxonomic assignments.
- The quantification of fungal 18S rRNA genes, bacterial 16S rRNA genes and denitrification genes was conducted with diluted and pooled DNA samples. Specific primers were used to detect fungal 18S rRNA genes and bacterial 16S rRNA genes as well as the functional genes of ammonia-oxidizing bacteria and archaea (*BamoA* and *AamoA*), Cu-nitrite reductase (*nirK*), cd_1 -nitrite reductase (*nirS*), and nitrous oxide reductase (*nosZ* clade I and II) in a quantitative PCR assay including fluorescence measurement.
- CO_2 and N_2O fluxes from the forest floor were measured using a four-point sampling method from a temporally closed chamber inserted in the soil organic layer. Gas samples were stored in evacuated glass exetainers and subsequently analyzed by gas chromatography. For the incubation experiment, incubators with mineral soil and different treatment additions (insect feces, needle litter and a mixture of both) were attached to an automated gas chromatographic system to measure CO_2 and N_2O concentrations continuously during 31 days.
- Major element compositions (Al, Ca, Fe, K, Mg, Mn, Na, P, and S) of pine-tree lappet feces, larval cadavers and needle litter in study 4 were measured in mixed, dried and finely ground samples. Material was extracted by pressure digestion in HNO_3 and

total element concentrations were measured using inductively coupled plasma – optical emission spectrometry (ICP-OES).

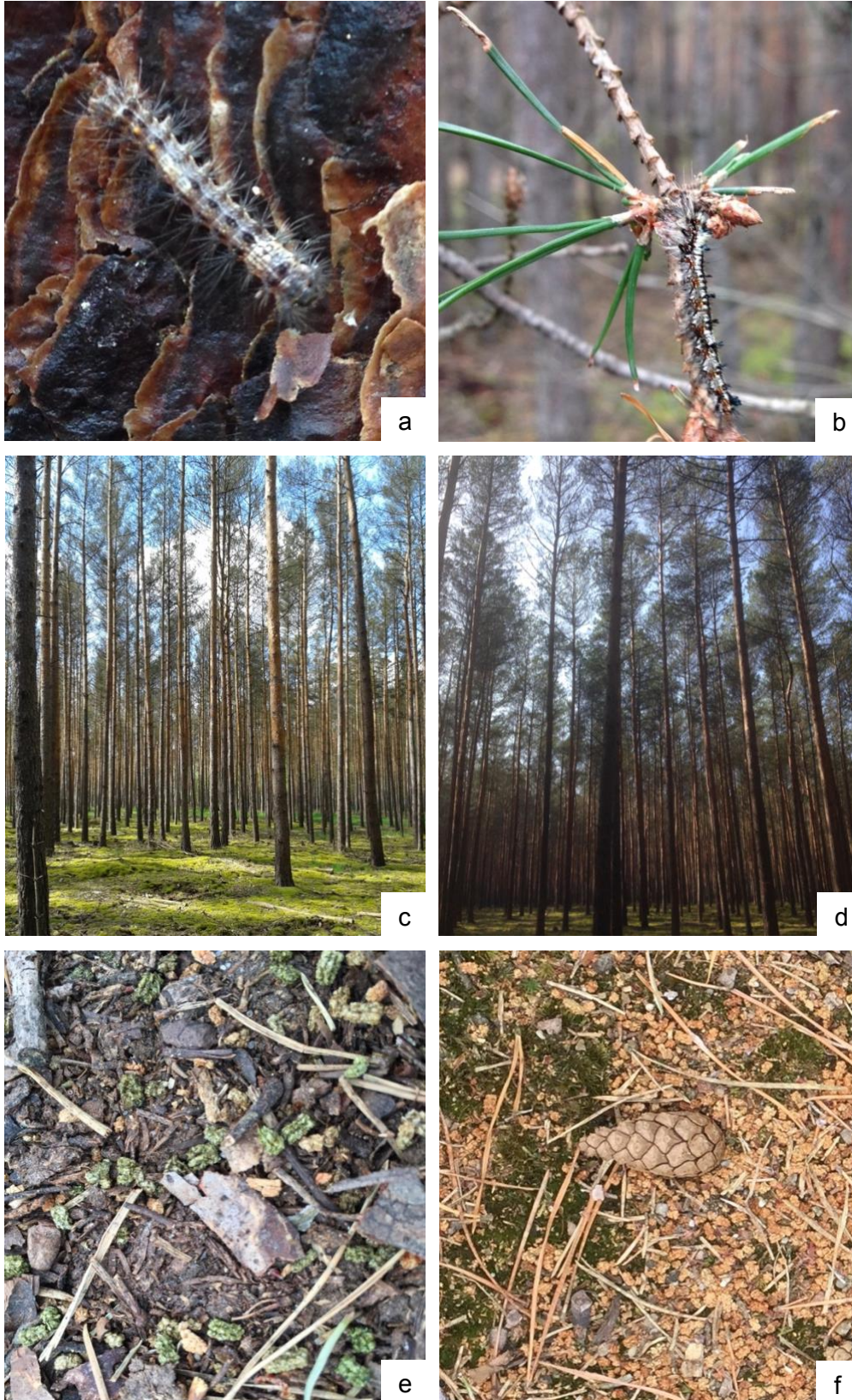


Figure 3: Nun moth larva (*L. monacha*) (a); Pine-tree lappet larva (*D. pini*) (b); Scots pine (*P. sylvestris*) forest defoliated by the nun moth (c) and by the pine-tree lappet (d) Feces of the pine-tree lappet on the forest floor during main defoliation (May) (e) and in Autumn (September) (f).

3. Results and Discussion

3.1. Overview: Main findings

Study	Main findings on infested forests	Conclusion
Study 1: “ <i>Defoliating Insect Mass Outbreak Affects Soil N Fluxes and Tree N Nutrition in Scots Pine Forests</i> ”	<ul style="list-style-type: none"> - Increased N input (feces, litter, throughfall) - Increased N losses via soil solution (mostly nitrate) - Reduced inorganic and organic net N uptake capacity - Compensatory N accumulation in fine roots and needles 	<ul style="list-style-type: none"> → Soil N availability is increased via surplus N input → Scots pine does not respond with an increased N acquisition → Investment in defense by accumulation of amino acid-N and protein-N as a survival strategy
Study 2: “ <i>Changes of Scots Pine Phyllosphere and Soil Fungal Communities during Outbreaks of Defoliating Insects</i> ”	<ul style="list-style-type: none"> - Change of fungal community in soil and the phyllosphere - Decrease of ectomycorrhiza fungi - Potential pathogenic fungal taxa in the phyllosphere - No change in bacterial community in the phyllosphere and soil 	<ul style="list-style-type: none"> → Bacterial community is more resilient toward insect disturbances than fungal community → Interference of the ectomycorrhiza-tree symbiosis → Threat to tree health by pathogenic fungi (secondary pests)
Study 3: “ <i>The Abundance of Fungi, Bacteria and Denitrification Genes during Insect Outbreaks in Scots Pine Forests</i> ”	<ul style="list-style-type: none"> - Increase of bacteria in needles, vegetation layer and organic layer - Increase of fungi in vegetation and organic layer - Increased <i>nirK</i> gene abundance in soil - Decreased soil C/N 	<ul style="list-style-type: none"> → Organic input fosters short term bacterial and fungal growth in soil → Litter, Feces and Larval Cadavers serve as habitat for Microorganisms and soil N Source → <i>nirK</i> increase indicates an increased genetic potential of N₂O emissions → Denitrification is the dominating process
Study 4: “ <i>Increased Forest Soil CO₂ and N₂O Emissions during Insect Infestation</i> ”	<ul style="list-style-type: none"> - Increased CO₂ emissions from soil in the forest - Indications of increased N₂O emissions on one sampling date - Incubation experiment: <ul style="list-style-type: none"> - Increased CO₂ and N₂O in the feces treatment compared to the needle litter and soil only treatment - Increased soil C/N after the experiment 	<ul style="list-style-type: none"> → Insect outbreaks enhances demineralization and causes increased CO₂ emissions from soil → Increased soil N₂O emissions are possible (experiment) but do not necessarily have to occur (field) → Decomposition of feces leads to rapid soil responses compared to needle litter decomposition

3.2. N loss or N recycling? The fate of insect derived N in soil

Several studies assume that the N input during insect outbreaks is primarily recycled within the soil system (Lovett et al. 2002; Frost and Hunter 2007; Kaukonen et al. 2013). Other studies suppose a net N loss from the ecosystem during insect outbreaks (Ritchie et al. 1998; Pitman et al. 2010). Even though most results on insect outbreaks are study site dependent and therefore not directly comparable, the general trend is either an accelerated nutrient turnover (often associated with increased N losses) or a decelerated nutrient turnover (with N redistribution and recycling or storage within the system) (Burghardt et al. 2018).

→ **Our findings illustrate that timing and scale of the flux and pool changes are critically important when evaluating C and N cycling within one forest ecosystem. Acceleration of turnover processes in soil dominates in infested Scots pine forests but the severity and persistence of the ramifications of each process and flux distinctly varies over time and in intensity.**

While some ecosystem variables show an immediate but short-term reaction to the insect outbreak (e.g., microorganisms), others respond belated with maximal fluxes in autumn (e.g., CO₂-C emissions, total N and NO₃⁻ in soil solution). To comprehend the different timings of the soil reactions and to draw appropriate conclusions about ecosystem turnover processes a closer look on the changes of quality and quantity of the input composition during insect outbreaks is essential, which was covered in study 1 of this thesis.

The needle litter N input during the nun moth outbreak year was constantly high with mean fluxes of 6.4 kg ha⁻¹ month⁻¹ (+320.5% compared to the non-infested site). These high fluxes were caused not only by the higher total quantity of the needle litter but also by the increased total N contents in infested needles as compensatory response to the defoliation (see study 1). Scots pine needle litter (and also some compounds of the insect feces especially secondary metabolites such as tannins or lignin) can retain long-term in recalcitrant organic matter when introduced to the soil (Hicke et al. 2012). These recalcitrant organic polymers are more resistant towards decomposition compared to labile input sources (e.g., feces, throughfall) and thereby preventing immediate leaching or plant uptake during insect outbreaks (Lovett et al. 2002). Thus, high inputs of needle litter, twigs, and branches often result in a delayed decomposition of the material regarding the actual outbreak event (Hicke et al. 2012). For example, increased rates of soil respiration were observed for a forest defoliated by the Siberian moth (*Dendrolimus superans sibiricus* Tschtrk.) even three years after the outbreak (Baranchicov et al. 2002). On our nun moth infested study site, soil CO₂ emissions were still increased in

autumn after the main defoliation period, which can be seen as a belated response towards the actual defoliation activity (study 4). Like needle litter, throughfall N concentrations remain high during almost the entire vegetation period. Compared to the control site, throughfall N content remains increased at all sampling dates with average total N fluxes of $15.3 \text{ kg ha}^{-1} \text{ month}^{-1}$. Especially DON, which can make up to 63% of the total N in the throughfall during main defoliation, is an easily available N source for the microorganisms in soil and thereby triggers microbial growth and turnover processes constantly throughout the measured vegetation period (le Mellec et al. 2011).

Feces amounts as the third major component of organic input fluxes vary within the vegetation period in dependency to the larval development (le Mellec et al. 2009). While no measurable inputs arise during winter and spring before hatching of insect, the feces amount reached maximum fluxes of $11.3 \text{ kg ha}^{-1} \text{ month}^{-1}$ during main defoliation which was almost twice as much as the needle litter input on the same date of the infested site (study 1). In general, feces can make up to 46% of the total organic input during insect outbreaks, but on our study site it had a share of only 18% of the total input in 6 months (Fogal and Slansky, 1985). Notwithstanding of its amount, insect feces has diverse and distinct effects on the soil C and N cycling because of its physical and chemical composition (Frost and Hunter 2007). Feces from insects feeding on Scots pine have an inconsistent C/N ratio with an average of 60 but peaks up to 90 due to varying assimilatory requirements of the insect in its larval development (le Mellec and Michalzik 2008). In contrast, Scots pine needle litter and throughfall C/N ratio is about 34 – 40 and 5 – 40, respectively, during the vegetation period (le Mellec and Michalzik 2008). However, C/N ratio of inputs as an indicator for demineralization in soil is not always useful since concentrations of molecules can be different with identical N content (Lind and Barbosa 2012). Therefore, N input via feces can have simultaneously accelerating (e.g., amino acids) and decelerating (e.g., alkaloids) impacts on soil microbial abundance (Lind and Barbosa 2012). Feces, despite the wide C/N ratio, increases total content of C and N in soil and can lead to fast microbial and thereby soil respiration responses (see study 3 and 4). Labile compounds of the feces (e.g., urea, soluble sugars, simple carbohydrates; Russel et al. 2004) and the dissolved and particular C and N in the throughfall directly accelerated the microbial abundance (+583% and +145% bacteria and fungi, respectively, during main defoliation, study 3). The microbial breakdown of the additional C goes ahead with N incorporation from the microbial biomass and increasing CO_2 emissions compared to pre-defoliation conditions (study 4). This temporal microbial N immobilization may prevent even higher physicochemical N losses through leaching or gaseous emissions during times of maximum organic inputs, as occurring during main defoliation. In insect infested forests, particularly in sandy soils as they are found in our

research area, this process could be an important short-term buffer especially during periods of high labile C and N inputs and reduced root N uptake by the vegetation (Kuzyakov and Xu 2013). On the other hand, microbial N immobilization hampers root N uptake during increased N supply by the insect derived inputs. During late summer and autumn, the replenishment of feces and thereby labile N decreases to nearly the same level as the needle litter input with fluxes of $5.7 \text{ kg ha}^{-1} \text{ month}^{-1}$ which is evoked by ceasing larval abundance due to their metamorphoses to moths in July. With decreasing feces replenishment, bacterial abundance in soil decrease to the level of the non-infested site. Fungal populations in the organic layer of the infested site remain slightly increased, probably due to their specialization on the turnover of complex organic compounds as found in the needle litter (Poll et al. 2008). Nevertheless, their abundance in the OI layer is 8 times less compared to main defoliation conditions. With the microbial dieback in autumn forest soil C and N losses reach their maximal extent: $\text{CO}_2\text{-C}$ emissions (+85% compared to the non-infested site) and N fluxes in the soil solution (especially DON fluxes in the soil solution with +800% compared to the non-infested site) increase in autumn, as we could observe in study 1 and 4. In relation to the total N input, the highest N losses via soil solution occur in autumn. At the same time, the trees N uptake capacity in autumn was still decreased, which implies that this conceivable alternative pathway for N translocation from the soil was restricted.

Consequently, the major proportion of the high C and N input fluxes in autumn got lost via different pathways: C mostly as gaseous CO_2 emissions and N primarily as dissolved NO_3^- and DON in the soil solution. Finally, the post defoliation microbial dieback towards the initial level of pre-defoliation conditions and the losses of C and N via soil solution and gaseous emissions result in a decreased C/N ratio of the ground vegetation and the organic layers as well as the mineral soil in autumn. To summarize, feedbacks on the forests N cycling during insect outbreaks are strongly correlated to the quality and quantity of the organic input compounds which's amounts in turn depend on the insects feeding behavior and life cycle (see Figure 4). Whereas during main defoliation maximal amounts of feces input cause microbial immobilization as well as accelerated turnover, in autumn C and N remobilization from the microbial biomass and the further breakdown of the recalcitrant input compounds and thereby N losses of the ecosystem are the dominating processes.

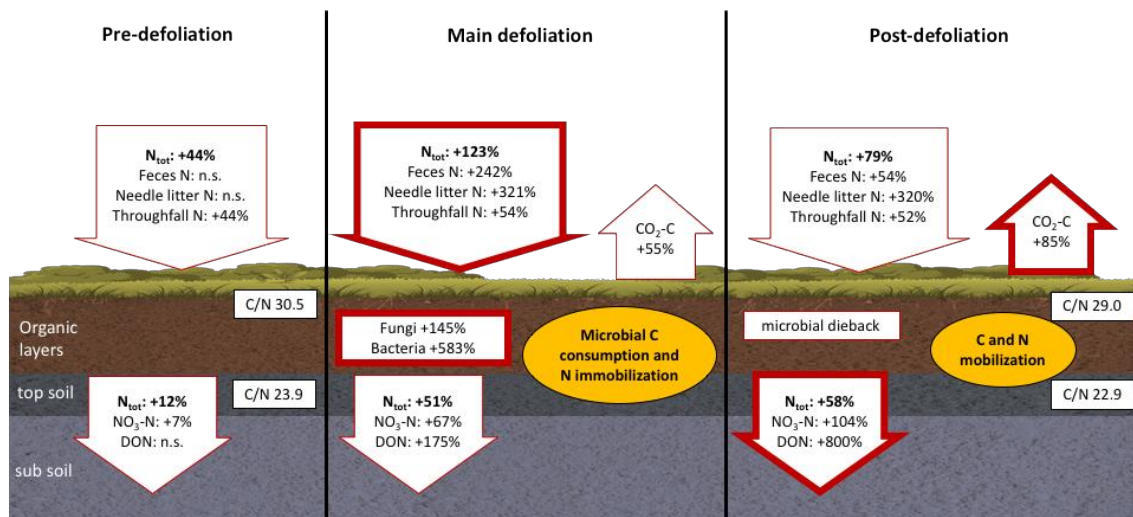


Figure 4: Schematic overview of the major N input components, N losses via soil solution, CO₂-C fluxes, fungal and bacterial biomass and C/N of the organic layer and the Ah layer during an outbreak of the nun moth. Values as percentage change compared to the non-infested site during the vegetation period, thick red borderlines mark maximum fluxes (inputs, losses) or amounts (fungal and bacterial rRNA gene abundance). Data based on study 1, 3, and 4; n.s.= no significant differences between infested and non-infested were detectable.

3.3. N nutrition and health – insect outbreaks interfere the trees N balance

N is an important element for many metabolic processes in plants and the development and functioning of vital cells (Hirsch and Mauchline 2015). Therefore, the N uptake of available N from the soil is essential for the trees N supply. Increased mineralization rates during insect mass outbreaks, resulting in increased availability of N in soil, are believed to favor plant regeneration and growth following insect outbreaks (Frost and Hunter 2007; le Mellec and Michalzik 2008). A fertilizing effect of the insect derived input on tree recovery is therefore a possible pathway often assumed following an insect outbreak (cf. Christenson et al. 2002). Previous studies on the N uptake capacity of defoliated trees are rare, showed contrasting results, focused only on inorganic N sources, and investigated seedlings instead of adult trees, which are different in their metabolic processes and N requirements (e.g., Lovett and Tobiessen 1993; Kosola et al. 2001; Rubino et al. 2015). These knowledge gaps were targeted in study 1.

→ **Infested adult Scots pines are inhibited in their NH₄⁺, NO₃⁻ and glutamine net N uptake capacity by up to 65%, especially following the main defoliation by insects. In contrast, arginine uptake was unaffected by the insect defoliation and was the preferred N source of all investigated trees. These outcomes are of special interest since they provide first insights on organic N uptake by adult trees defoliated by insects.**

These findings in combination with the recorded high N losses via soil solution and potential gaseous emissions (study 1 and 4) indicate that trees infested by the nun moth cannot directly benefit from the organic input during insect infestations. Debilitated by the defoliation, trees seem to be inhibited in their competitiveness for soil N. Particularly in the rhizosphere, trees directly compete with microorganisms for N compounds (Kuzyakov and Xu 2013). During times of high C supply caused by the relatively wide C/N ratio of the feces, soil microorganisms use N for their metabolisms to break down the additional C input. Microorganisms are often described as 'superior competitors', especially on a short-term scale following organic inputs (Näsholm and Persson 2001). At the same time, this interspecific competition can lead to a N deficiency of the trees by being outcompeted by the soil microorganisms (Hirsch and Mauchline 2015).

Study 2 demonstrates another possible effect associated with the reduced N uptake capacity of trees during insect outbreaks. The abundance of an ectomycorrhizal fungus assigned to the genus *Russula* was strongly reduced in the upper organic layers during the pine-tree lappet outbreak. The defoliation by the insect probably decreased the rate of photosynthesis and consequently the carbohydrate supply to the symbiotic association between tree and mycorrhiza (Markkola et al. 2004). As a consequence, ectomycorrhiza abundance in soil decreases. Another explanation for the loss of ectomycorrhiza can be seen in the increased N input. Abundance and diversity loss of ectomycorrhiza following anthropogenic N additions were previously observed but until now not in case of insect derived organic inputs (Palátová 2002; Tarvainen et al. 2003). In times of sufficient N supply within the soil, the root colonization with ectomycorrhiza is associated with increased N uptake, N contents, and tree growth rates (Marschner and Dell 1994; Quoreishi and Timmer 1998; Ahangar et al. 2012; Heinonsalo et al. 2015). Therefore, the observed decrease of the ectomycorrhizal fungus could have contributed to the reduction in N uptake capacity.

Further, the observed decrease in N uptake capacity can be evoked by impaired fine root functioning due to the general debilitation of the infested tree. As response to heavy defoliation a decreased growth and dieback of trees fine roots is often found (Gieger and Thomas 2002). Impaired functioning of fine root uptake can be a first indicator of beginning root mortality. However, a decrease in fine root biomass also occurs following to N depositions through sufficient N supply of the roots and therefore preferential N investment in aboveground plant parts (Palátová 2002). In the end, a probably a combination of impaired root conditions, ectomycorrhiza dieback, weakened competitiveness against microorganisms, and the general reduction of the trees energy supply by photosynthesis have contributed to the diminution of the N uptake capacity during the insect outbreak. Based on these findings and in contrast to previous

assumptions, we can exclude a fertilizing effect of the insect feces for the infested trees. This is supported by an experiment by Frost and Hunter (2007) who used ^{15}N labeled feces to demonstrate that deposited N in feces usually contributes only by <1.0% – 1.2% to the tree's foliage N.

The high uptake capacity for arginine and glutamine by all investigated Scots pines supports the hypothesis that organic N sources might be preferred by trees over inorganic N sources. Particularly arginine is a storage component of many soil fungi, which is released to the soil solution during microbial breakdown (Finlay 1992). Since seasonal controlled microbial breakdown is a process which is distinctly elevated following the main defoliation in autumn, Scots pine trees are probably well adapted to this periodical release of arginine to the soil solution. The observed dieback of ectomycorrhizal fungi (study 2) may have served as easy allocatable arginine source for the infested trees. The premise for this assumption is that infested trees still have an intact and active transporter system for arginine in the plasma membranes of the roots which is not completely dependent of mycorrhiza occurrence. In contrast, the transporter system for glutamine and the inorganic N sources seems to be impaired by the dieback of ectomycorrhizal fungi or the defoliation. Another explanation for the trees preference of organic N sources is the less energy requirement of the plant for its utilization compared to inorganic N sources, especially during times of inhibited energy supply by photosynthesis (Gruffmann et al. 2013). In nutrient limited sites preferences of organic N sources for the tree's N uptake are frequently reported (Stoelken et al. 2010; Li et al. 2015). Study 1 demonstrated that this is also the case for Scots pine growing on poor podzols, at least regarding arginine uptake, regardless if infested by insects or not.

Despite the observed reduction in N uptake capacity, nun moth infestation stimulates changes in foliar chemistry, particularly of the N metabolites. N in foliage is an essential component for the generation of amino acids, nucleic acids, and chlorophyll and is therefore an indicator of the tree health status (Hirsch and Mauchline 2015).

