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Seeing the forest for the trees: Tree species effects on soil microbial communities and nutrient cycling dynamics

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Seeing the forest for the trees: Tree species effects on soil microbial communities and nutrient cycling dynamics

A thesis for a double degree of

Doctor of Philosophy

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Summary

Tree species influence soils above and belowground communities through leaf litter and root inputs. Soil microbial communities can directly influence tree growth and development through processes such as decomposition of leaves, and indirectly through chemical transformation of nutrients in soils as an influence of altered C:N ratios due to leaf litter and root inputs. This thesis aims to document some of the mechanisms by which trees influence soil microbial communities and nitrogen cycling processes like gross and net ammonification and nitrification. This thesis also aims to determine the role of site nitrogen status in modulating those tree species effects. The effects of tree species on ammonification and nitrification rates in forest floors and mineral soils were explored, and related to functional genetic markers for ammonia-oxidation by archaea and bacteria (*amoA* AOA and AOB), bacterial denitrification (*nirS* and *nirK*), and the general markers for bacteria (*16S)* and fungi (*ITS)*. Two paired high-resolution laboratory methods were used to investigate the relationships between trees, soils, and the microbial communities, including molecular techniques such as quantitative polymerase chain reaction (qPCR) to target gene abundances in soils, and 15N pool-dilution experiments to understand how ammonium and nitrate are produced and consumed in soils. Soil samples were collected from two common garden experiments, named EP571 in Canada (Ribbons et al. 2016), and in Denmark, and both 15N and qPCR-based techniques were used to determine tree species effects and attribute N cycling processes to the abundances of functional genes. At EP571, western red cedar *(Thuja plicata*) forest floor nitrogen transformation rates differed from Douglas-fir *(Pseudotsuga menziesii)*, Sitka spruce *(Picea sitchensis)*, and western hemlock *(Tsuga heterophylla)*, which corresponded with western red cedar having highest abundances of bacterial *16S* and *amoA* AOA genes.

A manipulative mesocosm (the Rhizotron) in Wales was used to determine how mixtures and monocultures of seedling species influenced tree growth, soil physical properties and soil microbial community structure and function within the first three years of growth. Within the Rhizotron experiment both alder (*Alnus rubra)* and Douglas-fir grew taller when grown in mixtures compared with monocultures of each species. Sycamore maple (*Acer pseudoplatanus)* acquired slightly greater aboveground biomass when planted in mixtures with common oak (*Quercus robur*), which attained less biomass in mixtures than monocultures. C:N ratios of leaves, stems, roots, and rhizospheric soils were determined to see if mixtures influenced C:N in trees. Rhizospheric soil microbial communities (including bacterial and fungal markers and the 4 genes tied to N cycling) were compared among the 4 tree species in the Rhizotron. Soil samples for microbial analyses were collected before seedlings were planted, and just before the experiment was harvested. These data show differences in height, biomass and C:N ratios between species can be observed at a seedling growth stage, but microbial communities may require longer exposure to develop. Lastly, the Bangor Diverse experiment was used to further explore diversity and mixture effects on soil microbial communities and N transformations. We found few mixture or monoculture tree species effects on mineral soil microbial communities or net nitrification or ammonification rates. Collectively, these stories shed light on the important functional role of soil microbes in forest soil N cycling. This thesis also highlights the use of isotope and microbial techniques for parsing out relationships between site, tree species identity and ecosystem functions, with the largest links observed between gross ammonification and microbial communities.

Danish Resume

Træarter påvirker de mikrobielle samfund i jorden igennem løvfald og input fra rødder. Jordens mikrobielle samfund kan direkte påvirke trævækst og udvikling igennem processer som nedbrydning af blade, samt indirekte igennem kemiske transformationer af næringsstoffer i jorden som en effekt af ændret C:N-forhold pga. input af løvfald. Denne afhandling har til formål at dokumentere nogle af de mekanismer, hvorved træer påvirker jordens mikrobielle samfund og processer i kvælstofkredsløbet som f.eks. brutto- og nettoammonifikation og -nitrifikation. Afhandlingen har bl.a. til formål at bestemme, hvilken rolle stedspecifik kvælstofstatus har på moduleringen af disse træartseffekter. Træarters effekt på ammonifikation- og nitrifikationsrater i det organiske lag og mineraljorden blev undersøgt og relateret til funktionelle genmarkører for ammoniumoxidation af archæa og bakterier (*amoA* AOA og AOB), bakteriel denitrifikation (*nirS* og *nirK*), og de generelle markører for bakterier (*16S)* og svampe (*ITS)*. To metoder blev anvendt til at undersøge sammenhængene mellem træarter, jordbund og de mikrobielle samfund, inklusiv molekylære teknikker som kvantitativ polymerase kædereaktion (qPCR) til at bestemme genforekomst i jord og 15N pulje-fortyndings for at forstå, hvordan ammonium og nitrat bliver produceret og forbrugt i jorden. Jordprøver blev indsamlet fra to træartsforsøg, i Canada og Danmark, hvor 15N og qPCR-baserede teknikker blev brugt til at bestemme træartseffekter på kvælstofskredsløbsprocesser og relatere disse til mængden af funktionelle gener. I Canada var kvælstoftransformationsraterne idet organiske lag for thuja højere end i Douglasgran *(Pseudotsuga menziesii)*, Sitkagran *(Picea sitchensis)* og tsuga *(Tsuga heterophylla)*, hvilket korresponderede med at thuja havde *(Thuja plicata)* den højeste tilstedeværelse af bakterielle *16S* og *amoA* AOA gener.

Et mesokosmosforsøg (rhizotron) i Wales blev brugt til at bestemme, hvordan træartsmonokulturer og -blandinger af påvirkede trævækst, jordens fysiske egenskaber og jordens mikrobielle samfundsstruktur og –funktion i de første tre år efter plantning. I rhizotroneksperimentet voksede både el (*Alnus rubra)* og Douglasgran sig højere ved dyrkning i blandinger sammenlignet med monokulturer af hver art. Ær (*Acer pseudoplatanus.*) havde produceret en lidt større biomasse over jorden når den blev plantet i blanding med almindelig eg (*Quercus robur*), der til gengæld fik en mindre biomasse i blandingerne fremfor i monokulturer. C:N-forholdet for blade, stammer, rødder og rhizosfærejord blev bestemt for at se, om blandinger påvirkede C og N lagring i træer. De mikrobielle samfund i rhizosfærejord (baseret på bakterie- og svampemarkører og de 3 gener relateret til kvælstofkredsløbet) blev sammenlignet iblandt de fire træarter i rhizotronen. Jordprøver til mikrobielle analyser blev indsamlet før træerne blev plantet og igen lige før træerne blev høstet. Disse data viser at forskelle i højde, biomasse og C:N-forhold imellem arterne kan observeres indenfor de første tre år, men at mikrobielle samfund formentlig kræver længere tids eksponering for at udvikles. Til slut blev Bangor Diverse eksperimentet brugt til yderligere at undersøge diversitets- og blandingseffekter på jordens mikrobielle samfund og kvælstofstransformationer. Vi fandt få træartsrelaterede diversitets eller identitetseffekter på mineraljordens mikrobielle samfund, eller på nettonitritifikation- eller nettoammonifikationsrater. Samlet set har afhandlingens enkeltstudier kastet lys på vigtige funktionelle rolle, som jordens mikrobielle samfund spillerr i skovjordes kvælstofskredsløb. Resultaterne fremhæver også brugen af isotop- og mikrobielle teknikker til at analysere sammenhænge imellem lokalitet, træartsidentitet og økosystemfunktioner.

Acknowledgements

This work would not have been possible without the unwavering support, advice, and encouragement of my supervisors Morag McDonald and Lars Vesterdal. Without their efforts, this thesis would still be a twinkle in my eye, rather than the full document you readers now have in your hands. I cannot express my gratitude enough, and will continue to be grateful for the time they have invested in me over the years.

I relied on a supervisory team including Cindy Prescott, Sue Grayston, Andy Smith, and John Healey. Cindy and Sue guided me through my first field season, and provided me with my first exposure to soil microbial and isotopic research techniques. Andy and John were helpful in the design of the Rhizotron project quite early on in my Ph.D. career. This PhD and research would not have been possible without the generous financial support from the European Commission through the FONASO Joint Doctorate Programme.

The Rhizotron work was also lovingly supported by Nigel Brown and Natalie Chivers, the curators of Treborth Botanic Gardens (the permanent home to the Rhizotron itself), as well as the Friends of Treborth community. I am so grateful to have been welcomed into your community at Bangor.

Thank you to my colleagues and fellow PhD students, who cheered me on along the way, and helped me puzzle through the difficult moments over cups of tea. Thank you to Mette for assisting with the Danish summary translation. Thank you to Karen, Hannah, and the Tyn Y Ffridd family who made living in Bangor a delight. Thank you to Chelsea and Michael for the laughter we shared, and the many thoughtful conversations over cheese. Thank you to Joe Panci for starting me down this path over a decade ago, and to Hannah Panci for keeping me laughing along the way. Thank you to Allison, Deanna, Connie, Corynn, and the many other Appleton friends for your support right at the end of this dissertation journey. Thank you also to the many communities including LURun4Fun that helped me balance work with another passion of my life: running.