→ **We could demonstrate in study 1 that infested Scots pines intensively invest resources into the accumulation of total soluble protein-N, amino acid-N, and structural N in needle and root biomass.**

In times of insect attacks and foliage biomass loss accumulation of N metabolites (especially amino acids) in plants most active parts can serve as osmoprotectants to maintain the water balance of the tree or as storage against short-term fluctuations in N supply (Rennenberg et al. 2006). Further, the observed accumulation of N in fine roots and needles can arise from the production of defense compounds (e.g., phenolics, alkaloids) in these tree parts (Millard and Grelet 2010; Fang et al. 2016). Potential

pathogenic fungi were found on the needle samples in study 2 during both outbreaks. Since they were not detectable or occurred in distinct lower abundance in the non-infested needles, the insect outbreak has contributed to the dispersal of the observed fungi in the phyllosphere. This higher abundance can be a trigger for the production of defense compounds in the infested needles which in turn evokes increased N contents in needles. Therefore, the increased N metabolites in the needles were likely defense compounds as a response to the pathogenic fungi growing in and on the needle surface. Likewise needles, roots often produce organic exudates such as amino acids against secondary infestations with pathogenic fine root feeding bacteria, fungi, and insects (Bezemer and van Dam 2005; Oliva et al. 2016). The increase in total soil microbial DNA during the nun moth outbreak observed in study 3 can possibly contain such pathogenic microorganisms for trees. However, sequencing of the same samples in study 2 only gave results on family level of the soil organisms, which is too imprecise to conclude on distinct interferences between soil microorganisms and root nutrition. Nonetheless, presumably a combination of defense compounds, osmoprotectants, and (to minor parts) root N uptake may have caused the N accumulation in fine roots of infested trees. To synthesize, decreased N supply by the roots, evoked by the defoliation and impaired ectomycorrhizal symbiosis as well as coincident internal reallocation of N in fine roots and needles as reaction to the defoliation is accompanied by the risk of secondary infestations and thereby health impairments by pathogenic fungi. Thus, we could demonstrate that insect outbreaks have metabolic and physiological consequences at the whole-tree level, from the fine roots up to the needles.

3.4. Microbial dynamics during insect outbreaks – from the phyllosphere to the mineral soil

Microorganisms in forests can respond quickly to natural disturbances such as insect outbreaks (Pal et al. 2012). During the nun moth outbreak, the bacterial and fungal abundance of the upper organic layers as well as in the phyllosphere increased in comparison to non-outbreak conditions (study 3). At the same time, a fungal community shift in both forest compartments was detected during the main defoliation period (study 2). Interestingly, alterations in the fungal community and microbial abundance between insect infested sites and non-infested sites particularly occurred during main defoliation and are non-existent or less pronounced on the pre- and after-defoliation sampling dates (e.g., in spring during pine-tree lappet outbreak). Hence, the observed increase in microbial abundance as well as the shift in the fungal community structure is a direct and rapid response towards the highest input fluxes of feces, needle litter and throughfall N

that were measured during main defoliation. The lack of detectable microbial changes in autumn can be associated with a reduction of the feces supply from maximal fluxes of $11.3 \text{ kg ha}^{-1} \text{ month}^{-1}$ to $5.7 \text{ kg ha}^{-1} \text{ month}^{-1}$ while the other two input compartments remain almost constant (study 1). Therefore, the feces input played an important role within the total organic inputs during the outbreak regarding microbial dynamics. Taken together, our main outcomes on soil fungi and bacteria are that (a) only intensive defoliation (80% defoliation by nun moth) has an effect on fungal and bacterial abundance in forest soils, whereas a change in the fungal community structure is already measurable during lower defoliation intensities (50% defoliation by the pine-tree lappet) (b) even though C/N in the mineral soil decreased during insect outbreaks, the increase of fungi and bacteria and their fungal community shift is limited to the upper organic layers (VL, OI) and occurs only short term and consequently (c) independent of the defoliating insect or defoliation intensity, fungal and bacterial abundance in the lower organic layer (Of and Oh) and the mineral soil (Ah) remains unaffected.

→ **Hence, study 2 and 3 demonstrate the stability of the bacterial community structure in the soil and the phyllosphere, despite a general increased bacterial abundance in both compartments. This indicates that either the bacterial community is already well adapted or has a high resistance towards changes in the quality of the nutrient supply as occurring during the insect outbreaks.**

The relevance of the insect species on microbial dynamics during insect outbreaks is perceivable in the soil. Only the intensive nun-moth defoliation caused a response in the microbial biomass during main defoliation while during the less intensive pine-tree lappet defoliation comparatively small differences to the non-infested site were measurable, and these only in spring. This finding supports the hypothesis of Hillstrom et al. (2010) that only high insect derived input levels cause changes in soil C and N mineralization processes whereas medium and low input rates can be buffered without measurable effects.

→ **Further, the occurrence of alterations in the microbial community which were limited to the main defoliation exhibit that effects of insect caused defoliation on the microorganisms are rather short-term and reinforce the resilience of the soil microorganisms towards biotic disturbances.**

Additionally, it demonstrates that the microbial populations are relatively well adapted to endemic and high numbers of pest insects even though the internal fungal community structure can be altered periodically (study 2). For example, the fungal community shifts during main defoliation towards a reduction of saprophytic and saprotrophic fungi, but it adjusts back to pre-outbreak conditions in autumn. The ectomycorrhizal fungi of the genus *Russula* were distinctly decreased during main defoliation. This temporary

decrease of the ectomycorrhiza can be caused by a C limitation due to unbalanced symbiosis with the infested tree or fine root dieback which is frequently observed during insect outbreaks (Britton 1988; Markkola et al. 2004). Probably, the symbiosis balance between tree and ectomycorrhiza was reestablished with ongoing tree recovery following the main defoliation. The Scots pine is able to regenerate its needle biomass partly after herbivory infestation by the development of secondary needles later in the year from the defoliated recent year needle basis (Bartels 1993). The recovery of the ectomycorrhiza seems to benefit from that regrowth and thereby regaining photosynthesis products in late summer as well.

The observed substantial alterations in the soil microbial community and abundance on the insect infested sites during the main defoliation were likely a synergy between favorable environmental conditions during the summer months (warmer temperatures) and the insect-induced beneficial modifications of the habitat characteristics (high organic input). Seasonal changes in microbial abundance were previously observed but showed contrasting results (Mergel et al. 2001; Prevost-Boure et al. 2011; Grantina et al. 2012). Population peaks in summer and spring were measured for forests under different quantities of leaf litter (Prevost-Boure et al. 2011) while highest population sizes of cultivable bacteria occurred in spring and autumn (Mergel et al. 2001). Grantina et al. (2012) measured increasing fungal DNA contents going along with decreasing temperatures. Seasonal dynamics in microbial abundance are highly site specific and dependent on soil nutrient availability and moisture, organic C content, and pH (Ekelund et al. 2001; Poll et al. 2008; Rousk et al. 2009), which makes comparisons with non-infested reference sites of similar climatic conditions a key prerequisite for any interpretation of infestation effects. Under comparable site conditions, the diverse effects of the insect outbreaks on the soil microbiota during and their development during the year became distinct.

In the phyllosphere the bacterial abundance increase was more pronounced at the pine-tree lappet infested site compared to the nun moth infested site. This is surprising, since the population density of the insect was lower on the pine-tree lappet infested site. Possibly, the greater availability of food resulted in a more wasteful feeding behavior compared to the nun moth feeding, where food supply was already scarce due to progressed intensive defoliation. This wasteful feeding behavior promotes the leaching of energy rich C and N substances through the damaged needle surface (Müller et al. 2003). Leaching products provide a good food resource for bacteria since they are specialized in the turnover of easily decomposable compounds (Wardle et al. 2002; Poll et al. 2008). Thus, needle leaching can be the explanation for the increase in total

bacterial abundance in the phyllosphere. The fungal community changed and potential pathogenic fungal taxa increasingly arised in the phyllosphere. This can be an additional health threat for the weakened and stressed trees. Secondary infestation by pathogens following insect infestations often increases tree mortality significantly following herbivory caused defoliation (Wenk and Apel 2007). The increase of Xylariales (family: Amphispheariaceae), a fungal order that mostly colonizes dead plant parts, in the infested phyllosphere already indicates an increased dieback of needles or needle remains (Gäumann, 1964).

During heavy insect infestation, the canopy can become the largest source of C and N compounds in the throughfall (le Mellec et al. 2011). Our results corroborate the assumption of le Mellec et al. 2011, who found increased DON and NH₄-N concentrations in the throughfall under infested forests and expected an enhanced growth and accelerated turnover of phyllospheric microorganisms as response to these inputs. Thus, the bacterial increase in the phyllosphere may partly have contributed to the observed high N fluxes in throughfall (study 1) by wash-offs of the phyllosphere microorganisms during the outbreak event.

3.5. N₂O fluxes during insect outbreaks and the involvement of denitrifying microorganisms

The transformation of N in soils is mediated by microorganisms through reduction–oxidation reactions between N types of various oxidation states, where N losses can occur in gaseous forms e.g., as N₂O (Hallin et al. 2018). N₂O is a potent greenhouse gas and the proportion of microbial N₂O to N₂ production of soils under denitrifying conditions gains importance in terms of global warming (Ravishankara et al. 2009). The abundance of nitrifying and denitrifying genes in soil can be seen as a proxy for determining a soils N₂O emission potential and thereby valuate N loss pathways (Morales et al. 2010).

The measured N₂O-N soil emissions were rather low with an annual average flux of 0.80 µg m⁻² h⁻¹ for the nun moth infested and 0.42 µg m⁻² h⁻¹ for the non-infested site but showed no clear significant differences between infested and non-infested sites (study 4). As comparison, N₂O-N emissions from different forest sites in Germany vary between 3 – 11 µg m⁻² h⁻¹ (Schmidt et al. 1988). Nevertheless, denitrification seems to be the major source of N₂O in our research areas since the *amoA* gene abundances of ammonia-oxidizing bacteria (*BamoA*) and archaea (*AamoA*) were below the quantification limit on all sampling dates while denitrification genes (*nirS*, *nirK*, *nosZ I*, and *nosZ II*) were always present in varying amounts. The dominance of denitrification is generated by the forest soil properties: First, anaerobic microsites within the podzol in

our research area can form regions with high denitrification activity. Second, patchy distribution of organic C rich material forms hot spots of denitrification because the CO₂ production causes high O₂ consumption which in turn results in anaerobic microsites when a thin film of water prevents the O₂ diffusion (Parkin 1987).

N reduction during denitrification is a complex process and controlling factors of microbial N₂O production are still poorly understood (Šlmeč and Cooper 2002; Hirsch and Mauchline 2015). Therefore, the quantification of denitrifying genes investigated in study 3 contributes to the understanding of the controlling factors for denitrification in terms of insect derived soil inputs. There is growing evidence that organic inputs tend to increase denitrification activity in relation to the input amount (Philipot et al. 2007; Hallin et al. 2018). Hence, denitrification can be a major source for N losses following organic inputs (Hirsch and Mauchline 2015). The only sink for N₂O in the biosphere that is identified so far is the N₂O reductase encoded by the *nosZ* I and II genes (Hallin et al. 2018). About 83% of the genomes which comprise *nosZ* I also comprise *nirS* or *nirK* genes, while 51% of *nosZ* II are non-denitrifying N₂O reducers which means *nosZ* II organisms are distinctly associated with potential of N₂O reductions (Graf et al. 2014; Domeignoz-Horta et al. 2015). Further, *nirS*-type denitrifying organisms which were found in only 14% and 19% of all samples in 2014 and 2015, respectively, in contrast to *nirK* type denitrifying organisms perform complete denitrification which means *nirS* accounts less to N₂O emissions compared to *nirK* (Hallin et al. 2018). In contrast to that, *nirK* abundance often correlates with N₂O emissions and occurred in 59% and 57% (in 2014 and 2015, respectively) of all samples (Clark et al. 2012; Philipot et al. 2011).

→ **For our study site infested with the nun moth this means: increased *nirK* gene abundance in combination with the decreased *nosZ* II and consistent *nosZ* I and *nirS* gene abundance in the organic layers of the study site indicate potential increased N₂O emissions. This observation is of great ecological importance since N₂O emissions during insect outbreaks have not been in the scientific focus so far. We could demonstrate that insect outbreaks can be a trigger for this pathway of ecosystem N losses (study 3 and 4, see also Figure 5).**

However, the reduction of N₂O to N₂ is not only controlled by the existence of enzymes but also by environmental factors (Hallin et al. 2018). The extremely low soil pH in the research area favors N₂O emission since the reduction of N₂O to N₂ by the nitrous oxide reductase is inhibited under acidic conditions (Thomsen et al. 1994; Dannenmann et al. 2008). In addition, enhanced soil respiration, as we could monitor in increased CO₂-C emissions during main defoliation (study 4), can lower the soil oxygen content and thereby trigger denitrification rates (as described above) (Butterbach-Bahl et al. 2013).

Furthermore, a high availability of labile C and NO_3^- as an energy source favors denitrification while decreasing C availability increases the N_2O to N_2 ratio (Betlach and Tiedje 1981; Barnard et al. 2005). Therefore, the increased N input in study 2 may have contributed to the observed increase in the genetic potential of soil N_2O emissions (study 3). These field observations were supplemented by an incubation experiment with mineral soil and different organic treatment additions (study 4). In this experiment insect feces increased N_2O -N emissions from the soil by 6 times compared to needle litter. The increase of N_2O emissions after feces addition measured in the incubation experiment strongly supports the assumption of increased N_2O emissions during insect outbreaks. In the experiment, an increased C/N ratio in the soil of in the feces treatment (in contrast to the decrease recorded in the field study) were found. However, the underlying conditions for mineralization in the experiment were different (moister, warmer, no organic layer or mesofauna, no plant roots) compared to field conditions and the feces input was disproportionately high, which may explain the contrasting results of both datasets. In general, N additions to sandy soils are believed to promote rather NO_3^- leaching than N_2O emission, which could be confirmed with the results from the nun-moth infested study site (Butterbach-Bahl et al. 2013). Nevertheless, the incubation experiment demonstrates that under controlled conditions high N_2O emissions from forest soils can be one consequence of insect feces input. At the same time, the increase in *nirK* gene abundance in the field study underlines that this process can potentially occur under field conditions too but was de facto not measurable with the used method due to the complex interaction of N availability and denitrifying conditions over time. The field observation of the denitrifying gene abundance in combination with the gas measurements from the incubation experiment provide an improved understanding of the mechanisms behind the impacts of insect derived N input on N_2O emissions.

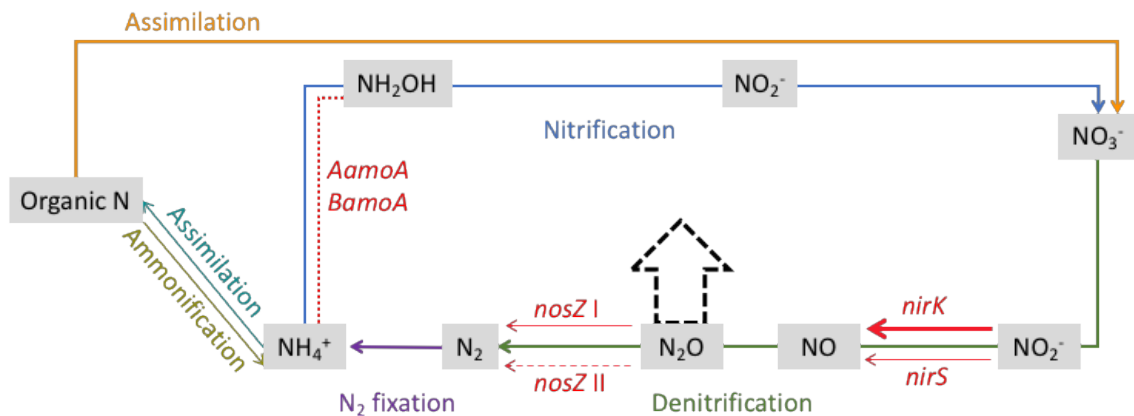


Figure 5: Simplified schematic overview of microbial N turnover pathways in forest soils (without anaerobic ammonium oxidation and dissimilatory nitrate reduction to ammonium). Red text displays functional genes involved in each modification step investigated in this thesis; bold red arrows indicate an increase; dashed red arrows indicate a decrease and ordinary red arrows indicate no change between soil gene abundance of the infested and non-infested sites. Dotted lines indicate functional genes below the quantification limit at both study sites. The black dotted arrow marks genetic potential for losses based on the investigated N cycling gene abundance. Depiction based on the findings from the main defoliation of the nun moth outbreak in 2014.

4. Conclusion and Outlook

Outbreaks of phytophagous insects are predicted to increase in extent, intensity, and distribution as a consequence of direct and indirect global warming effects (Vanhanen et al. 2007; Kurz et al. 2008; Mitton and Ferrenberg 2012; Walter et al. 2018). Altered reproduction and survival of the insects on the one hand and increased forest predisposition to insect attacks caused by climatic plant stressors on the other hand both contribute to this development (Kurz et al. 2008). The reproduction of univoltine insects can shift to a bivoltine reproduction and even polyvoltine insects benefit from warmer temperatures with increased reproduction rates (Netherer and Schopf 2010; Mitton and Ferrenberg 2012). Besides, a poleward shift of pests and pathogens and an increased spread of invasive species is predicted (Bebber et al. 2013). A lack of precipitation and warmer temperatures does not only inhibit the regeneration process of already damaged trees, it also increases their predisposition towards defoliating as well as wood- and bark-boring insects by weakening their defense mechanisms (Wenk and Apel 2007). This development empathizes an increasing endangerment of forests by insect outbreaks and with that a progressive disturbance of ecosystem C and N turnover processes.

With this thesis it was elucidated that ecological interactions within the soil – atmosphere – organism interconnection, which control major C and N turnover pathways in forests, are distinctly affected by insect mass outbreaks. The increased organic input did not only result in enhanced microbial abundance with promoted soil CO₂ emissions but additionally caused timely belated N leaching via soil solution. For the first time it could be demonstrated that insect feces are a potential trigger for forest soil N₂O emissions, however, further long-term studies are needed to reconfirm these findings under outbreak conditions in the field. Tree–microbial interactions change with inhibited ectomycorrhizal symbiosis as well as increased abundance of bacteria and appearances of potential pathogenic fungal taxa in the phyllosphere, which is an additional health threat for the infested tree. Trees themselves react to the infestation with decreased N uptake and a compensatory allocation of N metabolites and structural N in needles and fine roots as a defense and/or survival strategy. Further research on the origin and the function of these N compounds will elucidate trees compensatory metabolic processes towards insect caused defoliation stress.

The results of this thesis illustrate the complexity of the alterations insect infestations provoke in all forest compartments, above and below ground. Furthermore, the relevance of the outbreak intensity and the insects' developmental stage is revealed: almost all detected changes in soil and tree properties vary in intensity, duration, and occurrence which can be directly linked to the insect's life cycle and population density. Altogether, an increase of insect mass gradations driven by climate change will not only negatively affect net ecosystem production but can change forests internal microbial patterns of nutrient turnover and nutrient fluxes, causing progressive N losses. This, in turn, affects other important ecosystem compartments. For example, the NO₃⁻ losses via soil solution of infested forest stands have potential implications for nitrate enrichment in groundwaters. Further, the increased risk of greenhouse gas emissions from the soil (CO₂ and N₂O) and thus the impairment of the C sink potential of the forest reveals potential adverse feedbacks to climate change. Therefore, insect outbreaks with its implications for nutrient cycling, tree nutrition and health, and forests functioning as a C sink are considerable future challenges. Knowledge about ecosystem-specific temporal and spatial alterations of C and N cycling during insect outbreaks is urgently needed to assess feedback links on ecosystem functioning and to provide a basis for C modeling approaches.

With regard to the expected exacerbation of insect caused effects on forest ecosystems, forest protection including monitoring, prediction, management, and mitigation of insect outbreaks gains relevance. Considerate forest management and policies that aim healthy and stable forests are the key strategy to mitigate future biotic disturbances by

insects. In the region of Brandenburg this would include a forest conversion of the predominant non-site-specific Scots pine monoculture stands towards natural and diverse forest structures. Currently, more than 70% of the forest area in Brandenburg is planted with Scots pine while only about 13% of the forests are close to nature in terms of their tree species composition (Ministry of Rural Development, Environment and Agriculture of the Federal State of Brandenburg (MLUL) 2012). The forestries' medium-term aim to reduce pure Scots pine stands by 35% in the next 20 to 30 years is an appropriate and necessary first measure to create stable and less vulnerable forests (MLUL 2012). However, the frequency and intensity of insect outbreaks in Brandenburg is already increasing (Hentschel et al. 2016). Therefore, proactive mitigation strategies like the usage of appropriate silvicultural management plans which comprise the reduction of forest vulnerability and the enhancement of forest recovery are necessary approaches (Dale et al. 2001). This includes to support of the expansion of natural antagonists as well as the development and accreditation of suitable insecticides on the one hand and to cultivate forests that are unattractive for mass progradation of insects on the other hand (Möller et al. 2007). Thus, structural and age class diversity, habitat fragmentation and hence the preservation of the forest stand stability in order to reduce the susceptibility to insect disturbances should become priorities of future forest management and policies. Simultaneously, the use of forecasting methods to identify potential risk areas and predisposed forest structures based on prediction models or GIS-based analyzes is a preventive approach (Möller et al. 2007). These strategies may mitigate or even prevent the possible consequences of insect disturbances to forest ecosystems illustrated in this thesis, thereby helping to obtain several essential forests functions and an unimpeded C and N cycling of the forest ecosystems.