Of course, all of this work is possible in part thanks to my mother, Joan. For obvious biological reasons I wouldn't be here today without her, but her foresight and encouragement throughout my whole life is one of the greatest gifts she gave to me. My passion and enthusiasm for life and learning is clearly something I inherited from her. My siblings also deserve their fair share of credit for my influential childhood antics shaped my personal resilience and tenacity, and for this I thank my brother Ted, and sisters Elyse, Jenna, and Hannah.

My deepest thanks go to my partner in science and in life: Israel. You have braved this adventure with grace, compassion, strength, and perseverance at every step of the Ph.D. process. From digging up the Rhizotron, fieldwork in Vancouver Island, to grinding samples at Henfaes, your contributions to my Ph.D. in terms of physical labour were above and beyond. Your ability to make me laugh even when Murphy's Law has taken over is consistently impressive.

CONTENTS

LIST OF TABLES

LIST OF FIGURES

Chapter I. Introduction

1.1 Benefits of forest biodiversity

"Forests are the lungs of the planet" is often quoted, and scientifically somewhat substantiated in terms of global carbon dynamics, but lingering questions remain about the role of individual species identities in refining this relationship with another essential element of life: nitrogen. The overarching aim of this dissertation is to examine the relationships between the role of tree species identity on soils, ecosystem functions, and microbial communities. From an ecosystem function perspective, what difference does it make to have a forest full of common oak (*Quercus robur)* or Douglas-fir (*Pseudotsuga meziesii)*? Which aspects of these species identities influence ecosystem functioning, and can they be characterized by the functional traits these species possess? Under what conditions are tree species influenced the most pronounced? To address these questions, and the many cascading questions that are revealed in an effort to get to the essence of what matters, one first requires a thorough understanding of the fundamentals of ecosystem functions, as well as the common parlance and terminology used in ecological research.

There are numerous benefits and reasons to focus on the selection of proper tree species and tree species diversity effects (Cardinale et al. 2007). One prominent example of a benefit is increasing biodiversity as a means to increase resilience to the numerous negative effects of climate change (Pachauri et al. 2014), and disturbances more broadly (Gazol and Camarero 2016). These include changes in precipitation and drought-stress, which is relevant for forest ecosystems as water-stress could reduce tree growth (Pretzsch and Dieler 2011). One study in pure and mixed stands of Norway spruce, sessile oak, and European beech (*Fagus sylvatica* L), found evidence to suggest tree resistance and resilience to drought could be modified directly through species mixtures in Europe (Pretzschet al. 2012). Norway spruce stands had the lowest drought-resistance, but fastest recovery time whereas oak and beech were more drought-resistant and slower to recover after drought-stress. For both Norway spruce and oak in mixtures the trees performed similarly to those in monocultures, whereas when in planted in mixtures beech trees increased in resilience and resistance. Their findings in relation to drought-tolerance were consistent with previous studies on each individual tree species (Zang et al. 2012), but what

made the Pretzsch et al. (2012) study compelling was the documented mixture effects that could be teased apart between those three tree species.

1.2 Tree species effects on ecosystem processes

Recently, Forrester and Pretzsch (2015) set out to determine how, when, and where tree species diversity influences ecosystem functions and ecosystem services, as well as the magnitude for these effects in certain species combinations. They outlined key approaches for designing tree species mixture studies, and evaluated evidence in forest mixture studies already established, and highlighted the need to pay attention to stand density and stock levels across experiments. Amoroso and Turnblom's (2011) study on productivity in pure and mixed Douglas-fir and western hemlock plantations demonstrated that stand density levels influence overall productivity. They found monocultures of Douglas-fir were more productive and attained greater heights and diameters than western hemlocks across all stand density levels. Only when planted at the highest stand density were the mixtures and Douglas-fir monocultures equally productive, which Amoroso and Turnblom (2011) attributed to complementary site resource use and reduced intraspecific competition that would have been high in the monocultures.