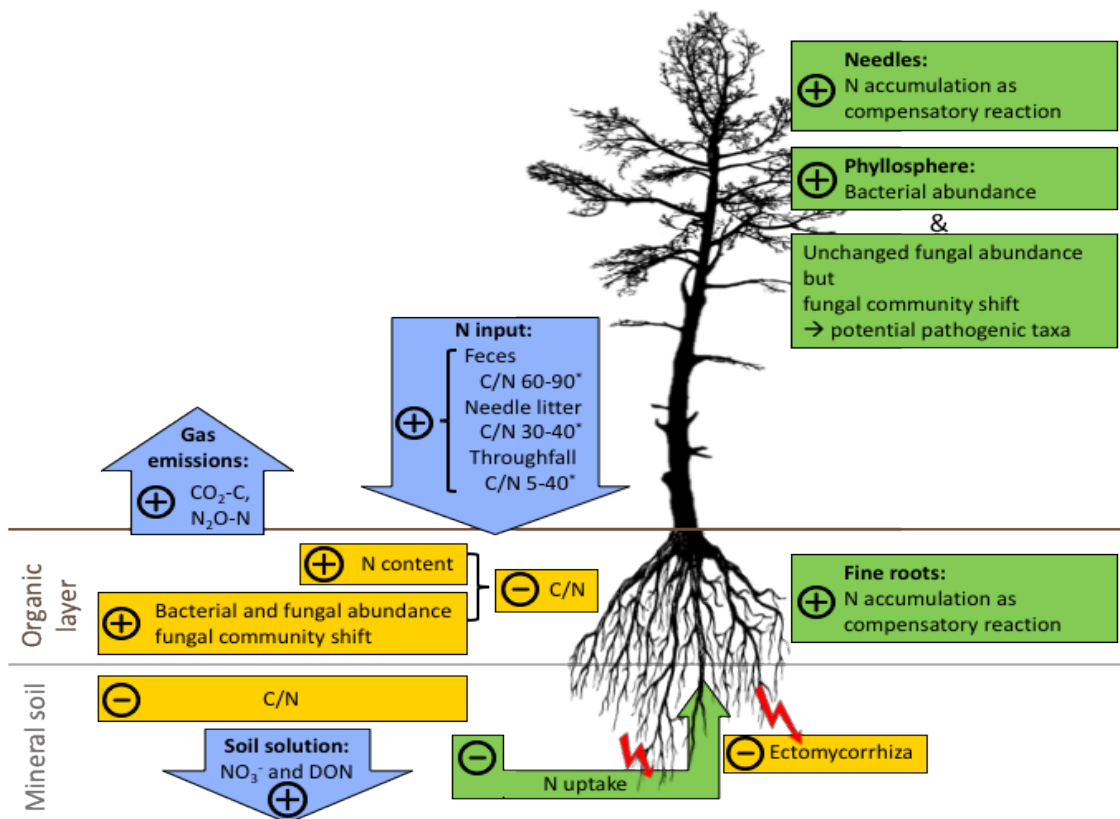


Figure 6: Overview of the main findings on process and flux changes during insect mass outbreaks. Blue: ecosystem fluxes; yellow: soil processes; green: tree processes; plus symbols indicate an increase and minus symbols a decrease of the particular flux or process. *C/N values based on le Mellec and Michalzik (2008).

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6. Manuscripts

6.1. Defoliating Insect Mass Outbreak Affects Soil N Fluxes and Tree N Nutrition in Scots Pine Forests

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Abstract

Biotic stress by mass outbreaks of defoliating pest insects does not only affect tree performance by reducing its photosynthetic capacity, but also changes N cycling in the soil of forest ecosystems. However, how insect induced defoliation affects soil N fluxes and, in turn, tree N nutrition is not well-studied. In the present study, we quantified N input and output fluxes via dry matter input, throughfall, and soil leachates. Furthermore, we investigated the effects of mass insect herbivory on tree N acquisition (i.e., organic and inorganic ¹⁵N net uptake capacity of fine roots) as well as N pools in fine roots and needles in a Scots pine (*Pinus sylvestris* L.) forest over an entire vegetation period. Plots were either infested by the nun moth (*Lymantria monacha* L.) or served as controls. Our results show an increased N input by insect feces, litter, and throughfall at the infested plots compared to controls, as well as increased leaching of nitrate. However, the additional N input into the soil did not increase, but reduce inorganic and organic net N uptake capacity of Scots pine roots. N pools in the fine roots and needles of infested trees showed an accumulation of total N, amino acid-N, protein-N, and structural N in the roots and the remaining needles as a compensatory response triggered by defoliation. Thus, although soil N availability was increased via surplus N input, trees did not respond with an increased N acquisition, but rather invested resources into defense by accumulation of amino acid-N and protein-N as a survival strategy.

Keywords: feces, inorganic N, litter, N fluxes, nitrate, N metabolites, organic N, throughfall

1. Introduction

In forest ecosystems, nitrogen (N) cycling is influenced by insect herbivory (e.g., Kurz et al. 2008; Le Mellec and Michalzik 2008; Morehouse et al. 2008; Cobb et al. 2010) already at low to moderate levels via loss of foliage, tree growth, throughfall leaching, litterfall, and litter decomposition (Schowalter et al. 1991; Chapman et al. 2003; Cunningham et al. 2009). With rapid defoliation by phytophagous insects, large amounts of tree biomass and thus nutrients are turned first into insect biomass and are subsequently released to the soil (Russel et al. 2004). To date, only few studies have analyzed the direct links between insect mass outbreaks and N fluxes from canopy to soil as well as nutrient dynamics and partitioning in the rhizosphere (e.g., Pederson and Bille-Hanssen 1995; Le Mellec and Michalzik 2008; Pitman et al. 2010). Consequently, only little is known about alterations of resource allocation patterns by herbivory (Kaitaniemi et al. 1999; Sampedro et al. 2009).

In forest ecosystems, insect mass outbreaks result in the mobilization of large amounts of organic N previously stored in otherwise long-living needles, which reenter ecosystem N cycling within a relatively short amount of time as a consequence of foliage loss. Thus, N entry to the soil is increased directly and indirectly via decomposition processes (Hunter 2001; Lovett et al. 2002; Keville et al. 2013) influencing microbial community structure, GHG flux, and N turnover (Michalzik and Stadler 2000; Stremińska et al. 2006; Le Mellec et al. 2011). This organic N input originates from both dissolved N from throughfall (Le Mellec and Michalzik 2008) as well as solid deposition of insect feces, dead larvae, and leaf fragments (Christenson et al. 2002; Le Mellec et al. 2009; Kaňa et al. 2013). Focusing on organic N, increasing amounts are based on two processes: (1) Partly eaten needle fragments are dropped by the feeding larvae, adding additional N to the needle litter. (2) Total N concentration in the needles of infested trees is increased compared to non-infested trees as a compensatory response to frass. For example, needles of Masson pine (*Pinus massoniana* Lamb.) infested with Masson pine moth (*Dendrolimus punctatus* Walker) accumulate secondary metabolites as chemical defense measures (Fang et al. 2016). Consequently, total needle N content is increased, in turn changing the composition of future litter (Millard and Grelet 2010). Furthermore, compared to leaf litter, insect feces often have an altered chemical as well as physical quality due to larvae digestion which causes inhomogeneous and porous surfaces and a wider C/N ratio (Le Mellec et al. 2009). In the soil, this surplus of N in combination with

quality changes can accelerate important processes in N cycling, i.e., (1) soil respiration (Reynolds and Hunter 2001; Frost and Hunter 2004), which might lead to increased soil CO₂ emissions (Lovett and Ruesink 1995; Michalzik and Stadler 2000), (2) mineralization, especially in N-limited ecosystems (Belovsky and Slade 2000; Chapman et al. 2003; Le Mellec and Michalzik 2008; Heinzdorf 2013), and (3) N leaching, particularly of nitrate, causing further N losses to the system (Swank et al. 1981; Pitman et al. 2010; Le Mellec et al. 2011). In contrast to these effects accelerating N turnover, other studies suggest a redistribution of N in the soil with no detectable ammonia volatilization, nitrous oxide emission, or nitrate leaching (Russel et al. 2004) and/or soil microbial N immobilization due to a wider C/N ratio. This might be observed especially when organic material, such as feces, are supplied to the soil, indicating slowed decomposition rates due to limited N availability (Le Mellec et al. 2009; Katayama et al. 2014).

The loss of major amounts of biomass as a consequence of insect mass outbreaks not only affects ecosystem N cycling, but also mediates tree internal changes (Dale et al. 2001; Kurz et al. 2008), such as nutrition, especially with regard to N and water relations (Kosola et al. 2001; Morehouse et al. 2008). However, contradictory results on the consequences of herbivory on N nutrition and water relations of forest trees have been reported. For example, external input of N via atmospheric deposition or fertilization over several years, and increased N storage were observed in eastern hemlock (Gómez et al. 2012; Rubino et al. 2015) and red oak (Frost and Hunter 2008). N storage was mostly a result of free amino acid accumulation (especially arginine and glutamine; Vestgarden 2001; Throop and Lerdau 2004; Kos et al. 2015). Increased soil N availability can lead to increased N uptake by tree roots (e.g., Stoelken et al. 2010; Li et al. 2015). However, in response to insect mass outbreaks, inorganic N uptake capacity was reduced in hybrid poplar (*Populus × canadensis* cv. Eugeneii) defoliated by gypsy moth (*Lymantria dispar* L.) (Kosola et al. 2001). In contrast, studies investigating eastern hemlock (*Tsuga canadensis* L.) and red oaks (*Quercus rubra* L.) found no effect of defoliation on inorganic N uptake (Lovett and Tobiessen 1993; Rubino et al. 2015). Loss of leaf biomass also changes tree internal water relations via a reduction in leaf transpiration, thereby negatively affecting tree water balance and nutrient uptake (Aroca et al. 2012). Until today, it is unknown whether organic N uptake capacity is affected by insect mass outbreaks and if infested trees have altered preferences for inorganic or organic N sources. However, organic N sources contribute significantly to tree N nutrition, particularly in N-limited forest ecosystems (Stoelken et al. 2010; Dong et al. 2015, 2016; Li et al. 2015). Tree internal changes in physiology often result in decreasing growth rates and higher mortality (Kosola et al. 2001; Morehouse et al. 2008). However, also

compensatory effects are observed including increased biomass production and higher growth rates (Russel et al. 2004) or N accumulation in different plant parts, mostly as proteins and amino acids (Gómez et al. 2012; Rubino et al. 2015), thereby increasing the nutritional value for herbivores. Particularly with regard to N, external uptake and internal allocation of N, but also a shift to C in the C/N ratio can support defense measures of trees (e.g., via the production of phenolics; Frost and Hunter 2008), and thereby directly affect insect population levels, survivorship, as well as outbreak frequency (Throop and Lerdau 2004).

Here, we study the effects of a mass outbreak of nun moth (*Lymantria monacha* L.) – a member of the Lymantriidae family of Lepidoptera – on N fluxes in the soil and the consequences for N nutrition of Scots pine (*Pinus sylvestris* L.) over the course of a year. In Germany, the nun moth is widely distributed in the pine forest of the north-eastern lowlands (Majunke et al. 2002). In spring 2013, 5,800 ha of forest in Brandenburg were affected by mass outbreaks of the nun moth including 366 ha of total defoliation (Möller and Heydeck 2013). At that time, more than 11,000 ha of Scots pine forest were treated with insecticides (Möller and Heydeck 2013). The study aimed to characterize the effects of insect mass outbreaks (1) on soil N fluxes (i.e., in litter, insect feces, throughfall, leachates), (2) on inorganic and organic N acquisition strategies of Scots pine, (3) on compensatory processes in the N nutritional status of fine roots and needles, (4) In addition, it was assessed how these responses shift over time. For this purpose, we quantified N contents in throughfall, dry matter input, soil leachates, and metabolites in fine roots and needles (i.e., total soluble amino acid, total soluble protein, and structural N levels). In addition, we quantified inorganic and organic ¹⁵N net uptake capacity of fine roots. We hypothesize that (1) N input via solid and wet depositions and N leaching via the soil solution will increase during the insect mass outbreak, especially during the main defoliation period (Le Mellec et al. 2009; Kaňa et al. 2013; Keville et al. 2013). (2) Infested trees are physiologically unable to take advantage of the fertilization effect of additional litter, because the uptake of nutrients and water is restricted by the loss of needle biomass, thereby leading to a decreased N uptake capacity (Kosola et al. 2001). (3) In fine roots and needles, amounts of total soluble proteins and amino acids increase as a compensatory effect to needle biomass loss (Frost and Hunter 2008; Gómez et al. 2012; Rubino et al. 2015).

2. Materials and Methods

2.1 Field Site Description

To study the consequences of insect mass outbreaks on soil N fluxes and tree N nutrition in Scots pine forests, two forest districts were chosen. One district was infested by the nun moth (*L. monacha* L.), the other served as control. Within each district, three plots of 300 – 350 m² were selected. The infested district was located 3 km north of Märkisch Buchholz, Brandenburg, Germany (52°8'38"N, 13°45'14"E, 42m a. s. l.); the control district – a stand with comparable initial site conditions but with an uncritical abundance of insect pests – 10 km west of Teupitz, Brandenburg, Germany (52°9'29"N, 13°36'47"E, 35 m a. s. l.). Following the administrative procedure of forest protection for monitoring of pests (nun moth of 25.05.93) in the study area, nun moth calamity was classified "heavy" (based on 1,185 counted moths on four representative stems) in 2013 with a needle loss of ~80% according to the Eberswalde Forestry State Center of Excellence (Landeskompetenzzentrum Forst Eberswalde, unpublished data). These mass outbreaks of nun moth can be favored by the semi-arid conditions of the study area, because increased frequencies of nun moth mass outbreaks are related with drought stress (Bejer 1988). All six plots were located in 65-year-old white moss pine forest (*Leucobryo-Pinetum*) stands with a total abundance of 96% Scots pine (*P. sylvestris* L.) and 4% beech (*Fagus sylvatica* L.) as well as isolated seedlings of pedunculate oak (*Quercus robur* L.) in the understorey. However, beech seedlings are only found at locations with increased soil water content, whereas small oak seedlings are very rare. Both species did not occur at the study plots. At all plots, the soil type was classified as podzol (FAO classification) on Aeolian sand with mostly fine to medium sand (0.2 – 0.63 mm) of glacial origin as parent rock material with little gravel. The average annual air temperature at the weather station "Lindenberg" [~40 km distance to the field sites is 9.2°C with an average annual total precipitation of 576 mm (1981 – 2010, German Federal Meteorological Service (DWD)]. See also Table 1 for more details on stands and soil (Ah horizon) of the study sites. Sampling and field measurements were conducted in 2014, 1 year after nun moth population culmination, at four time points related to the developmental stages of the nun moth: (1) pre-defoliation stage in early May (spring), (2) main defoliation stage in late May, (3) post-defoliation stage in July (summer), and (4) later post-defoliation stage in early October (autumn). For N in throughfall, dry matter input, and soil leachates, samples were additionally taken at two winter dates: pre-defoliation in February and post-defoliation in November. The chosen sampling times during one vegetation period were based on the nun moth's development cycle: Adult

nun moths fly from mid-July to the beginning of September and lay 70 – 300 eggs in bark alcoves with larvae hatching at the beginning of May and go through 5 – 7 larvae stages before pupation in July (Lipa and Glowacka 1995). Newly hatched nun moth larvae prefer young, soft needles while older larvae also feed on old needles (Lipa and Glowacka 1995). The feeding activity is very destructive because the needles upper half is cut off and then the remaining part is consumed (Lipa and Glowacka 1995). A mass outbreak of the nun moth usually implies the economic end of the stand because often at least 50% of the trees die as a consequence of enormous needle loss (Eberswalde Forestry State Center of Excellence 2013). Even after the insect mass outbreak, an increased vulnerability to storm events and drought stress remains, and under the current climate conditions in the northern German lowland, surviving trees usually require 5 – 6 years to regenerate completely (Eberswalde Forestry State Center of Excellence 2013).

Table 1: Stand and soil details at the field sites. Average tree height (m), tree age (years), stem density per ha, and diameter at breast height (m) in the year of the study (forester, personal communication), C and N content (%), C/N ratio, and pH were determined in mixed soil samples of the Ah horizon in early May (n = 3). C and N were quantified in oven-dried, finely ground soil samples using an Elementar Vario EI analyser (Elementar Analysensysteme GmbH, Langenselbold, Germany). pH was measured on air-dried soil samples.

		Infested site	Control site
Stand	Tree high, average (m)	19	18
	Tree age (years)	65	65
	stem density/ha	480	470
	Diameter breast heigh (DBH, cm)	23	23
Soil-Ah horizon	C content (%)	21.71%	13.81%
	N content (%)	0.74%	0.46%
	C/N ratio	29.42	30.25
	pH (H ₂ O)	3.4	3.3
	pH (KCl)	2.4	2.6

2.2 ¹⁵N Uptake Experiments

¹⁵N-labeling experiments were conducted in the field to quantify net N uptake capacity of infested and uninfested Scots pine trees. Per plots, 12 adult trees were randomly chosen. Intact fine roots (<2 mm diameter) still attached to the tree were carefully dug out, cleaned with water and incubated in 5 ml of an artificial soil solution according to the method described by Simon et al. (2010). The artificial soil solution contained 100 μM KNO₃, 90 μM CaCl₂* 2 H₂O, 70 μM MgCl₂* 6 H₂O, 50 μM KCl, 24 μM MnCl₂* 4 H₂O, 20 μM NaCl, 10 μM AlCl₃, 7 μM FeSO₄* 7 H₂O, 6 μM K₂HPO₄, 1 μM NH₄Cl, 25 μM glutamine,

and 25 μM arginine (pH 6.5), mimicking the conditions at a low N availability field site (Simon et al. 2013). Arginine and glutamine were chosen, because they represent the most abundant free amino acids in most plant parts (Griffin et al. 1991; Gessler et al. 1998). To quantify inorganic and organic net N uptake capacity, four different solutions were used for each individual tree, each containing all four N sources: NH_4^+ , NO_3^- , as well as the amino acids glutamine and arginine. However, only one N form was labeled with ^{15}N . Furthermore, a control solution without ^{15}N -labeled compounds was used to determine the natural abundance of ^{15}N in the roots. To avoid diurnal variation in N uptake (Gessler et al. 2002), incubation experiments were conducted for 2 h between 10 a.m. and 2 p.m. Following incubation, the submersed root parts plus an additional 10 – 15 mm were cut off, washed twice with 0.5 μM CaCl_2 , dried with cellulose paper, and the fresh weight was determined. After drying at 60°C for at least 48 h, the dry weight was determined.

2.3 Sampling of Fine Root and Needles

For the quantification of root and needle N metabolites, samples were taken from the same individuals used for ^{15}N uptake experiments. Approximately 2 – 3 g of fine roots were sampled from three roots. In addition, 50 – 60 fresh previous-year needles were taken from different tree branches from the outer middle crown. All samples were immediately shock-frozen in liquid nitrogen and stored at -80°C on return from the field until further analyses.

2.4 Sampling of Insect Feces, Needle Litter, Throughfall, and Soil Solution

N input into the soil was measured as dry matter input (i.e., total N in insect feces and needle fragments), and in throughfall as well as soil solution [i.e., total N, nitrate-N, and dissolved organic N (DON), respectively] according to Le Mellec et al. (2011). At all plots, 10 randomly distributed throughfall samplers (diameter 20 cm) were set up. At each sampling date, samplers from each plot were pooled to five mixed samples. For collection of soil percolates, zero tension humus lysimeters were established underneath the humus layer at each plot according to Le Mellec et al. (2011). Sampling of throughfall and soil solution was conducted at biweekly intervals, during main defoliation at weekly intervals. Insect derived fragments (i.e., feces, leaf debris) and (green) litter fall were collected using nylon tree nets (mesh size 300 \times 300 μm) with a net size between 15 and 17 m^2 according to the canopy diameter (Le Mellec and Michalzik 2008). Net sampling was conducted weekly.

2.5 Quantification of ^{15}N , ^{13}C , and Total N and C

To quantify ^{15}N , ^{13}C , as well as total N and C contents in the fine roots and total N content in needles, plant tissues were finely ground using a ball mill (Retsch TissueLyser, Haan, Germany). Aliquots of 0.8 – 2.5 mg were weighed into 4 × 6 mm tin capsules (IVA Analysentechnik, Meerbusch, Germany). Samples were analyzed with an elemental analyzer (NA2500, CE Instruments, Milan, Italy) coupled to an isotope ratio mass spectrometer (Delta Plus, Thermo Finnigan MAT GmbH, Bremen, Germany). As a working standard, glutamic acid was used, for $\delta^{13}\text{C}$ calibrated against the primary standards USGS 40 (glutamic acid, $\delta^{13}\text{C}_{\text{PDB}} = -26.39$) and USGS 41 (glutamic acid, $\delta^{13}\text{C}_{\text{PDB}} = 37.63$) and for $\delta^{15}\text{N}$ against USGS 25 (ammonium sulfate, $\delta^{15}\text{N}_{\text{Air}} = -30.4$) and USGS 41 ($\delta^{15}\text{N}_{\text{Air}} = 47.600$). To detect a potential instrument drift over time standards were included after every 12th sample. Net N uptake capacity was calculated according to Gessler et al. (1998): net N uptake capacity = $((^{15}\text{N}_l - ^{15}\text{N}_c) * N_{\text{tot}}^* \text{dw} * 10^5) / (\text{MW} * \text{fw} * t)$, where $^{15}\text{N}_l$ and $^{15}\text{N}_c$ represent the atom% of ^{15}N in labeled (N_l) and n control roots (i.e., natural abundance), respectively. N_{tot} is total N%, dw the dry weight, and fw the fresh weight of the root. MW is the molecular weight (^{15}N g mol $^{-1}$) and t stands for the incubation time. For each amino acid, the $^{15}\text{N}/^{13}\text{C}$ ratio of root fresh weight was compared to the total C/N ratio to determine whether amino acids were taken up as intact molecules (data not shown). Because net C uptake capacity based on ^{13}C incorporation in root fresh weight differed from the ^{15}N incorporation, it can be assumed that amino acids were either partially degraded in the artificial soil solution or on the root surface, or that amino acid-derived carbon was respired inside the roots (Simon et al. 2011).

2.6 Quantification of Total Soluble Protein-N, Total Amino Acid-N, Ammonium-N, Nitrate-N, and Structural N Content in Fine Roots and Needles

Before analyses of N metabolites, fine root and needle samples were ground to a homogeneous powder in liquid N_2 . Total soluble protein, amino acids, ammonium and nitrate concentrations were quantified according to Simon et al. (2010). For total soluble proteins content, ~0.05 g ground frozen plant material were extracted in 1.5 ml buffer containing 50 mM Tris-HCl (pH 8.0), 1 mM EDTA, 15% glycerol (v/v), 5 mM dithiothreitol, 0.1% Triton X-100 (v/v), 2 tablets of protease inhibitor cocktail (EDTA-free, Complete, Roche Diagnostics, Mannheim, Germany) and quantified using Bradford Reagent (Amresco Inc., Solon, Ohio, USA). The absorption was determined at 595 nm with a spectrophotometer (Ultrospec 3100 pro, Amersham Biosciences, Piscataway, USA). Bovine serum albumin (BSA) was used as standard. For total amino acids content,

~0.05g finely ground plant tissue was extracted as previously described in 1 ml methanol-chloroform (3.5:1.5, v:v) and 0.2 ml Hepes buffer (20 mM Hepes, 10 mM NaF, 5 mM EGTA, pH 7.0) (Winter et al. 1992). The concentration of total amino acids was quantified using the method of Liu et al. (2005). Absorption was measured at 570 nm in a spectrophotometer (Ultrospec 3100 pro, Amersham Biosciences, Piscataway, USA). L-glutamine was used as standard. Ammonium and nitrate contents were quantified with the method reported by Simon et al. (2010): Approximately 0.04 g of plant tissue were soaked in 0.1 g washed polyvinylpyrrolidone (PVP, Sigma-Aldrich Inc., Steinheim, Germany) prepared in 1 ml distilled water to bind phenols. Ammonium and nitrate concentrations were determined using an ion chromatograph (DX 120, Dionex, Idstein, Germany) coupled to an autosampler (AS 3500, Thermo Separation Products, Piscataway, NJ, USA) equipped with the PeakNet software (version 4.3, Dionex). Anion mixtures of NO^- , PO_3^- , SO_2^- , and SO_2^- or cation mixtures of NH^+ , K^+ , Mg^{2+} , and Ca^{2+} , both in distilled water, were used as standards. Ammonium and nitrate contents of fine roots and needles were negligible (data not shown). Thus, structural N was calculated by subtracting total soluble protein-N, and amino acid-N from total N.

2.7 Quantification of N in Insect Feces, Needle Litter, As Well As Throughfall, and Soil Solution

N input into the soil was measured in dry matter (i.e., total N in insect feces and needle litter), throughfall as well as soil solution (i.e., total N, nitrate-N, and dissolved organic N each). For determination of total N in dry matter, insect feces were separated from needle litter and both dried at 45°C. Fresh and dry weight was determined. Total N content was quantified in aliquots of the dry material (3 technical replicates per net) by thermal oxidation using a Leco CHN 1000 analyser (LECO Enterprise, Mönchengladbach, Germany). Aliquots of throughfall and soil solutions were filtered with 0.45 µm cellulose-acetate membrane filters (Sartorius, Göttingen, Germany) and analyzed for dissolved N (DN) via thermal oxidation (Dimatoc 100, Dimatex, Essen Germany) and for nitrate by ion chromatography (761 Compact IC, Methrom, Filderstadt, Germany) according to Le Mellec et al. (2011). DON was calculated as difference between DN and nitrate-N. Total N was quantified by thermal oxidation (Dimatoc 100, Dimatec, Essen, Germany) in solutions of 0.45 and 500 µm particle size.

2.8 Statistical Analyses

Prior to comparisons between treatments, all data were tested for normal distribution and homogeneity of variances. Statistical analyses for inorganic and organic net N uptake capacity as well as N metabolites in roots and needles were performed using R package version 1.3.1 (R Development Core Team 2008). Differences between infested and control sites were detected using Kruskal-Wallis test on ranks. For multiple pairwise comparison within one treatment at different sampling dates between the different N sources as well as N metabolites and infested vs. control plots at each sampling date, post-hoc Dunn's test was performed using the Bonferroni correction for p-value adjustment (Dinno 2015). For statistical analyses of throughfall, dry matter, and soil leachates, SPSS (SPSS Statistics for Windows, version 22.0., IBM, Armonk, USA) was used. Comparisons between infested and control sites, as well as sampling dates were tested using ANOVA.

3. Results

3.2 Insect Mass Outbreak Affects N Fluxes in Dry Matter Input, Throughfall, and Soil Leachates

In general, mean and accumulated N input into the soil (i.e., total N in insect feces, litter, and throughfall, as well as DON in throughfall) was significantly higher at the infested compared to the control plots at all sampling times (

Table 2). Similarly, N output (i.e., total N, nitrate-N, and DON in soil leachates) was significantly higher with insect infestation regardless of sampling time. Only DON in soil solution (winter I and II) and nitrate- N fluxes in throughfall (entire year) did not differ significantly in response to insect infestation. With regard to N allocation in throughfall, DON fluxes were generally higher than nitrate-N ($p \leq 0.050$), whereas soil leachates showed a reversed pattern with higher nitrate-N compared to DON ($p \leq 0.050$). Comparison with N fluxes across the sampling times showed no significant differences for the control plots. In contrast, for the infested plots, total N, nitrate-N, and DON in throughfall as well as DON in soil leachates increased significantly from the previous winter sampling to the main defoliation event ($p \leq 0.050$). For total N and nitrate-N in soil leachates, a significant increase was delayed (i.e., from previous winter to autumn sampling; $p \leq 0.050$).

Table 2: Mean N fluxes (kg/ha) per month and cumulative N fluxes (kg/ha) per 6 months. N_{tot} N=total; DON=dissolved organic N; Mean average N fluxes over a 6 months period. Asterisks indicate level of significance between control and infested plots (* $p < 0.050$, ** $p < 0.010$). Different capital letters indicate significant differences between sampling times within one treatment ($p \leq 0.050$). n.a. = not available.

Treatment	Mean fluxes of N (kg ha ⁻¹)							
	N _{tot} feces	N _{tot} litter	N _{tot} throughfall	NO ₃ -N throughfall	DON throughfall	N _{tot} soil solution	NO ₃ -N soil solution	DON soil solution
Infested								
Winter I	n.a.	n.a.	14.3±6.5* A	3.1±0.8 A	6.5±2.9* A	7.5±4.3* A	6.3±2.1* A	0.7±0.1 A
Spring	n.a.	n.a.	16.8±7.9* AB	3.7±1.3 AB	6.8±3.2* AB	9.7±4.9* AB	8.1±3.1* AB	0.8±0.1* AB
Main defoliation	11.3±2.9** A	5.9±1.9** A	17.9±8.1* B	4.7±1.6 B	11.3±2.9* B	10.7±5.1* AB	9.5±3.4* AB	1.1±0.2* B
Summer	8.9±3.6* A	6.7±2.4** A	15.4±8.3* AB	3.9±1.3 AB	8.7±3.6* AB	10.8±4.8* AB	9.6±3.4* AB	1.0±0.1* AB
Autumn	5.7±3.4** A	6.3±2.1** A	14.9±7.5* AB	4.4±1.7 AB	7.8±3.4* AB	10.9±4.9* B	10.0±3.6** B	0.93±0.3* AB
Winter II	n.a.	6.5±1.9** A	12.3±7.1* AB	3.3±0.8 AB	6.9±2.3* AB	9.7±3.9* AB	8.3±2.9** AB	0.7±0.2 AB
Mean	8.6±3.3**	6.4 ±2.1**	15.3±7.5*	3.9±1.3	8.0±3.1*	9.9±4.7*	8.6±3.1*	0.9±0.2*
Sum	25.9±2.8**	25.4±0.3**	91.6±2.0**	23.1±0.6	48.0±1.8**	59.3±1.3*	51.8±1.4*	5.2±0.2*
Control (non-infested)								
Winter I	n.a.	n.a.	9.9±3.9* A	4.3±1.8 A	5.3±1.9* A	6.7±3.4* A	5.9±1.9* A	0.6±0.1 A
Spring	n.a.	n.a.	11.1±3.3* A	4.6±1.9 A	5.4±1.3* A	6.9±3.2* A	5.6±1.6* A	0.5±0.1* A
Main defoliation	3.3±0.5** A	1.4±0.6** A	11.6±3.6* A	4.5±2.0 A	5.6±1.2* A	7.1±3.2* A	5.7±1.8* A	0.4±0.0* A
Summer	3.6±0.7** A	1.4±0.4** A	10.6±3.4* A	4.6±1.3 A	5.3±1.3* A	6.3±3.5* A	5.6±1.7* A	0.1±0.1* A
Autumn	3.7±0.7** A	1.5±0.5** A	9.8±3.4* A	4.1±2.3 A	5.1±0.9* A	6.9±3.0* A	4.9±1.9** A	0.1±0.1* A
Winter II	n.a.	1.4±0.2** A	10.1±3.9* A	4.7±2.3 A	4.9±2.0* A	6.9±3.3* A	4.9±1.2** A	0.1±0.1 A
Mean	3.5±0.6**	1.4±0.4**	10.5±3.6*	4.5±1.9	5.3±1.4*	6.8±3.3*	5.4±1.6*	0.5±0.1*
Sum	10.6±0.2**	5.7±0.1**	73.6±0.7**	26.8±0.2	31.6±0.2**	40.8±0.3*	32.6±0.4*	3.0±0.3*

3.2 Insect Mass Outbreak Reduced Organic and Inorganic Net N Uptake Capacity of Fine Roots in Scots Pine

Mass outbreaks of defoliating nun moth affected net N uptake capacity of Scots pine differently depending on N source (Figure 7). Net uptake capacity of ammonium-N, nitrate-N, and glutamine-N (i.e., only for main defoliation and summer) was significantly reduced in trees of the infested stands compared to the control stands ($p < 0.001$), whereas arginine-N net uptake capacity was not influenced at all. Mean reduction of net ammonium-N, nitrate-N, and glutamine-N uptake capacity was 29.6, 65.3, and 65.4%, respectively. Furthermore, net N uptake capacity changed over the vegetation period depending on N source. Net ammonium-N uptake capacity regardless of insect infestation and net glutamine-N uptake capacity at the infested plots were highest in spring ($p \leq 0.007$), however, decreased during main defoliation and summer with another increase in autumn ($p < 0.020$). Net arginine-N uptake capacity decreased regardless of insect infestation from summer to autumn ($p \leq 0.042$). For net nitrate-N uptake capacity no differences over the vegetation period were found. With regard to preference for certain N sources, we found differences between treatments: At the control plots, arginine-N was preferred over nitrate-N, glutamine-N, and ammonium-N ($p \leq 0.010$); at the infested plots arginine-N was preferred over glutamine-N and nitrate-N over ammonium-N ($p \leq 0.001$).

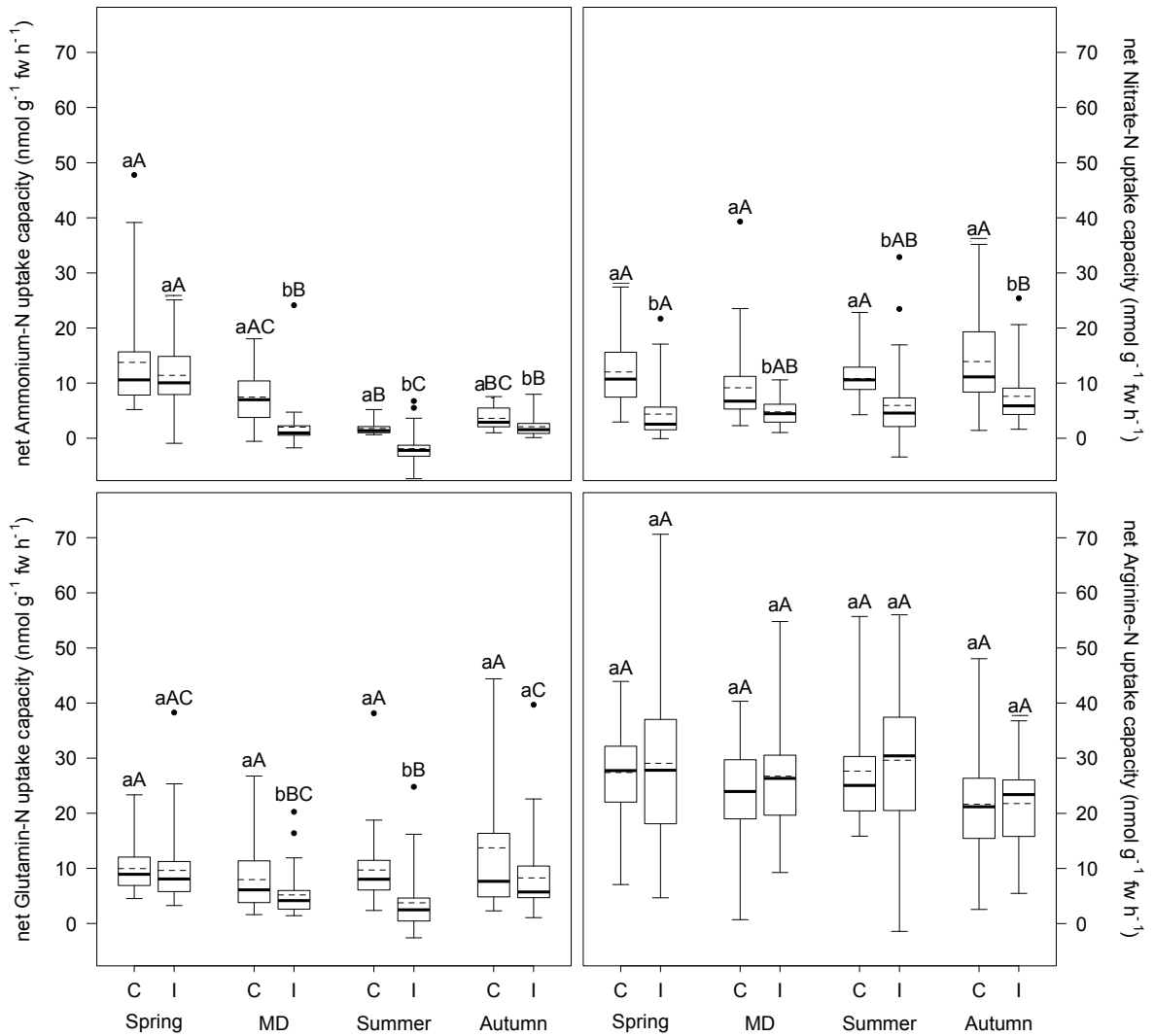


Figure 7: Inorganic and organic net N uptake capacity (nmol g⁻¹ fw h⁻¹) at infested and non-infested pine forests over the vegetation period. I, infested; C, control; MD, main defoliation. Box plots show means (dotted lines) and medians (straight lines) ($n = 12$ for each plot). Whisker extension equals 3x interquartile range distance. Different small letters indicate significant differences between infested and control plots within one sampling time ($p \leq 0.050$). Different capital letters indicate significant differences between the sampling times within one treatment ($p \leq 0.050$).

3.3 Insect Mass Outbreak Enhanced Total N, Soluble Protein-N, Amino Acid-N, and Structural N in Fine Roots and Needles of Scots Pine

In the fine roots (Figure 8), N metabolite concentrations were significantly higher at infested compared to control plots for total N (regardless of sampling time; $p \leq 0.009$), structural N (only in autumn; $p < 0.001$), total soluble protein-N (TSP-N, only at the main defoliation event; $p < 0.001$), and total soluble amino acid-N (TAA-N, at all sampling times

except spring; $p \leq 0.001$). Needles showed higher concentrations at infested compared to control plots for total N and structural N (both regardless of sampling time; ($p \leq 0.001$ and $p \leq 0.031$, respectively), TSP-N (only in spring and at the main defoliation event ($p \leq 0.003$), and TAA-N (only in spring and autumn; ($p \leq 0.016$).

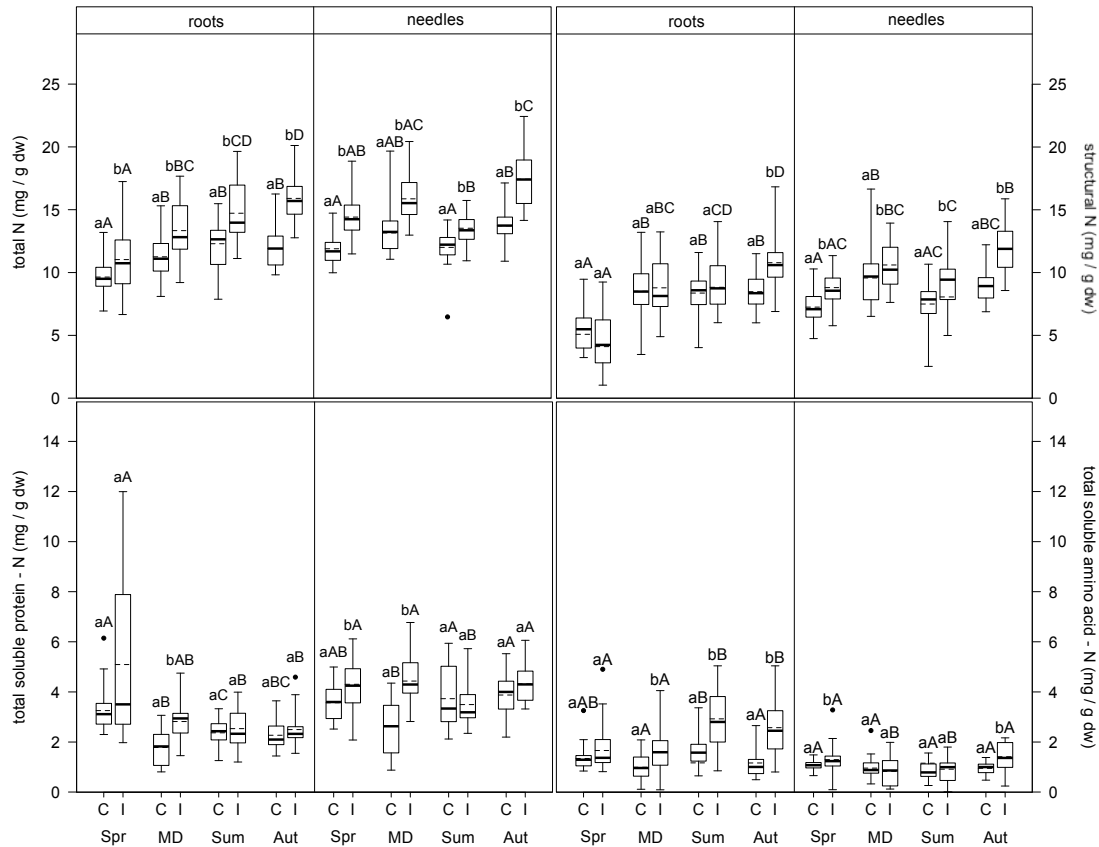


Figure 8: Total N, structural N, soluble protein-N, and soluble amino acid-N content in fine roots and needles (mg/g dw) at infested and non-infested pine forests over the vegetation period. I=infested; C=control; MD=main defoliation. Box plots show means (dotted lines) and medians (straight lines) ($n = 12$ for roots, $n = 8$ for needles for each plot). Whisker extension equals 3x interquartile range distance. Different small letters indicate significant differences between infested and control plots within one sampling time ($p \leq 0.050$). Different capital letters indicate significant differences between the sampling times within one treatment ($p \leq 0.050$).

Comparing sampling times within each parameter, levels of N metabolites differed between sampling dates (Figure 8). In fine roots of control plots, both total N and structural N concentrations did not differ significantly between the main defoliation event, summer, and autumn, but were significantly higher at these time points compared to spring ($p \leq 0.046$ and $p \leq 0.001$, respectively). TSP-N concentration was highest in spring > summer/autumn > main defoliation ($p \leq 0.001$). Similarly, TAA- N concentrations were

highest in summer compared to the main defoliation event and autumn ($p \leq 0.008$) with no differences between spring and the other sampling times. For the infested plots, fine roots showed different patterns: Total N concentration was higher in autumn > main defoliation event > spring ($p \leq 0.001$), with concentrations in summer only higher compared to spring ($p \leq 0.001$) and no differences between the other sampling dates. Structural N levels were highest in spring > frass > autumn ($p \leq 0.001$) and lower in summer compared to spring ($p \leq 0.001$). TSP-N concentrations were highest in spring > summer/autumn ($p \leq 0.001$), but no significant differences between any of the other time points. TAA-N concentrations were significantly higher in summer/autumn compared to the main defoliation event and spring ($p \leq 0.007$). Levels of N metabolites showed different patterns over the vegetation period in the leaves. At the control plots, total N concentrations were higher in autumn compared to summer and spring ($p \leq 0.016$), with no differences at the main defoliation event compared to the other sampling times. Structural N concentration was highest, whereas TSP-N concentration was lowest at the main defoliation event ($p \leq 0.016$). TAA-N concentration did not change at all between sampling times. At the infested plots, needles showed different patterns with regard to their N metabolites: Total N content in the needles was lower in summer compared to the main defoliation event and autumn and lower in spring compared to autumn ($p < 0.008$), but no other significant differences were found. Structural N concentration was higher with frass and in autumn compared to spring ($p \leq 0.019$), and lower in summer compared to autumn ($p = 0.005$). TSP-N was lowest in summer ($p \leq 0.040$) with no differences between the other sampling times. TAA-N levels were higher in spring and autumn compared to the main defoliation event and summer ($p \leq 0.043$).

Comparing composition of N pools in fine roots and needles, total N mainly consisted of structural N, with $59 \pm 10.8\%$ in roots of infested and $65 \pm 8.4\%$ control trees, and $64 \pm 4.2\%$ in needles of infested and $61 \pm 10.4\%$ of control trees. The second largest contribution was TSP-N: $24 \pm 12.4\%$ in roots of infested and $21 \pm 7.9\%$ in control trees, and $26 \pm 2.9\%$ in needles of infested and $30 \pm 9.1\%$ of control trees. The lowest contribution was from TAA-N with $15 \pm 3.1\%$ in roots of infested and $11 \pm 2.4\%$ of control trees, and $9 \pm 4.3\%$ in needles of infested and $8 \pm 1.5\%$ of control trees. Comparing N metabolites in fine roots and needles over the vegetation period, total N and structural N contents in needles were higher in summer and autumn compared to spring and main defoliation in both, infested and control trees ($p \leq 0.001$ and $p \leq 0.004$). TSP-N in the needles was higher compared to the roots at the main defoliation event, summer and autumn sampling times ($p \leq 0.001$). In contrast, TAA-N levels in the roots of infested

trees were higher than in needles ($p \leq 0.001$), except for spring. However, in spring and summer root TAA-N levels of the control trees were higher than in needles ($p \leq 0.004$).

4. Discussion

4.1 Consequences of Insect Mass Outbreaks on Soil N Fluxes

4.1.1 Insect Mass Outbreaks in Scots Pine Stands Lead to Enhanced N Input and Nitrate Leaching

Insect mass outbreaks can affect N cycling directly via changes in quantity and quality – specifically that of organic input of feces and dead insect biomass as well as changes in throughfall composition (Stadler et al. 2005; Müller et al. 2006; Le Mellec and Michalzik 2008) – and indirectly via changes in rhizodeposition, modified nutrient uptake rates by trees and altered root-soil microbe-interactions (Zimmer and Topp 2002; Throop and Lerdau 2004). In the studied Scots pine forests, the insect mass outbreak altered soil N cycling resulting in enhanced N input (i.e., total N, nitrate-N, DON) via feces and litter as well as N output (i.e., total N, nitrate-N, DON) compared to the control plots. In other studies, higher total N fluxes in throughfall were mainly due to increased fluxes of DON originating from leaching of damaged leaves and washouts of branches and leaves with phyllosphere microorganisms (Hunter 2001; Le Mellec and Michalzik 2008). For example, growth of epiphytic heterotrophic microorganisms was increased on infested needles and leaves of spruce and oak trees which was triggered by sugars and carbon rich excretions from insects. In turn, microbial growth in the phyllosphere changed the N composition of the throughfall (Guggenberger and Zech 1994; Stadler and Michalzik 2000). The increase in total N of litter and feces at infested plots compared to the controls was due to quantitative higher organic entries. The additional N input via feces into the system might have further implications. Feces consist mainly of labile C as well as extractable N in form of proteins which can stimulate soil microbial activity resulting in increased CO₂ emissions from the soil (Lovett and Ruesink 1995; Zimmer and Topp 2002; Frost and Hunter 2004). Because the physical and chemical structure of feces is easily soluble compared to needle litter (Jung and Lunderstädt 2000), feces are likely to mediate faster N turnover in the soil. The increased N input with insect mass herbivory in the present study also explains the increased N output as total N, nitrate-N, and DON at the infested forest site, because additional organic input leads to an enhanced release of nutrients via the soil solution (Stadler et al. 2001; Chapman et al. 2003; Le Mellec et al. 2009, 2011). Enhanced N output during insect mass outbreaks was previously observed for other forests and pest insects (e.g., Swank et al. 1981; Näsholm 1994;

Houle et al. 2009; Pitman et al. 2010). For example, in a boreal forest, the inorganic N output via soil the solution was 30 times higher during a mass outbreak of the spruce budworm compared to an undisturbed forest site (Houle et al. 2009).

4.1.2 Consequences of an Insect Mass Outbreak on Soil N Fluxes are Linked to the Life Cycle of the Feeding Insects

Variation in N fluxes over the vegetation period were observed only at the infested, but not the control plots suggesting that changes at the infested plots were caused by the nun moth and the related biotic stress. The observed N input fluxes increased from winter to spring and peaked at the main defoliation event, during which nun moth larvae are most active, and declined again until autumn, while the nun moth pupated, metamorphosed, and mated. The higher total N and DON fluxes in throughfall at the beginning of the previous winter compared to the winter following the main frass activity at the end of the measuring period are likely a response to the previous year's infestation. Similar patterns of increased N input via litter and throughfall over the vegetation period as a response to insect mass outbreaks have been reported in other studies (Stadler et al. 2001; Le Mellec et al. 2009; Pitman et al. 2010), suggesting that this response to massive herbivory is strongly linked to the variation in the life cycle of the feeding insects over the vegetation period.

N fluxes in the soil solution showed a delayed response to insect mass outbreaks with fluxes of total N, nitrogen-N, and DON peaking in autumn. This can be explained by several processes: (1) The reaction-time of a soil system is mainly regulated by rainfall events and soil buffer capacity which depends on the soil N status prior to the outbreak event (Pitman et al. 2010; Griffin et al. 2011). Soils with low N availability, such as the present study site, often show a lower or delayed reaction with regard to N input in the soil solution compared to N-saturated soils due to higher microbial immobilization that incorporates N into stable organic matter (Frost and Hunter 2004, 2007). (2) Furthermore, the export of N via nitrate and DON depends on soil water availability. At our research site, water supply is limited during late spring and summer due to low precipitation and high temperatures (Grüning, personal observation; Gerstengarbe et al. 2003). Increasing precipitation and decreasing transpiration in autumn and winter contribute to the peak of N fluxes in the soil solution in autumn. These results show the significance of sampling time for soil N cycling. Overall, additional N input in response to insect mass herbivory exceeded N losses via leaching. Thus, insect mass outbreaks have the potential to cause long-term effects on soil N cycling by significantly increasing the total N load in soils (Vestgarden 2001), and thus providing an additional N source for

understorey vegetation and/or tree regeneration in the years following the insect mass outbreak (Griffin et al. 2011; Kaňa et al. 2013).

4.2 Consequences of Insect Mass Outbreaks on Tree N Nutrition

4.2.1 Reduction of N Acquisition of Inorganic N and Glutamine-N by the Roots

Tree N uptake strongly depends on soil N availability (Stoelken et al. 2010; Simon et al. 2013; Dong et al. 2016). N supply in soils of infested forests is often increased (Belovsky and Slade 2000; Chapman et al. 2003; Le Mellec and Michalzik 2008) as also found in the present study (see above). Still, inorganic N and glutamine-N net uptake capacity of Scots pine trees were strongly reduced (30 – 65% reduction depending on N source) under massive herbivory by the nun moth, especially at the main defoliation event. A reduced inorganic N uptake capacity was found also for hybrid poplar (*Populus x canadensis* cv. Eugeneii) defoliated by gypsy moths (Kosola et al. 2001). In contrast, for defoliated oak seedlings (Lovett and Tobiessen 1993) and hemlock saplings (Rubino et al. 2015) a difference in inorganic net N uptake was not detected. Three aspects differed in the latter two studies compared to the present study: (1) Rubino et al. (2015) applied ¹⁵N directly to the soil, (2) both studies used only inorganic N sources, and (3) both studies investigated seedlings instead of adult trees. However, N acquisition strongly varies with tree age (Simon et al. 2011). For example, woody seedlings take up inorganic and organic N preferably in spring, whereas adult beech trees show highest N uptake in autumn (Simon et al. 2011). Furthermore, loss of needle biomass, and thus photosynthetic tissues, due to massive insect herbivory raises the relative costs of root N uptake (Jacquet et al. 2014; Fang et al. 2016). For the utilization of amino acid-N, less energy is on average required compared to inorganic N sources such as nitrate and ammonium (Zerihun et al. 1998; Gruffman et al. 2013). The preference for arginine-N in the present study supports the view that organic N sources might be preferred over inorganic N sources at limited energy generation by photosynthesis. However, under these conditions internal reallocation of N might even be a better strategy for survival than organic N uptake. Therefore, it is not surprising that infested Scots pine trees did not use the additional N available in the soil upon infestation in the present study.

Previous assumptions that Scots pine has a reduced affinity toward glutamine and prefers ammonium as N source (Persson and Näsholm 2003; Simon et al. 2013) were not confirmed by the present results. The shift of N uptake toward glutamine-N by infested trees was mainly caused by a reduction in nitrate rather than an increase in glutamine-N uptake capacity with infestation. The observed preference of Scots pine for organic N sources has been described previously by Persson et al. (2006) and Simon et

al. (2013). It is also relevant for other species, such as European beech (Dannenmann et al. 2009; Stoelken et al. 2010; Simon et al. 2011; Li et al., 2015), oak, hemlock (Gallet-Budynek et al. 2009), willow, and black spruce (Kielland et al. 2006).

4.2.2 Consequences of an Insect Mass Outbreak on Organic and Inorganic N Acquisition Depend on Sampling Time over the Vegetation Period

The uptake of ammonium-N and glutamine-N by infested Scots pine is influenced by sampling time during the vegetation period and thereby strongly linked to the life cycle of the nun moth. N uptake capacity was reduced during the insect mass outbreak. Changes of inorganic and organic N uptake at different times during the vegetation period have been investigated in previous studies (Simon et al. 2011; Dong et al. 2016). However, in the present study the observed changes were only found for infested trees, but not for control trees, suggesting a link to the increasing population size of the nun moth with a population peak at the main defoliation event. Rising spring and summer temperatures and increased evapotranspiration might exacerbate the decline of external N acquisition, thereby adding stress to the already physiologically impaired trees (Heinzdorf 2013). N uptake is strongly related to water as well as N availability (Gessler et al. 1998; Stoelken et al. 2010; Li et al. 2015; Dong et al. 2016). Furthermore, N acquisition is species-specific and depends on current tree N nutrition (Näsholm et al. 2009; Gruffman et al. 2014). The high ammonium net uptake capacity found in the present study in spring – when nun moth activity is still relatively low – suggests that insect mass herbivory already interacts with tree internal regulation of N acquisition at early insect infestation. The present data also suggest that infested trees might face difficulties replenishing their N reserves from external sources after winter by N uptake in case of an insect mass outbreak.

4.2.3 Enrichment of Total N in Roots and Remaining Needles of Infested Trees as a Compensatory Response to Insect Defoliation

In response to insect herbivory and defoliation, Scots pine trees accumulated N in fine roots and remaining needles. This increase in total N concentration is not a result of enhanced N acquisition from the soil (see above), but rather due to an increase in levels of total soluble protein-N, amino acid-N, and structural N that is mediated by tree internal sources. Second-year needles are the major storage pool for N in Scots pine trees (Millard et al. 2001). N stored in these needles might serve as buffer against short-term fluctuations in N supply (Millard and Grelet 2010), for example when N acquisition by the roots is reduced during an insect mass outbreak. N in needles covers up to 60% of the

tree's N demand for the next vegetation period, thus playing a key role in tree regeneration after partial defoliation (Millard and Grelet 2010; Polacco and Todd 2011). For example, a 50% defoliation of mountain beech (*Betula pubescens* ssp. *tortuosa* Ehrh.) by autumnal moth (*Epirrita autumnata* Borkhausen) lead to a N accumulation of the remaining leaves, most likely in form of Rubisco (Ribulose-1,5-bisphosphate carboxylase; Hoogesteger and Karlsson 1992; Palacio et al. 2012), thereby stimulating the rate of photosynthesis of the remaining leaves as compensatory reaction (Lovelock et al. 1999). In general terms, the increase in N pools in needles and fine roots might serve different purposes: (1) Amino acid levels might increase in direct response to N fertilization in different plant parts of adult Scots pine (bark, wood, foliage: Nordin et al. 2001; needles: Näsholm and Ericsson 1990; roots: Ahlström et al. 1988). (2) As a consequence of defoliation, tree water balance is disturbed (Bréda et al. 2006), because of an imbalance between root water uptake and leaf transpiration (Aroca et al. 2012). Accumulation of amino acids serving as osmoprotectants (Griffin et al. 1991) enables the tree to maintain water uptake and, consequently N supply, even when water-stressed (Fotelli et al. 2002; Rennenberg et al. 2006). (3) The N stored in the remaining needles in the canopy is also invested in the production of defense compounds, such as phenolics and lignin (Millard and Grelet 2010; Fang et al. 2016). On the other hand, higher nitrogen contents can benefit herbivores by improving the nutritional quality of the host plant. However, plant defense compounds also depend on the trees N content, which in turn has negative impacts on the insect's growth and survival (Kytö et al. 1996). Furthermore, roots produce organic exudates (e.g., amino acids) against secondary infestations with pathogenic root feeding bacteria, fungi, and insects (Bezemer and van Dam 2005; Oliva et al. 2016). Metabolites involved in aboveground defense are also synthesized in the roots and then transported aboveground (Van der Putten et al. 2001). In the study, the increase in structural N, i.e., lignin, in the fine roots that can also be considered a means of defense was seen only in autumn indicating a time-delayed response to defoliation. Similar to the present study, increased concentrations of total N, TAA-N, and TSP-N in needles were found in studies investigating eastern hemlock infested with hemlock woolly adelgid (e.g., Stadler et al. 2005; Gómez et al. 2012; Rubino et al. 2015; Soltis et al. 2015). With 72% we measured an even stronger increased TSP-N content in infested Scots pine needles during main defoliation compared to control trees. In contrast, TAA-N concentrations in needles were significantly higher only in spring and autumn, which could be the result of protein breakdown of infested needles during the main defoliation period (Krasensky and Jonak 2012). The amino compounds produced could be allocated to non-infested needles thereby contributing to its enhanced N content. Apparently, defoliation by the nun moth has consequences at the whole-tree

level and mediates responses not only in the most affected plant tissues (i.e., needles). When N acquisition by the roots is reduced as a response to insect mass herbivory, although soil N availability is high, internal reallocation of N seems to be a means to counteract the biotic stress.

Besides internal reallocation of N, tree carbon (C) resources and/or internal allocation might change in course of defoliation as net photosynthesis decreases when major parts of a tree's green tissue are lost (I-M-Arnold et al. 2016). Together with an accumulation of N in response to defoliation (as observed in the present study), C storage compounds might increase as well, as has been found for various tree species, such as black pine (*Pinus nigra* J.F.Arn., Palacio et al. 2012), eastern hemlock (*T. canadensis* L., Soltis et al. 2015), balsam fir (*Abies balsamea* L., Deslauriers et al. 2015), and red oak (*Q. rubra* L., Frost and Hunter 2008). This increase in C might support re-growth after defoliation (Palacio et al. 2012) or serve as defense by thickening cell walls via accumulation of C-rich cellulose and lignin (Soltis et al. 2015). As a result C/N ratio would be more or less constant, and indeed this was found in a study by Le Mellec et al. (2009) for a Scots pine forest comparable to our study site, both belonging to the same region of periodically reoccurring insect mass outbreaks. Contrasting this, lower needle C/N ratios were observed in three pine species (*Pinus pinaster* A., *P. nigra* J.F.Arn., *P. sylvestris* L.) defoliated by the pine processionary moth (*Thaumetopoea pityocampa* Den.Schiff.) indicating an overbalance of N accumulation in comparison to C accumulation (Hódar et al. 2015).

Overall, nun moth defoliation has been shown to reduce annual tree growth of Scots pine, which can be explained by inhibited water and nutrient supply and reduced photosynthetically active tissue (Beker 1996). With >90% needle loss in one vegetation period, a threshold is reached for Scots pine leading to significantly decreased tree growth and increased mortality rates (Cedervind and Langstrom 2003), which might become even more severe with multiple consecutive years of severe defoliation (Van Asch and Visser 2007).

5. Conclusions

The studied insect mass outbreak had significant impact on forest soil N cycling as well as N nutrition of Scots pines. Both, N input and output of the humus layer in the forest soil were strongly related to the biomass loss in response to the massive insect herbivory. To compensate for the aboveground losses of biomass, trees can either increase root N acquisition for the stimulation of growth processes (Lovett and Tobiessen 1993) or can reallocate N from internal sources. The present study suggests reallocation

of N from internal sources, because inorganic and organic N acquisition of tree roots was reduced in spite of increased soil N availability, whereas total N, structural N, soluble amino acid-N, and soluble protein-N levels were increased in fine roots and the remaining needles. However, N in- and output fluxes in the soil and within trees vary depending on environmental factors, such as climate, soil type, insect population dynamics, and outbreak intensity (Jung 2008; Gallet-Budynek et al. 2009; Keville et al. 2013). Our study investigated the consequences of an insect mass outbreak only for the duration of one vegetation period, but not potential recovery of the surviving trees. Thus, further studies are required to gain further insights into these complex processes.

Author contributions

JS and AI conceived and designed the study. MG and JS conducted the ¹⁵N uptake experiments and analysis of N uptake capacity and N metabolites. AI sampled and analyzed N in throughfall, dry matter, and soil leachates. Data evaluation and manuscript writing was performed by MG, AI, and JS. HR contributed the IRMS analyses and ideas to the study and the manuscript. All authors agreed on the final version of the manuscript.

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Conflict of Interest Statement

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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6.2. 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 (van Lierop et al. 2015). There is evidence that increasing intensity, frequency, duration, and extent of insect outbreaks are closely related to climate and global change (Dale et al. 2001; van Lierop et al. 2015). Pest insects like the nun moth (*Lymantria monacha* L.) can evidently benefit from these changing environmental conditions (Majunke et al. 2008). 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 (Möller and Heydeck 2013). 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 (Langström et al. 2001; Armour et al. 2003; Hicke et al. 2012).

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) (Vorholt 2012). Next generation sequencing (NGS) technologies offer the highest sampling depth, allowing the detection of low-abundant taxa (Knief 2014), 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., Nakatsu et al. 2000; Díez et al. 2001; Yang et al. 2001; González-Toril et al. 2003; Li et al. 2006; Brons and van Elsas 2008). In a pioneering study by Yang et al. (2001), 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 (Dik and van Pelt 1992; Stadler and Müller 1996). Beside excreta, the growth of microorganisms inhabiting the phyllosphere is enhanced by nutrient leaching from leaves damaged by folivorous insects (Kimmins 1972; Schowalter et al. 1986; Stadler and Müller 2000; Müller et al. 2003).

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 (le Mellec et al. 2009). 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 (L-M-Arnold et al. 2016). Some studies show stimulation of the soil microbial activity accompanied by alterations in soil nutrient cycling during insect outbreaks (le Mellec et al. 2009; L-M-Arnold et al. 2016). 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 (Stremińska et al. 2006). 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 (Kuikka et al. 2003; Saravesi et al. 2015), while saprophytic and endophytic fungi species may benefit (Saravesi et al. 2015).

The effects of outbreaks of single pests either on phyllosphere or soil microbial communities have been studied already (e.g., Müller et al. 2003; Stremińska et al. 2006; Ferrenberg et al. 2014; Štursová et al. 2014; Treu et al. 2014; Menkis et al. 2015; Abdelfattah et al. 2016; Mikkelsen et al. 2016a and 2016b; Pec et al. 2017; Brouillard et al. 2017), 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, OI, 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 (Brandfass and Karlovsky 2008) downscaled to 50 mg material followed by phenol extraction (Sambrook et al. 1989). 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) polyvinylpyrrolidone, adjusted to pH 8.0 with NaOH), 1 μ L 2-Mercaptoethanol, and 1 μ L 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 μ L of chloroform/isoamylalcohol (24:1 (v/v)), the mixture was shaken, incubated on ice for 10 min, and centrifuged at 7380 \times g for 10 min. We mixed 700 μ L of the supernatant thoroughly with 700 μ L phenol and centrifuged at 7380 \times g for 10 min. The supernatant (650 μ L) was extracted with the same amount of chloroform/isoamylalcohol (24:1 (v/v)) in the same manner. Following this, 600 μ L of the liquid phase were transferred to a new Eppendorf tube containing 200 μ L of polyethylene glycol (PEG) 6000 (30% (w/v)) and 100 μ L of NaCl (5 M). After mixing the content thoroughly, the tubes were incubated at room temperature for 20 min and centrifuged at 16,000 \times g for 15 min to obtain DNA pellets. The supernatant was discarded and the pellet was washed with 500 μ L of 70% (v/v) ethanol twice. The remaining ethanol in the tubes was evaporated under vacuum, the pellets were resuspended in 50 μ L 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 μ L DNA solution were mixed with 2 μ L loading buffer (100 mM EDTA, 50% (v/v) glycerol, 0.025% (v/v) bromphenol blue) and loaded into a 0.8% agarose gel in 1 \times 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 μ L.

Bacterial 16S rDNA gene fragments were amplified using the primer set F968-GC and R1401-1a that included the hypervariable regions V6 – V8 (Brons and van Elsas 2008). PCR mixtures were composed of 16 mM $(\text{NH}_4)_2\text{SO}_4$, 67 mM Tris-HCl, 0.01% Tween-20, pH 8.8 (NH_4 reaction buffer, Bioline, Luckenwalde, Germany), 3 mM MgCl_2 , 200 μ M of each deoxyribonucleoside triphosphate, 0.8 μ M of each primer, 1 mg/mL bovine serum albumin (BSA), 0.25 u Biotaq DNA-polymerase (Bioline, Luckenwalde, Germany) and 1 μ L 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 (Vainio and Hantula 2000). The composition of PCR mixture was identical with PCR conditions for 16S rDNA except for 1.5 mM MgCl_2 , 0.5 μ M 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 Na_2EDTA , 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 (SILVER SEQUENCETM DNA Sequencing System. 2017) 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 OI) 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 (Voříšková et al. 2014). 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 μ L 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 μ L 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 (Basic Local Alignment Search Tool 2017) 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 (Sambrook et al. 1989), 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 (Fromin et al. 2002; Fry et al. 2006). 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 (Oksanen et al. 2013).

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 9). Taxonomic assignments based on the sequences of the phyllosphere community in both years revealed that all fungi were assigned to the phylum Ascomycota (Figure 10, Table 3 and Table 4).

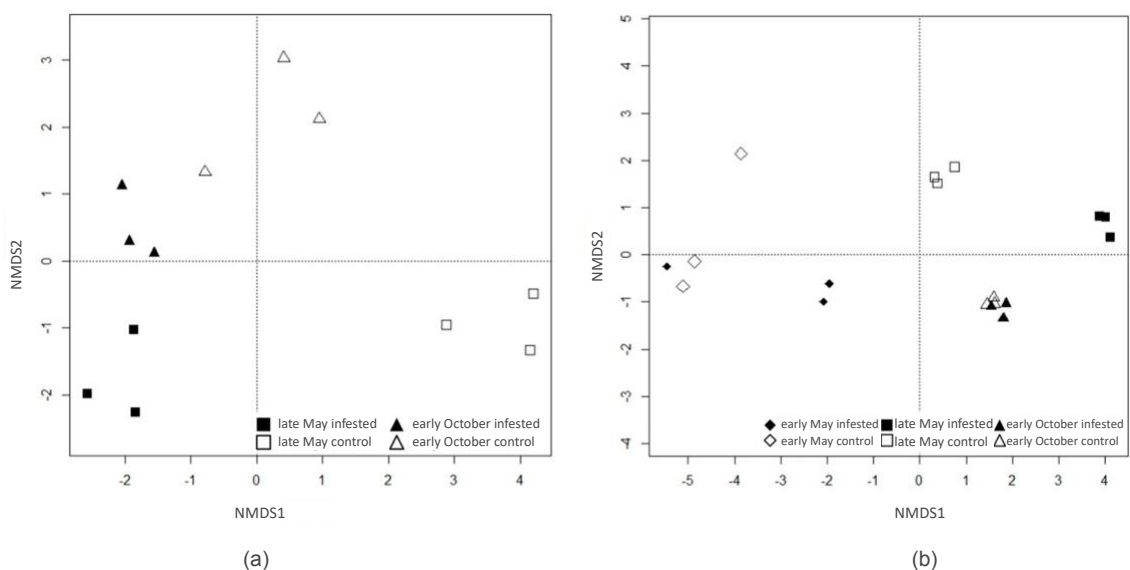


Figure 9: Non-metric multidimensional scaling ordination (NMDS) of Euclidean distance matrices of 18S rDNA denaturing gradient gel electrophoresis (DGGE) profiles of the phyllosphere fungal community in Scots pine (*Pinus sylvestris* L.) forests in 2014 (a) and 2015 (b).

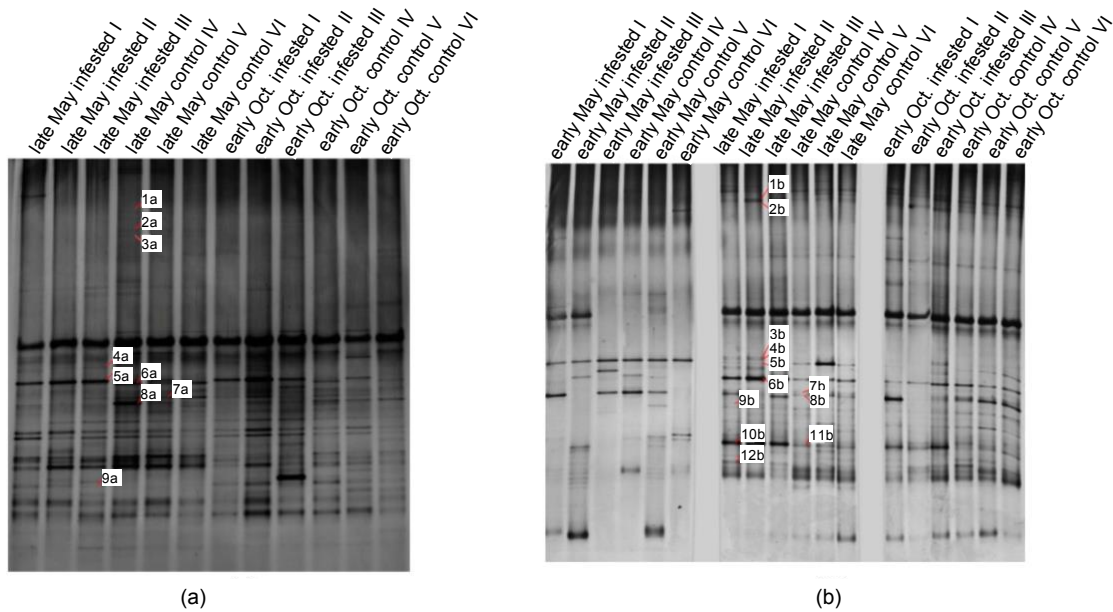


Figure 10: Denaturing gradient gel electrophoresis (DGGE) profiles of 18S rDNA gene fragments of the phyllosphere fungal community in Scots pine (*Pinus sylvestris* L.) forests in 2014 (a) and 2015 (b). Labelled Bands were excised for sequencing.

Table 3: 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 DGGE bands are shown in Figure 10 (a).

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 4: 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 10 (b).

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.

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: Amphisphaeriaceae), 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 identities: 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 exhibit different abundance between infested and uninfested samples (5 taxa). Both PCR products assigned to Pleosporales (family: Pleosporaceae) occurred only in samples of infested phyllosphere from 2015.

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 11). 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 12); 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 5). 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 6). This taxon was only detected in uninfested plots, despite a relatively low abundance in one of the three infested plots.

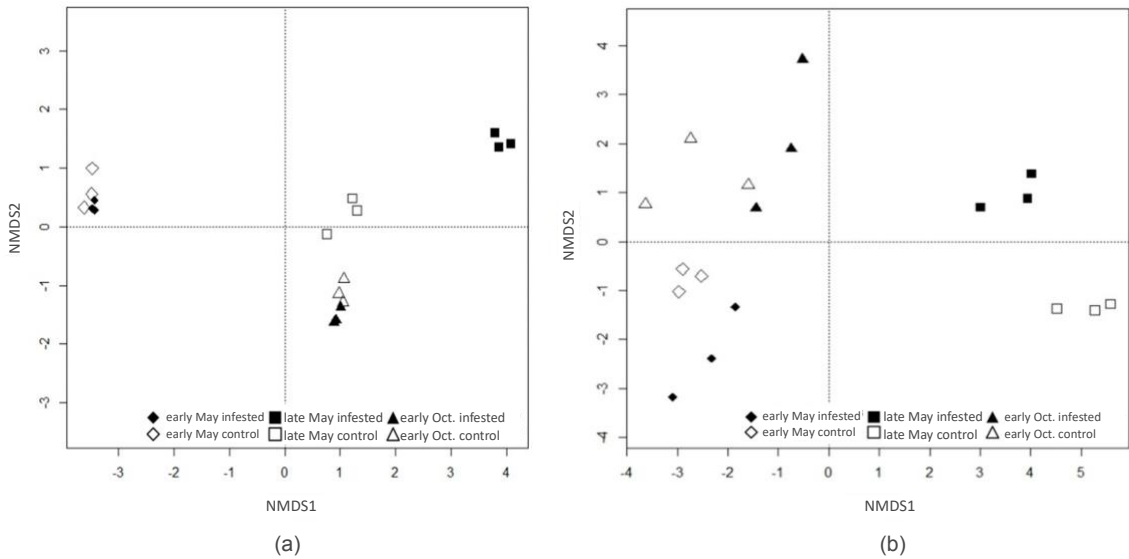


Figure 11: Non-metric multidimensional scaling ordination (NMDS) of Euclidean distance matrices of 18S rDNA denaturing gradient gel electrophoresis (DGGE) profiles of the phyllosphere fungal community of the L soil organic layer in 2014 (a) and OI soil organic layer in 2014 (a) and OI soil organic layer in 2015 (b).

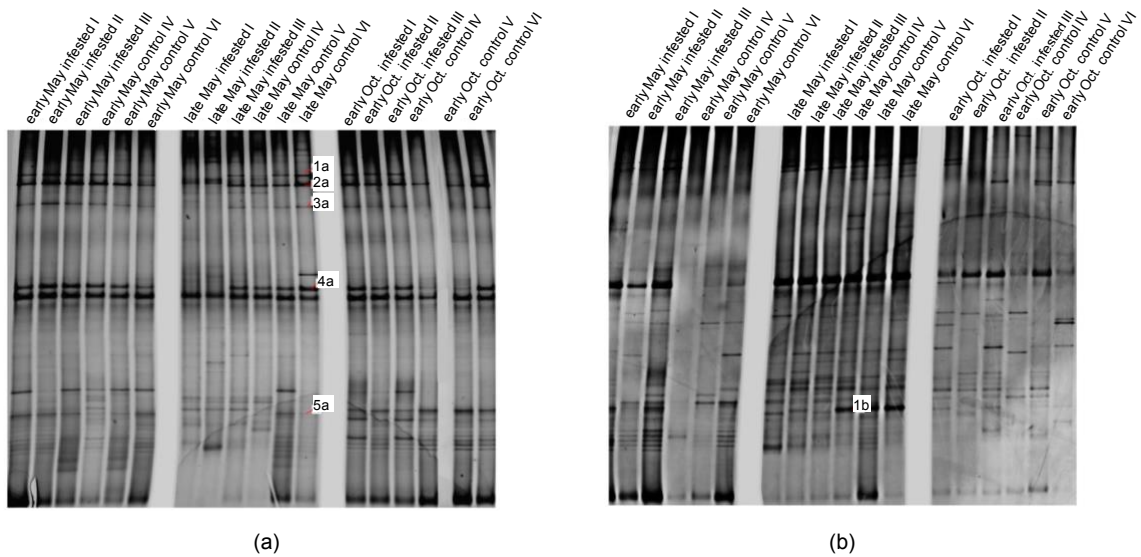


Figure 12: Denaturing gradient gel electrophoresis (DGGE) profiles of 18S rDNA gene fragments of the soil fungal community in Scots pine (*Pinus sylvestris* L.) forests of the L soil organic layer in 2014 (a) and the OI soil organic layer in 2015 (b). Labelled bands were excised for sequencing.

Table 5: 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 12 (a).

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 6: 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 12 (b).

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

+, ++ and +++ indicate increasing 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 (Lilley et al. 1997). Studies on honeydew-excreting aphids showed that C-rich insect excreta significantly increased the growth of phyllosphere bacteria, filamentous fungi and yeasts (Dik and van Pelt 1992, Stadler and Müller 1996). For example, heavy infestations of eastern hemlock (*Tsuga canadensis* L.) with hemlock wooly adelgid (*Adelges tsugae* A.) strongly stimulated bacteria, filamentous fungi, and yeasts in the phyllosphere (Stadler et al. 2005). A study conducted by Müller et al. (2003) 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 (Müller et al. 2003). 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 (Stadler and Müller 2000). 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 (Ferrenberg et al. 2014). 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 (Mikkelsen et al. 2016a). 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 (Stremińska et al. 2006). An increase of N content in needles as a compensatory response to defoliation was often observed (Gómez et al. 2012; Rubino et al. 2015, Grüning et al. 2017). 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 (Vestgarden 2001). In addition, the extent of infestation was found to determine the biogeochemical responses as well as the soil bacterial communities (Mikkelsen et al. 2016a, Brouillard et al. 2017). Mikkelsen et al. (2016b) 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. Menkis et al. 2015, Abdelfattah et al. 2016). 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*) (Menkis et al. 2015). These are known to cause damage to plant tissues. Therefore, alteration of fungal communities during pest outbreaks, additionally may threat tree health (Menkis et al. 2015). 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 (Kang et al. 1999, Jeewon et al. 2004). 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 (Cannon et al. 1995). Some taxa (e.g., *Scirrhia pini* F. & P.) are known to cause economically relevant damage to conifers (Funk and Parker 1966; Butin 1985). 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 (Dobbeler 2005; Ma et al. 2013). 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 (Barbee et al. 1999; Zhang et al. 2009), 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 (Simon et al. 2009), 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 (Battaglia et al. 2011; Houbraken and

Samson 2011; Kaur and Shweta 2013). 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., Štursová et al. 2014; Saravesi et al. 2015) 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. (2003) reporting reduced production of sporocarps as well as reduced diversity in ectomycorrhizal fungi under defoliated trees (see also Štursová et al. 2014; Treu et al. 2014; Pec et al. 2017). 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 (Pec et al. 2017) as well as in the abundance of ectomycorrhizal fungi (Treu et al. 2014). Bark beetle outbreaks in Norway spruce stands also reduced ectomycorrhizal fungi, but increased saprophytic fungi (Štursová et al. 2014). 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 (Saravesi et al. 2015). Ectomycorrhizal fungi depend on the supply of carbohydrates via host roots (Nehls et al. 2007; Smith and Read 2008). 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 (Gieger and Thomas 2002). 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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Author Contributions

A.I.-M.-A. and P.K. conceived and designed the experiments; L.B. and M.M.G. performed the experiments; L.B. analyzed the data; L.B. and M.M.G. wrote the manuscript; P.K. revised the manuscript.

Conflicts of Interest

The authors declare no conflict of interest.

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

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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 N₂O 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 (Stadler et al. 2005; Müller et al. 2006; le Mellec and Michalzik 2008). This results in high amounts of labile C and extractable N (Lovett and Ruesink 1995; Zimmer and Topp 2002; Stremińska et al. 2006), which can increase mineralization rates (Ritchie et al. 1998; Chapman et al. 2003). Because soil microorganisms can respond quickly to natural disturbances (Pal et al. 2012), increased soil microbial respiration rates are commonly observed following the input of organic material during forest pest outbreaks (Lovett and Ruesink 1995; Reynolds and Hunter 2001). 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) (Strickland and Rousk 2010; Weber et al. 2013; Jagadamma et al. 2014). While bacterial growth decreases following N fertilization or N deposition owing to C limitation (e.g., Demoling et al. 2008; Tang et al. 2016), the input of labile C compounds increases bacterial and fungal populations (Stremińska et al. 2006; De Graaff et al. 2010). For example, oak (*Quercus robur* L.) defoliation by the gypsy moth (*Lymantria dispar* L.) led to increased populations of fungi and N₂-fixing bacteria in soil (Oneř et al. 2016). 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.) (Stremińska et al. 2006). 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 (Štursová et al. 2014; Ferrenberg et al. 2014). 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 (Wardle et al. 2002; Poll et al. 2008). 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 (Martikainen 1984; Stremińska et al. 2006) and the nitrate reduction potential and nitrogen loss via denitrification are shown to be increased in these soils (Bárta et al. 2010). 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 (Dale et al. 2001).

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 NO₂-reduction were the Cu-nitrite reductase (*nirK*), the cd₁-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°80'38" 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 (Grüning et al. 2017; Beule et al. 2017). 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 (Supplementary Table 9 and Figure 14). 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 (Supplementary Table 10 and Supplementary Table 11). 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.

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 m² 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 m². 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. Beule et al. 2017). 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 (Sambrook et al. 1989) and subsequent polyethylene glycol precipitation (Brandfass and Karlovsky 2008). 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 ddH₂O. 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), *nosZI* from *Bradyrhizobium japonicum* (strain USDA 110), *nosZII* 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 (Beule et al. 2017) with ampicillin (50 µg/mL). For plasmid linearization, the restriction enzyme Sall 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 (Sambrook et al. 1989). 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 (Supplementary Table 10).

2.5. Real-time PCR

Primers, primer sequences, and the expected amplicon lengths are listed in Supplementary Table 11. Amplification of target genes was conducted in a CFX 384 Thermocycler (Biorad, Rüdigenheim, 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 MgCl₂, 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 ddH₂O for negative controls. The annealing and extension time and temperature for each primer pair are listed in Supplementary Table 10. 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 Larvae Chemical Analyses

The soil pH was measured in dH₂O (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 (Ogle 2018). 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 (R Development Core Team 2018).

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 Supplementary Table 12 and Supplementary Table 13. 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 OI layer ($p = 0.010$) (Figure 13). 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 OI 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 OI 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.

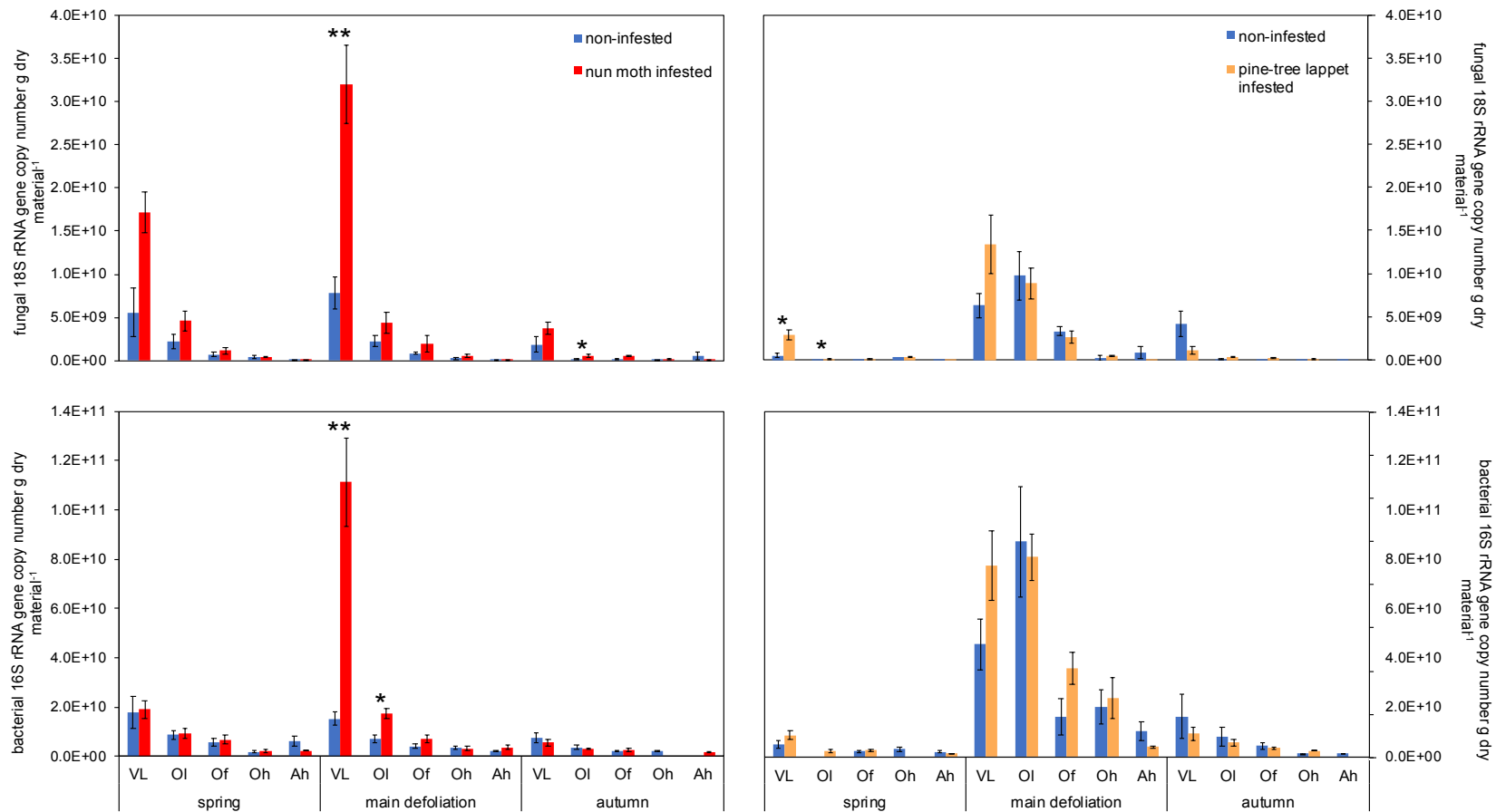


Figure 13: The mean fungal 18S rRNA and bacterial 16S rRNA gene copy numbers per gram of dry soil/plant material of the vegetation layers, organic layers, and the mineral soils of the nun moth (*Lymantria monacha* L.) and pine-tree lappet (*Dendrolimus pini* L.) infested and non-infested Scots pine (*Pinus sylvestris* L.) forest sites. VL = vegetation layer, OI = 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 Supplementary Table 13); 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, ** $p < 0.010$, * $p < 0.050$).

3.2. Denitrification Gene Abundance in Soil – *nirK*, *nirS*, *nosZI*, *nosZII*

The abundances of the denitrification genes in the soil are summarized in Table 7. During the nun moth's main defoliation, the VL and the OI 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 *nosZII* 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 *nosZI* during the nun moth defoliation.

During the pine-tree lappet defoliation, the *nosZI* abundance was higher in the Ah during the main defoliation, the Of during autumn, and the *nosZII* 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 OI during the main defoliation and *nosZII* 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.

Table 7: The mean \pm standard error of the *nirK*, *nirS* and *nosZI* and *nosZII* 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, OI= litter organic, Of= fibric organic, Oh= humic organic, Ah=humic mineral. (varying sample size, see Supplementary Table 13, 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	OI	Of	Oh	Ah	VL	OI	Of	Oh	Ah
<i>nirK</i>	non-infested	2.04×10^8 $\pm 1.07 \times 10^8$	1.36×10^8 $\pm 5.32 \times 10^7$	6.95×10^7 $\pm 2.15 \times 10^7$	4.09×10^7 $\pm 1.43 \times 10^7$	3.87×10^7 —	5.58×10^8 $\pm 3.17 \times 10^8$	1.89×10^9 $\pm 5.91 \times 10^8$	4.32×10^8 $\pm 1.89 \times 10^8$	3.61×10^8 $\pm 1.42 \times 10^8$	4.17×10^7 $\pm 2.06 \times 10^7$
	infested	$1.53 \times 10^{9**}$ $\pm 2.16 \times 10^8$	$4.73 \times 10^{8*}$ $\pm 2.20 \times 10^8$	1.64×10^8 $\pm 5.95 \times 10^7$	2.84×10^8 $\pm 1.90 \times 10^8$	1.40×10^8 $\pm 2.51 \times 10^7$	8.52×10^8 $\pm 1.81 \times 10^8$	$1.39 \times 10^{9*}$ $\pm 8.25 \times 10^8$	3.70×10^8 $\pm 1.19 \times 10^8$	1.47×10^8 $\pm 1.01 \times 10^8$	1.96×10^8 $\pm 1.28 \times 10^8$
<i>nirS</i>	non-infested	9.93×10^7 $\pm 3.66 \times 10^7$	n.d. —	8.37×10^7 —	n.d. —	n.d. —	1.66×10^8 —	1.50×10^8 $\pm 4.36 \times 10^5$	1.22×10^8 $\pm 1.29 \times 10^7$	8.42×10^7 $\pm 7.29 \times 10^6$	n.d. —
	infested	1.06×10^8 $\pm 1.73 \times 10^7$	7.47×10^7 $\pm 1.24 \times 10^7$	n.d. —	n.d. —	n.d. —	1.66×10^8 —	2.25×10^8 $\pm 3.68 \times 10^7$	8.25×10^7 $\pm 6.75 \times 10^6$	6.59×10^7 $\pm 2.84 \times 10^7$	6.05×10^7 —
<i>nosZI</i>	non-infested	8.53×10^5 $\pm 4.92 \times 10^5$	3.83×10^5 $\pm 9.62 \times 10^4$	2.16×10^5 $\pm 2.39 \times 10^4$	2.43×10^5 $\pm 3.73 \times 10^4$	5.33×10^5 $\pm 2.88 \times 10^5$	3.59×10^6 $\pm 3.22 \times 10^6$	8.58×10^5 $\pm 4.49 \times 10^4$	1.40×10^6 $\pm 5.13 \times 10^5$	9.74×10^5 $\pm 2.43 \times 10^5$	1.59×10^5 $\pm 4.06 \times 10^4$
	infested	8.02×10^5 $\pm 1.32 \times 10^6$	6.05×10^5 $\pm 7.86 \times 10^4$	5.44×10^5 $\pm 2.04 \times 10^5$	4.73×10^5 $\pm 1.01 \times 10^5$	4.60×10^5 $\pm 2.06 \times 10^5$	1.87×10^6 $\pm 1.31 \times 10^5$	3.21×10^5 $\pm 9.76 \times 10^4$	1.29×10^6 $\pm 5.55 \times 10^5$	6.04×10^5 $\pm 1.97 \times 10^5$	$5.89 \times 10^{5*}$ $\pm 1.48 \times 10^5$
<i>nosZII</i>	non-infested	2.53×10^7 $\pm 1.68 \times 10^7$	2.38×10^7 $\pm 1.04 \times 10^7$	9.37×10^6 $\pm 3.79 \times 10^6$	4.17×10^6 $\pm 2.69 \times 10^6$	2.62×10^6 $\pm 1.70 \times 10^6$	1.96×10^8 $\pm 8.85 \times 10^7$	4.75×10^7 $\pm 1.97 \times 10^7$	3.62×10^7 $\pm 2.47 \times 10^7$	1.51×10^6 $\pm 6.85 \times 10^5$	4.45×10^8 $\pm 5.77 \times 10^8$
	infested	3.26×10^7 $\pm 1.88 \times 10^7$	1.93×10^7 $\pm 9.95 \times 10^6$	1.13×10^7 $\pm 5.96 \times 10^6$	6.58×10^6 $\pm 3.22 \times 10^6$	1.32×10^6 $\pm 8.69 \times 10^5$	2.32×10^8 $\pm 1.99 \times 10^8$	3.75×10^8 $\pm 3.05 \times 10^8$	7.97×10^6 $\pm 4.40 \times 10^6$	2.94×10^6 $\pm 1.89 \times 10^6$	2.04×10^5 —

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 14). The soil pH did not differ between the infested and non-infested sites but decreased from the top to mineral soil layers (Supplementary Table 9).

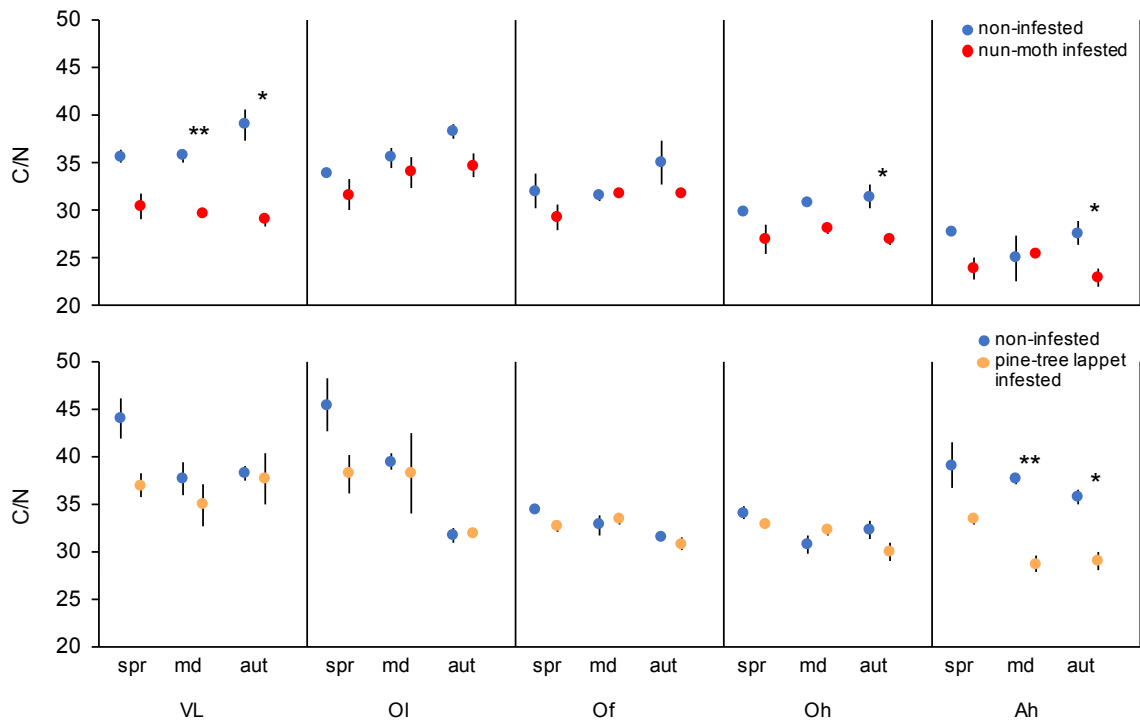


Figure 14: The mean \pm standard error of the C/N of the vegetation layers, organic layers, and the mineral soil of the nun moth (*Lymantria monacha* L.) and pine-tree lappet (*Dendrolimus pini* L.) infested and non-infested Scots pine (*Pinus sylvestris* L.) forest site. VL = vegetation layer, OI = 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, ** $p < 0.010$, * $p < 0.050$.

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% C 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 15). No difference in the 18S rRNA gene abundance between the infested and non-infested site nor between the two pests was detectable.

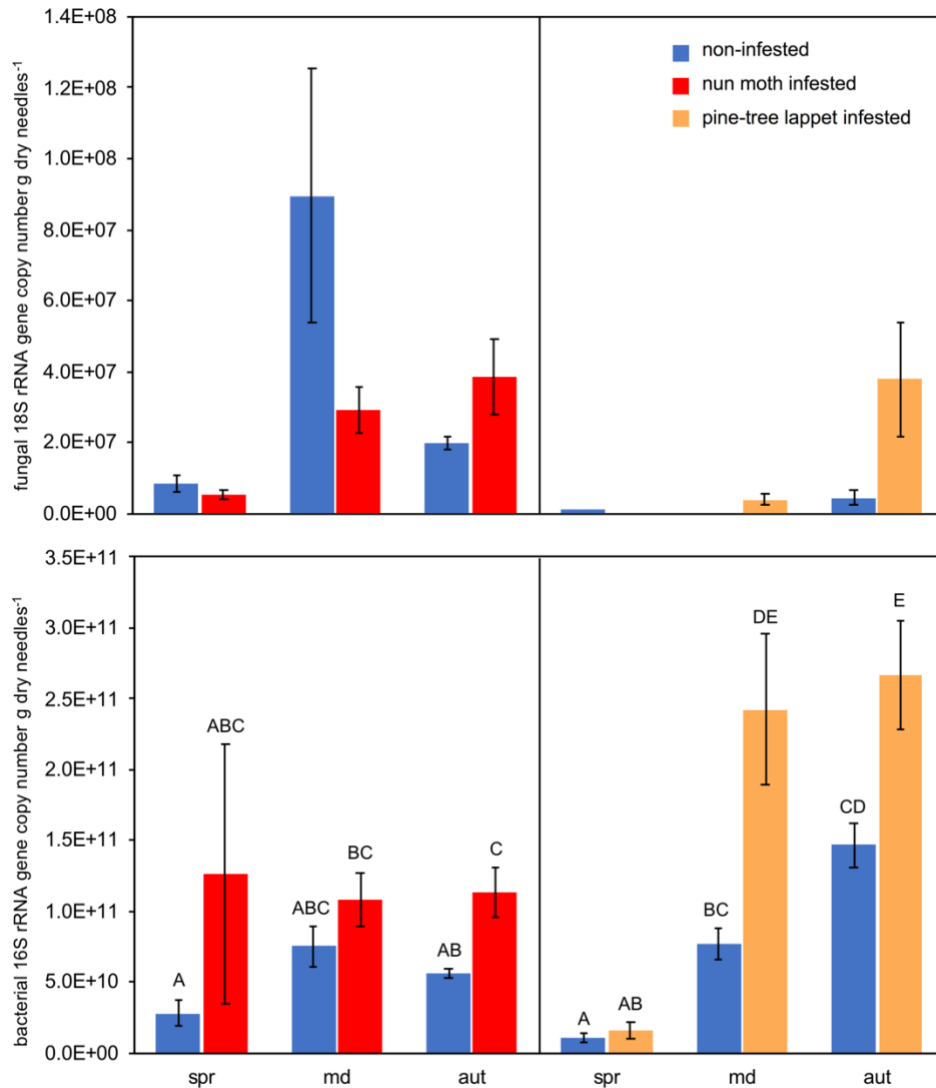


Figure 15: The mean fungal 18S rRNA and bacterial 16S rRNA gene copy numbers per gram of dry needles of the nun moth (*Lymantria monacha* L.) and pine-tree lappet (*Dendrolimus pini* L.) infested and non-infested Scots pine (*Pinus sylvestris* L.) forest site (the error bars represent standard error of the mean, spr = spring, md = main defoliation, aut = autumn. Varying sample sizes, see Supplementary Table 14, 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 8). 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 8: The mean \pm 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. Samples were collected after no longer than 48 h ($n = 3$, $\diamond n = 1$, asterisks indicate significant differences between the nun moth and pine-tree lappet infested site based on the Kruskal-Wallis test, * $p < 0.050$).

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} \diamond –	7.34×10^{11} $\pm 5.77 \times 10^9$

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 (De Vries et al. 2006; Strickland and Rousk 2010). 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 (Grüning et al. 2017). 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 (Stremińska et al. 2006). 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 (Poll et al. 2008; Rousk et al. 2009; Levy-Booth et al. 2014). 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 (Mikkelsen et al. 2017). 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. Grüning et al. 2017). 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 (Lovett and Ruesink 1995; Zimmer and Topp 2002; Poll et al. 2008; Güsewell and Gessner 2009). 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 (Berg et al. 1998). Additionally, it has been observed that only moderate to high amounts of organic input lead to changes in soil microbial population growth (Hillstrom et al. 2010). 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 (Zimmer and Topp 2002; Le Mellec et al. 2009). Even though the changes of fungal and bacterial population sizes were limited to the VL and OI 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.) (Mikkelsen et al. 2017; Brouillard et al. 2017). Therein, the biogeochemical response to the outbreak was most prominent in the mineral soil and dependent on the percentage of dead trees (Mikkelsen et al. 2017; Brouillard et al. 2017). 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 (Zimmer and Topp 2002) and during *Operophtera brumata* L. and *Erannis defoliaria* L. outbreaks in the organic layers of an oak forest (I-M-Arnold et al. 2016). 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 (Kopáček et al. 2010). 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 (Barnard et al. 2005; Tang et al. 2016). However, the response of the denitrification genes remains controversial since denitrification is determined by many interacting environmental and soil biological factors (Barnard et al. 2005; Levy-Booth et al. 2014). 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 (Tang et al. 2016). 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 (Kandeler et al. 2009). In our study, the *nosZI* and *nosZII* 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. Demoling et al. 2008; Levy-Booth et al. 2014).

Our findings are consistent with the assumption that denitrifying microorganisms do not show coherent responses to environmental changes (Boyle et al. 2006; Philippot et al. 2009). For example, *nirK* proved to be more responsive to changes such as land use practices (Dandie et al. 2011), vegetation cover (Smith and Ogram 2008), and water source quality (Zhou et al. 2011), compared to *nirS*. Furthermore, *nirK* has a higher sensitivity towards nutrient additions than *nirS* (Kandeler et al. 2006; Bárta et al. 2010). 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 (Kandeler et al. 2009; Gschwendtner et al. 2014; Kennedy et al. 2015) 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 (Nannipieri et al. 2003). 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 (Mikkelsen et al. 2017). Abundance and activity patterns of denitrifying microorganisms often do not respond congruently to environmental changes (Gschwendtner et al. 2014). 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 (Smith and Osborn 2009). Nevertheless, both *nir* genes are known to correlate with soil net N₂O fluxes (Morales et al. 2010; Rasche et al. 2011; Levy-Booth et al. 2014; Wu et al. 2017). *nirS*-possessing organisms usually are comprised of *nosZ* genes which enable complete denitrification, while *nirK* organisms mostly lack *nosZ* (Graf et al. 2014). This implies that soil N₂O emissions are predominantly determined by *nirK*-, rather than by *nirS*-type microorganisms. Thus, the *nirS/nirK* ratio may be negatively correlated with the soil N₂O sink capacity (Stadler and Michalzik 2000; Jones et al. 2014). 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 N₂O emissions from infested forest sites. However, to date, there is no long-term field study on the effects of insect outbreaks on N₂O 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. Stadler and Michalzik 2000; Müller et al. 2003). 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 (Stremińska et al. 2006).

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 (Vorholt 2012). 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 (Lindow and Brandl 2003). 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) (Strauss et al. 2002; Redford and Fierer 2009). 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 (Strauss et al. 2002). The leaching of substances from the destructed needle parts, as well as substances in insect saliva, can act as growth promoters of phyllospheric microorganisms (Steinbauer et al. 1997; Vorholt 2012). 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.) (Suda et al. 2009). Eastern hemlock (*Tsuga canadensis* L.) infested with Hemlock woolly adelgid (*Adelges tsugae* A.) showed an increased abundance of epiphytic microorganisms (Stadler et al. 2005). Bacteria can have parasitic, commensal and mutualistic interactions with trees (Vorholt 2012). 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 N₂O emissions during pest outbreaks yields in increased soil N₂O emissions.

Supplementary Materials

Supplementary Table 9: Mean [\pm standard error] of pH (soil:H₂O 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, OI= litter organic, Of= fibric organic, Oh= humic organic, Ah=humic mineral.

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

Supplementary Table 10: qPCR conditions for the different target genes; *BamoA* standards were generated from genomic DNA of *Nitrosomonas europaea* (strain DSM 28437), *AamoA* standards were generated from environmental clones.

Target Gene	Final MgCl ₂ Concentration (mM)	Primer Concentration (μM)	Initial Denaturation	Denaturation	Annealing	Extension
<i>nirK</i>	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
<i>nirS</i>	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
<i>nosZI</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 Initial Denaturation	Denaturation	35 cycles Annealing	Extension
<i>BamoA</i>	2.0	0.3	95 °C, 120 s	94 °C, 20 s	62 °C, 30 s	68 °C, 35 s
<i>AamoA</i>	30	0.3	95 °C, 120 s	94 °C, 20 s	61 °C, 30 s	68 °C, 45 s
<i>nosZII</i>	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

Supplementary Table 11: Target genes, oligonucleotide primers and amplicon size.

Target Gene	Primer Name	Primer Sequence (5'–3')	Amplicon Size (bp)	Reference
bacterial ammonia monooxygenase α subunit (<i>BamoA</i>)	amoA-1F	GGGGTTTCTACTGGTGGT	491	Rotthauwe et al. (1997)
	amoA-2R	CCCCTCKGSAAAGCCTTCTTC		
archaeal ammonia monooxygenase α subunit (<i>AamoA</i>)	CrenamoA23f	ATGGTCTGGCTWAGACG	628	Tourna et al. (2008)]
	CrenamoA616r	GCCATACABCKRTANGTCCA		
Cu-nitrite reductase catalytic subunit (<i>nirK</i>)	nirK876F	ATYGGCGGVAYGGCGA	165	Henry et al. (2004)]
	nirK1040R	GCCTCGATCAGRTRRTGGTT		
<i>Cd</i> ₁ -nitrite reductase catalytic subunit (<i>nirS</i>)	cd3aF	G TSAACG TSAAGGARACSGG	410	Michotey et al. (2000), Throbäck et al. (2004)
	R3cd	GASTTCGGRTGSGTCTTGA		
<i>nitrous oxide reductase catalytic subunit</i> (<i>nosZ</i> I)	NosZ2F	CGCRACGGCAASAAGGTSMSST	267	Henry et al. (2006)
	NosZ2R	CAKRTGCAKSGCRTGGCAGAA		
<i>nitrous oxide reductase catalytic subunit</i> (<i>nosZ</i> II)	1153 nosZ 8F	CTIGGICCIYTKCAYAC	698	Jones et al. (2013)
	1888 nosZ 29R	GCIGARCARAAITCBGTR		
fungal 18S rRNA (18S)	FR1	AICCATTC AATCGGTAIT	350	Vainio and Hantula (2000)
	FF390	CGATAACGAACGAGACCT		
bacterial 16S rRNA (16S)	341F	CCTACGGGAGGCAGCAG	194	López-Gutiérrez et al. (2004)
	534R	ATTACCGCGGCTGCTGGCA		

Supplementary Table 12: Detectable target genes in % of all samples during the outbreak of the nun moth (*Lymantria monacha* L.) and pine-tree lappet (*Dendrolimus pini* L.). Total sample number soil: n = 180, needle n = 48, litter, needle, feces: n = 3, NA = data not available.

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%
<i>nirK</i>	59.4%	57.2%	NA	NA	NA	NA	NA	NA	NA	NA
<i>nirS</i>	13.9%	19.4%	NA	NA	NA	NA	NA	NA	NA	NA
<i>nosZI</i>	32.8%	35.6%	NA	NA	NA	NA	NA	NA	NA	NA
<i>nosZII</i>	72.2%	63.9%	NA	NA	NA	NA	NA	NA	NA	NA

Supplementary Table 13: Samples above the detection limit for fungal 18S and bacterial 16S rRNA gene abundance, *nirK*, *nirS*, *nosZI* and *nosZII* 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		
	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	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
	<i>nosZI</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
	<i>nosZII</i>	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
	<i>nirK</i>	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
	<i>nirS</i>	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
	<i>nosZI</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
	<i>nosZII</i>	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
	<i>nirK</i>	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
	<i>nirS</i>	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.

Supplementary Table 14: 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

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.

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Conflicts of Interest

The authors declare no conflict of interest.

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6.4. Increased Forest Soil CO₂ and N₂O Emissions During Insect Infestation

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Abstract

Forest soils are major sinks of terrestrial carbon, but this function may be threatened by mass outbreak events of forest pests. Here, we measured soil CO₂-C and N₂O-N fluxes from a Scots pine (*Pinus sylvestris* L.) forest that was heavily infested by the nun moth (*Lymantria monacha* L.) and an adjacent noninfested (control) forest site during one year. In the infested forest, net emissions of CO₂-C were higher during main defoliation, summer and autumn, while indications of increased N₂O-N emissions were found at one sampling date. On basis of this, a microcosm incubation experiment with different organic matter treatments was conducted. Soil treatments with needle litter, insect feces plus needle litter, and insect feces showed 3.7-, 10.6-, and 13.5-fold higher CO₂-C emissions while N₂O-N of the insect feces plus needle litter, and insect feces treatment was 8.9-, and 10.4-fold higher compared with soil treatments without added organic matter (control). Hence, the defoliation in combination with high inputs of organic matter during insect outbreaks distinctly accelerate decomposition processes in pine forest soils, which in turn alters forests nutrient cycling and the functioning of forests as carbon sinks.

Keywords: CO₂; N₂O; insect outbreak; frass; litter; soil emissions; nun moth; disturbances; climate change

1. Introduction

The worldwide forest area affected annually by insect outbreaks amounts about 36.5 million hectares (Kautz et al. 2017), thereby representing a threat to the function of forests as a carbon sink (Kurz et al. 2008; Clark et al. 2010; Dymond et al. 2010; Hadden and Grelle 2017). Insect defoliation not only leads to decreased canopy biomass, tree growth, and inhibits the production new foliage, but can be accompanied by changes in the N nutritional status of infested trees (e.g., decreased net N uptake, N accumulation in fine roots and needles) (Stadler et al. 2005; Grüning et al. 2017). Defoliation of forest areas during forest pest outbreaks distinctly increase organic input into the soil in the form of insect feces, cadavers, litter, and other plant material (Stadler et al. 2005; Müller et al. 2006; Le Mellec and Michalzik 2008). Such changes of litter quality and quantity affect the soil organic matter composition (Chapman et al. 2003), and alter nutrient cycling (Belovsky and Slade 2000; Frost and Hunter 2004; Le Mellec et al. 2009). Depending on the outbreak intensity, insect feces can account for up to 46% of the total litterfall amount in Scots pine forests (Fogal and Slansky 1985). The easy soluble structure of insect feces, with high amounts of labile C and extractable N, can facilitate nutrient release in soils (Lovett and Ruesink 1995; Zimmer and Top 2002; Stremińska et al. 2006). Therefore, decomposition processes in the course of defoliations may be enhanced (Ritchie et al. 1998; Chapman et al. 2003), thereby triggering CO₂ emissions from forest soils (Lovett and Ruesink 1995; Hillstrom et al. 2010; L.-M.-Arnold et al. 2016). In contrast, findings from xylophagous insects (compared to phytophagous insects) showed contradictory results. For example, Ponderosa pine (*Pinus ponderosa* Laws.) stands infested by different bark beetle species (Scolytinae) (Morehouse et al. 2008) as well as temperate mixed forests infested by the emerald ash borer (*Agilus planipennis* F.) (Matthes et al. 2018) showed no effect on soil CO₂ emissions. Bark beetle induced tree diebacks in lodgepole pine (*Pinus contorta* Dougl. ex Loud.) forests caused even decreased CO₂ emissions (Mikkelsen et al. 2017; Brouillard et al. 2017).

N₂O flux changes during insect outbreaks are less well studied. No effects on N₂O emission and nitrate leaching were detected in a manipulation experiment with intensive defoliation of hybrid poplar (*Populus x euroamericana* cv. *Eugeneii*) stands by gypsy moth (*Lymantria dispar* L.) (Russel et al. 2004). A rather decelerating effect on nutrient cycling in the course of insect outbreaks, with reduced litter decomposition and accumulation of the soil nitrogen storage was assumed by Madritch and Lindroth (2015), Verkaik et al. (2006), and Ritchie et al. (1998), which may be related to high amounts of tannins in feces, that can build recalcitrant protein-tannin complexes, and impair soil

microbial activity. Therefore, nitrogen deriving from insect outbreaks is hypothesized to be rather redistributed in the ecosystem than to be lost (Lovett et al. 2002).

Carbon and nitrogen cycling is a function of complex effects and interdependencies between climate, topography, soil properties and microbial soil community structures (Russel et al. 2004). Moreover, forest soils seem to have a certain resistance to biotic disturbances. This may explain why effects of organic input from insect outbreaks on microbial respiration or nitrogen immobilization in soils are often only detectable at relatively high levels of defoliation (>70%) (Hunter et al. 2003, Hillstrom et al. 2010). Nevertheless, the effects of forests pests on greenhouse gas emissions are not sufficiently understood to predict environmental effects. The lack of knowledge about C and N balances at the soil–atmosphere interface during biotic disturbances contributes to this unpredictability.

In this study, we analyzed CO₂-C and N₂O-N fluxes in the course of a nun moth (*Lymantria monacha* L.) outbreak in Scots pine (*Pinus sylvestris* L.) forests in Germany. Moth larvae regularly hatch in April/May, feed on pine needles until July, and can completely defoliate trees during this period (Bejer 1988). We quantified net CO₂-C and N₂O-N fluxes from a forest soil in an infested compared with a noninfested forest stand. In a consecutive microcosm incubation experiment, we analyzed net CO₂-C and N₂O-N fluxes under controlled conditions with treatment additions of pine needles, insect feces, and pine needles plus insect feces. We expected that (i) decomposition processes would be accelerated by organic inputs in the course of the insect outbreak and (ii) CO₂-C and N₂O-N emissions from soils would respond to these biogeochemical changes.

2. Materials and Methods

2.1. Field Measurement

The measurements were conducted in a 65-year-old Scots pine forest (52°8'38" N, 13°45'14" E, 42 m above sea level) heavily infested by the nun moth (~80% crown defoliation) compared with a noninfested 65-year-old Scots pine forest growing on a site with similar site conditions (52°9'29" N, 13°36'47"; 35 m above sea level). Both forests are characterized as "white moss pine forests" (*Leucobryo-Pinetum* W.Mat.) grown on podzol (Food and Agriculture Organization of the United Nations (FAO) classification) on aeolian sand (0.2 – 0.63 mm), with little gravel and a pH (1:10 in H₂O) ranging from 3.2 to 3.9 in the mineral soil (Ah) horizon. Soil C/N ratio of the infested site was 29.4 and of the noninfested site 30.3. Both sites did not differ significantly in their soil microbial community composition in early May prior the nun moth population peak (Beule et al.

2017). Average annual temperature was 9.2 and 10.8°C and average annual precipitation was 611 and 474 mm for the study sites in 2013 and 2014, respectively (German Federal Meteorological Service (DWD) and Climate Data Center (CDC), Weather Station Lindenberg (Station ID: 3015), 13.07.2018, see also Supplementary Figure 20; for a description of site properties, see Grüning et al. (2017), Beule et al. (2017)). We measured the CO₂-C and N₂O-N fluxes across one year (August 2013 – July 2014), i.e., on three dates in 2013 (August – October with n = 18 for each date) and five dates in 2014 (March – July with n = 30 for each date). The study sites were arranged in a paired sample comparison of noninfested versus infested forests that were located in spatial proximity to each other with n = 9 in 2013 and n = 15 in 2014 for each plot. To quantify soil CO₂-C and N₂O-N emissions, a polyvinyl chloride (PVC) lid (25 cm diameter, 13 cm high) was applied to a cylindrical PVC-U-frame (25 cm diameter, 10 cm high) which was permanently inserted (7 – 8 cm deep) in the organic layer and Ah horizon of sampling sites. Litter was not removed within the frames. CO₂-C fluxes were measured with a four-point-sampling method, where 20 mL air samples were taken with a syringe via a septum from the closed chamber after 0, 20, 40, and 60 min following its sealing and stored in evacuated glass exetainers. CO₂-C and N₂O-N concentrations were determined by gas chromatography (ECD, Shimadzu, Duisburg, Germany). Fluxes were calculated from the linear change of the gas concentrations during chamber closure, the volume of the chamber and the enclosed surface area, according to Lessard et al. (1993) (Lessard et al. 1994). Values were corrected for air temperature and air pressure using the following equation:

$$factor = \frac{air\ pressure\ [pa] * molar\ weight\ [\frac{g}{mol}]}{gas\ constant\ [\frac{J}{mol\ K}] * temperature\ [K]} * 10^{-3}$$

and projected to one square meter and one hour. Simultaneously, temperature of the top 10 cm soil depth, gravimetric soil water content of the Ah, and sampling time were recorded for each plot.

2.2. Incubation Experiment

For the microcosm incubation experiment, randomly collected upper mineral soil (Ah) of the control site was used, homogenized, sieved at 2 mm and 200 g fresh weight were transferred to 20 glass incubators (1000 cm³). Incubators were attached to an automated gas chromatographic system (GC) with a ⁶³Ni electron capture detector (ECD) for the measurement of CO₂ and N₂O concentrations (Shimadzu, Duisburg, Germany) (for a

description, see also Lofffield et al. (1997)). The air space of the incubators was flushed with synthetic air and the flux was calculated by determining the differences between inlet air and exhausted air (12 measurements per incubator and day). Experimental runtime was 31 days, including six days of soil pre-incubation. Treatments were added on day seven in form of feces, Scots pine needle litter and a mixture of both with five biological replicates for each treatment. The feces was produced under laboratory conditions from *Dendrolimus pini* L. Feces was mixed, dried at 20 °C for 72 h and needle and bark residues were removed. Needle litter was collected from the noninfested control site in 2014 and dried at 20°C for 72 h. The total amount of needle and feces input was 5 g dry-weight-equivalent of total C, resulting in the addition of 49.2 mg feces (feces treatment), 48.9 mg needle (needle treatment) and 24 mg of both (feces plus needle litter treatment), respectively, per gram soil. Temperature was constant 20°C and no light exposure during the experiment. On day 4 and day 18, 60 mL dH₂O were added to the incubators. The amount of added water was adjusted to reach 70 – 80% of the soils maximum water holding capacity. The analyses of element contents of Aluminum (Al), Calcium (Ca), Iron (Fe), Potassium (K), Magnesium (Mg), Manganese (Mn), Sodium (Na), Phosphorus (P), Sulphur (S) of the used soil, feces, and needle litter samples are given in Supplementary Table 15. Values are based on HNO₃ extraction (described in Heinrichs et al. (1986)) and subsequent measurement by using an ICP-OES (iCAP 6300 Duo VIEW ICP Spectrometer, Thermo Fischer Scientific GmbH, Dreieich, Germany). For the measurement of total carbon (C_{tot}) and nitrogen (N_{tot}) content soil was dried at 105°C for 24 h, finely ground, and analyzed by a total organic carbon analyzer multi C/N (Analytik Jena, Jena, Germany).

2.3. Statistical Analyses

Statistical analyses were conducted in R 3.3.3 (R Development Core Team 2017). All data sets were tested for distribution of normality and homogeneity of variances by applying the Shapiro-Wilk test and Levene's test, respectively. CO₂-C and N₂O-N fluxes were analyzed separately for each of the seven sampling dates by paired Wilcoxon signed-rank tests, with n = 18 (for each of the three the dates in 2013), and n = 30 (for each of the five dates in 2014), respectively. The Kruskal-Wallis test was used to detect differences between the accumulated CO₂-C and N₂O-N fluxes from the four treatments of the incubation experiment. In addition, Spearman's rank correlations (r_s) were used to assess the relationships between soil greenhouse gas fluxes (CO₂-C and N₂O-N) and soil temperature as well as soil water content from the field study.

3. Results

3.1. Field Measurement

CO₂-C fluxes averaged 47.33 ± 7.83 mg CO₂-C m⁻² h⁻¹ in noninfested forests and 61.28 ± 21.98 mg CO₂-C m⁻² h⁻¹ in infested forests. They considerably increased during summer, early in the autumn and during main defoliation in the infested forest site with 1.8- ($p = 0.004$), 1.9- ($p = 0.016$) and 1.5-fold ($p < 0.001$) increased emissions in August and September 2013 and June 2014, respectively (Figure 16). N₂O-N fluxes averaged 0.27 ± 0.41 µg N₂O-N m⁻² h⁻¹ in noninfested forests and 0.91 ± 0.74 µg N₂O-N m⁻² h⁻¹ in infested forests. N₂O-N emissions were significantly increased in the infested forest in October 2013 ($p = 0.039$), while N₂O-N flux of the noninfested forest was negative from August 2013 to March 2014 (Figure 17).

CO₂-C fluxes correlated positively with soil temperature ($r_s = 0.738$, $p = 0.046$, Supplementary Figure 21), but not with soil water content ($r_s = -0.700$, $p = 0.233$). N₂O-N emissions were neither correlated with soil temperature ($r_s = -0.095$, $p = 0.840$) nor with soil water content ($r_s = -0.300$, $p = 0.683$).

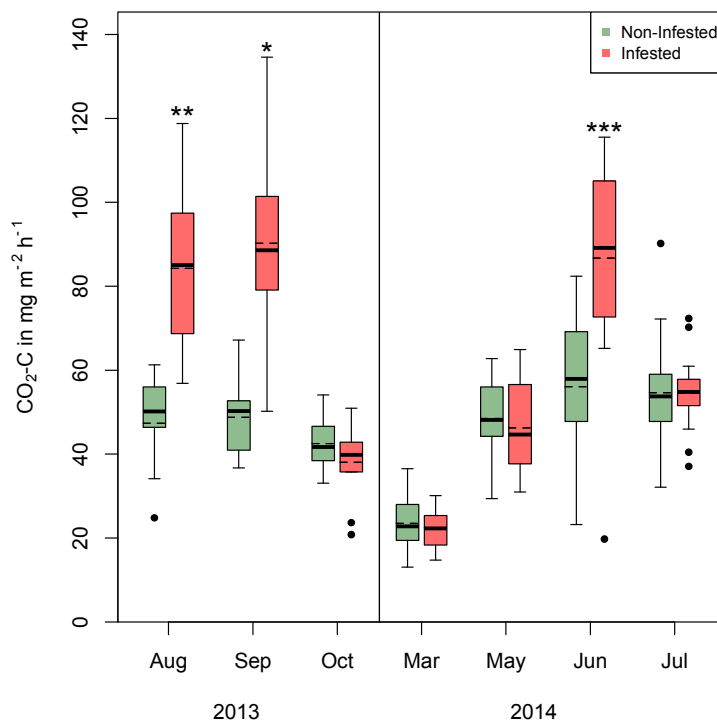


Figure 16: CO₂-C emissions (mg m⁻² h⁻¹) from the mineral soil during an outbreak of the nun moth (*Lymantria monacha* L.) in 2013 and 2014, and the adjacent non-infested control of Scots pine (*Pinus sylvestris* L.) forest sites. Infested = red, non-infested = green, Aug = August, Sep = September, Oct = October, Mar = March, Jun = June, Jul = July. Box plots show means (dotted lines) and medians (solid lines) ($n = 9$ in 2013 and 15 in 2014 for each plot). Whisker extension

equals 1.5x interquartile range distance. Asterisks indicate significant differences between infested and control plots within one sampling time (paired Wilcoxon signed-rank tests, $p \leq 0.050$).

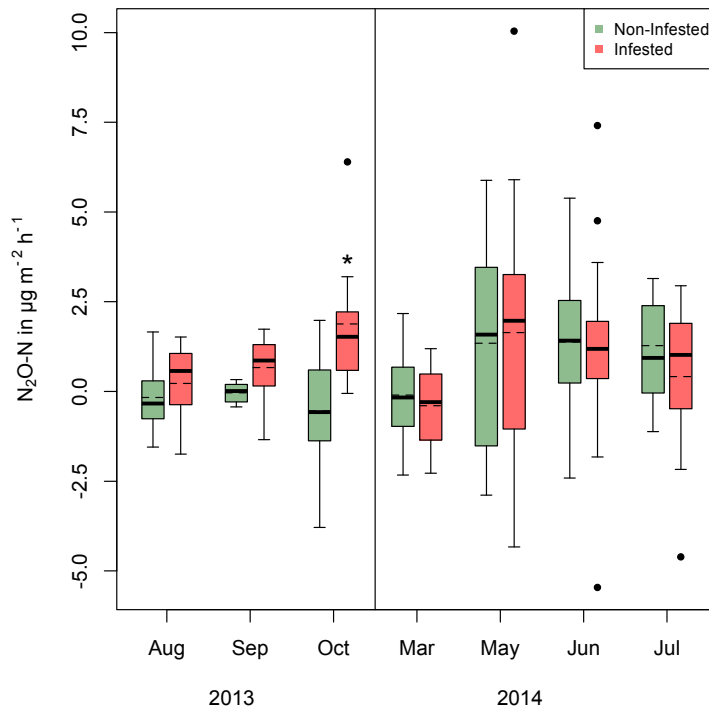


Figure 17: N₂O-N emissions (µg m⁻² h⁻¹) from the mineral soil during an outbreak of the nun moth (*Lymantria monacha* L.) in 2013 and 2014 and the adjacent non-infested control of Scots pine (*Pinus sylvestris* L.) forest sites. Infested = red, non-infested = green, Aug = August, Sep = September, Oct = October, Mar = March, Jun = June, Jul = July; Box plots show means (dotted lines) and medians (solid lines) ($n = 9$ in 2013 and 15 in 2014 for each plot). Whisker extension equals 1.5x interquartile range distance. Asterisks indicate significant differences between infested and control plots within one sampling time (paired Wilcoxon signed-rank tests, $p \leq 0.050$).

3.2. Incubation Experiment

The accumulated carbon and nitrogen fluxes across the 31-day study period were 54.04 ± 1.27 mg CO₂-C h⁻¹ in soil treatments without addition of organic matter (control), 202.11 ± 4.39 mg CO₂-C h⁻¹ in soil treatments with addition of needles, and 574.83 ± 37.85 mg CO₂-C h⁻¹ in soil treatments with addition of needles plus insect feces, and 731.46 ± 7.30 mg CO₂-C h⁻¹ in soil treatments with addition of insect feces (Figure 18). N₂O-N fluxes amounted 0.59 ± 0.34 µg N₂O-N h⁻¹ in soil treatments without addition of organic matter (control), 0.91 ± 0.13 µg N₂O-N h⁻¹ in soil treatments with addition of pine needles, 5.25 ± 0.45 µg N₂O-N h⁻¹ in soil treatments with addition of pine needles plus insect feces, and 6.14 ± 0.27 µg N₂O-N h⁻¹ emissions in soil treatments with addition of insect feces (Figure 19).

Maximum emissions of 10.98 mg CO₂-C h⁻¹ were reached by the insect feces treatment on the fourth day after treatment addition (with 48-fold higher emissions compared with the control). Similarly, maximum N₂O-N fluxes of 0.07 µg N₂O-N h⁻¹ were reached by the feces treatment at the fourth day after treatment addition (with 13-fold higher emissions compared with the control). From that day, fluxes of both gases, decreased slowly with time.

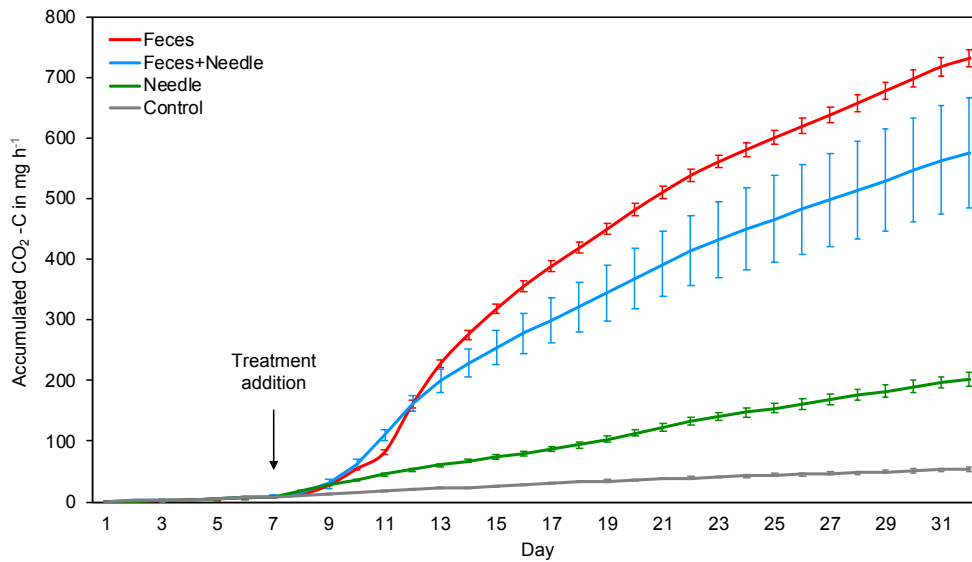


Figure 18: Accumulated CO₂-C flux (mg h⁻¹) of the incubators with treatments of feces from the pine-tree lappet (*Denrolimus pini* L.), feces plus Scots pine (*Pinus Sylvestris* L.) needle litter, needle litter, and a control with soil only during the 31 days of the incubation experiment. Treatments were added on day 7 with n=5. A total of 12 measurements per day were conducted.

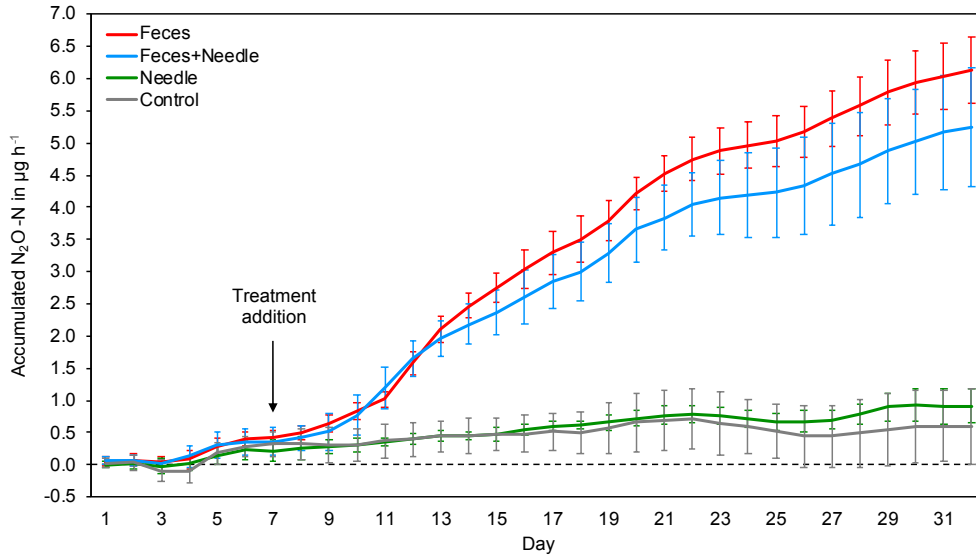


Figure 19: Accumulated N₂O-N flux (µg h⁻¹) of the incubators with treatments of feces from the pine-tree lappet (*Denrolimus pini* L.), feces plus Scots pine (*Pinus Sylvestris* L.) needle litter, needle litter, and a control with soil only during the 31 days of the incubation experiment. Treatments were added on day 7 with n=5. A total of 12 measurements per day were conducted.

Despite similar element contents of needles and feces (see Supplementary Table 15), inputs of feces significantly accelerated soil CO₂-C and N₂O-N fluxes. When compared with the soil treatment without addition of organic matter (control), the experimental inputs of C and N via needles and feces accelerated CO₂-C emissions 3.7-fold in treatments with addition of needles, 10.6-fold in treatments with addition of needles plus feces, 13.5-fold in treatments with addition of feces (all *p*-values < 0.009). N₂O-N emissions were accelerated averagely 8.9-fold in treatments with addition of needles plus feces (*p* < 0.010) and 10.4-fold in treatments with addition of feces (*p* < 0.010), while it was not increased compared to the treatments with addition of needles (*p* = 0.117).

Soil C/N ratio before treatment addition 32.06 in all incubators. At the end of the experiment, C/N ratio of the control almost stayed the same with 32.08, while in the feces treatment C/N increased to 32.23. In contrast, the needle treatment and the needle plus feces treatment decreased to 31.43 and 31.46, which was significant lower compared to the feces treatment (*p* = 0.018 and *p* = 0.024).

4. Discussion

Scots pine forests infested with the nun moth showed increased soil emissions of CO₂-C during several sampling dates and indications of increased N₂O-N emissions at one date, which both may be related to the altered quality and quantity of organic inputs

during the pest outbreak. In the incubation experiment, feces input rapidly accelerated CO₂-C and N₂O-N emissions from soil with up to 14- and 25-fold, respectively, higher fluxes compared to those from needle litter. The increased deposition of organic matter during the defoliation of a pine stand provides large amounts of labile organic C and N, which in turn may positively influence microbial decomposition processes (Christenson et al. 2002). An experiment with feces from *Melanoplus borealis* F. and *Chorthippus curtipennis* F. feeding on different diets demonstrated that up to 46% of the emitted CO₂-C from soil can originate from the added feces (Fielding et al. 2013). Considering that the organic input during nun moth outbreaks can be much higher compared to those from natural (noninfested) conditions (e.g., 300% higher feces and needle litter N input on our study site in 2014, Grüning et al. (2017)), as well as the easily biodegradable structure of feces can be an explanation for the higher CO₂-C emissions from the infested forest site (even following the outbreak when the actual defoliation activity has already ceased). For example, fir forests (*Abies spec.*) defoliated by the siberian moth (*Dendrolimus superans sibiricus* Tschtrk.) showed increased rates of soil respiration even three years after the pest outbreak (Baranchicov et al. 2002). Therefore, increased deposition of organic matter (especially feces) during our nun moth defoliation may have contributed to the enhanced greenhouse gas emissions from forest soils during the outbreak years.

The biogeochemical pathways by which carbon is transformed and moves through forest ecosystems are strongly coupled with those of nitrogen (Janssens et al. 2010). High inputs of labile carbon enhance microbial growth and nitrogen immobilization, while low C inputs rather promote N leaching (Hillstrom et al. 2010). The increased soil C/N ratios of the feces treatment in our incubation experiment may therefore be an indicator of microbial immobilization, and this is supported by the relatively slow decrease of the gas emission rates following peak emissions. In contrast, C/N ratios under field conditions are often observed to decrease during insect outbreaks (Kaukonen et al. 2013; L.-M.-Arnold et al. 2016; Mikkelsen et al. 2017; Brouillard et al. 2017), even on our sampling sites (Grüning et al. 2018).

The availability of organic inputs, microbial activity and emerging greenhouse gas emissions are influenced by soil aeration, fluctuation of the water table, nutrient availability, temperature, and favorable microclimatic conditions (e.g., temperature and precipitation) (Brady and Weil 1996; Sinsabaugh 2010; Jungkunst et al. 2004; Butterbach-Bahl et al. 2013). Our field study was conducted in a continental climate, with (temporally) semi-arid conditions during summer and autumn which can hamper a fast

microbial decomposition (Jung and Lunderstädt 2000; Riek and Stähr 2004). This might have a negative impact on the microbial decomposition of organic matter during pest outbreaks, and explain the relative differences in CO₂-C and N₂O-N emissions between noninfested and infested real forests compared with those from our microcosm experiment under optimized conditions (see also Lovett and Ruesink (1995)). Further, N₂O emissions from forest soils are spatial and temporal highly variable (“hot spots” and “hot moments” of N₂O emissions (Groffman et al. 2009), which makes measurement and comparability across sites difficult (Butterbach-Bahl et al. 2013). However, on our infested study site, the abundance of NO₂⁻ reducers (*nirK* genes) in the soil was also found to be increased (Grüning et al. 2018), indicating a genetic potential for accelerated N₂O emissions. To our knowledge, we show for the first time that both CO₂ and N₂O emissions can be triggered simultaneously by organic inputs deriving from pest insects.

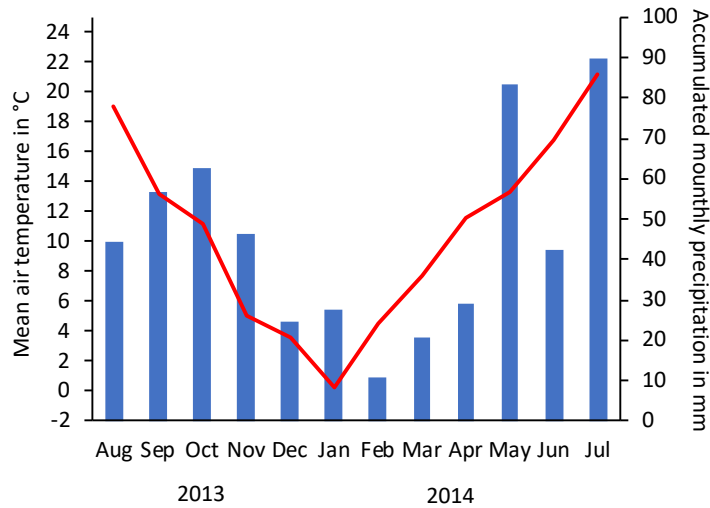
Nitrification and NO₃⁻ losses as well as denitrification and N₂O losses are expected to increase as the fraction of mineralized ammonium increases (Aber et al. 1998). These processes can take place simultaneously in the same soil, e.g., in large, air-ducting pores and inside large soil aggregates, respectively (Stevenson and Cole 1999; Jungkunst et al. 2004; Butterbach-Bahl et al. 2013). Further, accelerated tree growth and increased carbon storage in biomass as well as increased autotrophic respiration from the rhizosphere and decreased heterotrophic respiration from soil microorganisms are consequences of N inputs, thereby contributing to the carbon-sink potential of a forest (Janssens et al. 2010). However, infested forest trees are often physically impaired in their N nutrition, N uptake and reduced in biomass growth rates as consequence of the defoliation (Kosola et al. 2001; Simmons et al. 2004; Grüning et al. 2017). Additionally, our results suggest increased microbial decomposition and CO₂ emissions during insect outbreaks. All this can have the potential to reduce the forests carbon sequestration capacity or even switch the forest to a carbon source (Kurz et al. 2008; Clark et al. 2010; Dymond et al. 2010; Hadden and Grelle 2017).

5. Conclusions

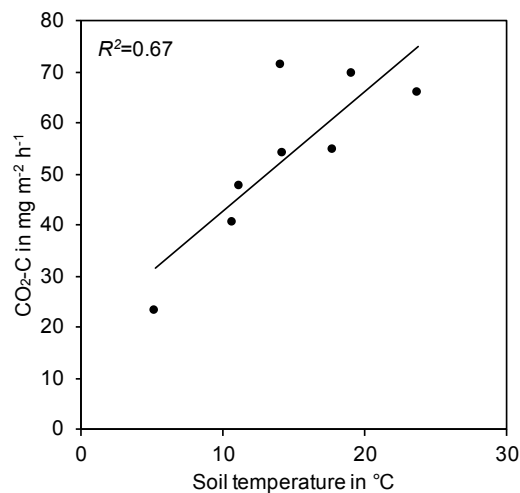
Climate change appears to be a major driver of forest pest outbreaks (Ayres and Lombardero 2000; Dale et al. 2001; Flower and Gonzales-Meler 2015) and most insects are expected to benefit from a high temperature to precipitation quotient (Wenk and Apel 2007; Mitton and Ferrenberg 2012). Our results indicate that organic input during a pest outbreak in a pine forest can trigger considerable CO₂ and N₂O emissions via a transformation of tree biomass into fast decomposable organic matter, which in turn has implications for nutrient cycling and forests functioning as a carbon sink. Future studies

on nitrifying and denitrifying microorganisms under variable conditions are necessary to better understand the effects of insect-derived input and the implications for soil C and N turnover processes.

Supplementary Materials:



Supplementary Figure 20: Monthly average precipitation (mm; blue bars) and mean air temperature (°C; red line) during the sampling period (August 2013–July 2014). Aug = August, Sep = September, Oct = October, Nov = November, Dec = December, Jan = January, Feb = February, Mar = March, Apr = April, Jun = June, Ju = July. Data from DWD (German Federal Meteorological Service and CDC (Climate Data Center, Weather Station Lindenberg (Station ID: 3015), 13.07.2018).



Supplementary Figure 21: Relationship between mean soil CO₂-C emissions (mg m⁻² h⁻¹) and soil temperature (°C of the top 10 cm of soil depth)

Supplementary Table 15: Elemental composition of the mineral soil, pine-tree lappet (*Dendrolimus pini* L.) feces, and Scots pine (*Pinus sylvestris* L.) needle litter used in the incubation experiment.

Element	Soil	Needle	Feces
N (%)	0.46	0.93	0.84
C (%)	13.81	61.04	60.59
Ca (%)	0.05	0.49	0.51
Fe (%)	0.36	0.03	0.02
K (%)	0.05	0.20	0.14
Mg (%)	0.02	0.06	0.05
Mn (%)	<0.01	0.03	0.02
Na (%)	0.02	0.01	0.01
P (%)	0.02	0.05	0.05
S (%)	0.05	0.06	0.05

Samples were dried at 40 °C for 48 h, finely ground and 100 mg material based on three subsamples was extracted under pressure in 2 mL 65% HNO₃ at 180 °C for 10 h.

Author Contributions

Investigation, M.M.G., F.G.; Formal analysis and Visualization, F.G., M.M.G.; Writing – Original Draft Preparation, M.M.G.; Writing – Review & Editing, M.M.G, C.T. and A.I.-M.-A.; Conceptualization and Supervision, A.I.-M.-A.

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Conflicts of Interest

The authors declare no conflict of interest.

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Declaration

I hereby declare, to the best of my knowledge and belief, that this thesis with the title „Effects of insect mass outbreaks on the C and N balance in forest ecosystems” contains no material previously published or written by another person, except where due reference has been made in the text of the thesis. This thesis contains no material which has been accepted or definitely rejected for the award of any other doctoral degree at any university.

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