Binkley and Giardina (1998) proposed three hypotheses for how to categorize tree species influences on soils, i.e. the tight-weave, loose-weave, and frayed hypotheses. The tight-weave hypothesis suggests there is a positive feedback from tree species to soils, leading to increased tree species fitness. For example, this could be achieved if trees increased the supply of a limiting resource (such as a nutrient, soil water, etc.), or conversely decreased the supply of other resources for competitor trees (e.g. reduced light). Another mechanism for the tight-weave strategy might employ decreased supply of resources to both the tree and its competitors beyond a competitor's ability to survive or a certain threshold. A real world example would be hemlock (*Tsuga canadensis)* and sugar maple (*Acer saccharum)* forests in Sylvania Woods, Michigan (Frelich et al. 1993). Hemlock reproduction is apparently inhibited on sugar maple dominated sites as a result of the physical factors of a maple forest floor. On the flip side, sugar maple reproduction in hemlock dominated sites is also inhibited by a low N supply characteristic of eastern hemlock forests.

Alternatively, they suggest the loose-weave hypothesis may hold true for other species (Binkley and Giardina 1998). The loose-weave hypothesis can be characterized by secondary effects of other fitness traits, including rapid growth, anti-herbivory, or drought tolerance. While this hypothesis cannot be explicitly connected to this dissertation research, it is likely that loose-weave connections exist in these same research forests. As a result, trees may be influencing soils in a way that may or may not benefit trees or their competitors. One example of the loose-weave hypothesis is nutrient removal from soils into tree biomass, which would reduce soil fertility but increase growth over a short time period, which would culminate in increased tree fitness overall. A real world example would be tissues with high concentrations of tannins, which discourage feeding by herbivores, which would in turn alter decomposition rates. Selective herbivory would perhaps slow decomposition as leaf litter that has high C:N ratios would accumulate (Bryant et al. 1989).

The third proposed connection is called frayed, which is characterized by indirect effects of processes not under direct selection pressures, such as food quality of leaf litter for worms, fungi, and bacteria (Binkley and Giardina 1998). The frayed hypothesis suggests that not all interactions between organisms and ecosystems are direct or indirect. For example, trees may have direct effects on soils, but those are often mediated by soil organisms, including the vast microbial community, all of which must act in concert in order for a tree's strategy to work. In Minnesota, Kienzler et al. (1986) reported 30% less aboveground litterfall in aspen vs. red pine or white spruce stands, but ten times more bacteria and twice the fungal biomass of red pine or white spruce. These findings were based on experimental plantations of aspen (*Populus tremuloides)*, red pine (*Pinus resinosa)*, and white spruce (*Picea glauca*). They also found greater abundance of worms, beetles, larvae, springtails, and arachnids within the aspen stand. This leaves the reader wondering whether the soil was uniquely adapted for aspen compared with red pine or white spruce, or whether the soil microbial community was opportunistically responding to the different litter inputs of aspen. Binkley and Giardina (1998) highlighted that "the role of soil communities in mediating or altering the effects of trees on soils needs much more investigation" which is the chief objective in this dissertation.

1.3 Research Questions

My research focused on forests at varying stages in development, with some experiments conducted in forests that are 40+ years in age (EP571 and the Danish tree species trials), at the initial development stage (first few years of seedling and sapling growth in the Rhizotron), to the stage of a young forest with canopy closure (Bangor Diverse). Are there consistent tree species effects, or do underlying site nutrient status play a more dominant role on ecosystem functioning? See Chapter 3 for a case study example and find out. Is former land-use history and soil pH a controlling factor in mediating tree species effects? Chapter 4 aimed to answer this questions with targeted research in the Danish tree species trials. Furthermore, if trees do influence nitrogen cycling, is that nitrogen then recycled into neighbouring trees, even at a young stand age? The Rhizotron experiment, more fully explained in chapter 6, was partially designed to address this same question. Do we see differences in ecosystem functions like nitrogen cycling in young forest stands, and corresponding differences in soil microbial communities in the bulk soil after a decade of development? Chapter 7 will address what was found in the Bangor Diverse experiment.

Do tree species influence soil N transformations?

A major theme in my Ph.D. research has been the exploration of tree species effects on nitrogen dynamics in soils. This started with a standard month long incubation for net mineralization and nitrification rates, which is a useful approach for determining tree species effects on ammonium and nitrate availability. A further refinement was the addition of 15N isotope based research techniques, which required great precision and consistency in fertilizer application and the resulting soil extractions. These techniques were especially useful to illuminate on the specific production and consumption of nitrate and ammonium within the tree species experiments, and these data were used to extrapolate on the tree species mediated effects on nitrate and ammonia cycling partitioned as separate forms of nitrogen. These aspects of my research are novel not only in their contribution of these datasets but also in their paired application with functional genetic markers, to examine connections with microbial communities.

Are there links between tree species, soil microbial communities, and N cycling?

Another major theme throughout my research is to determine the links between microbial communities and ecosystem processes. A suite of techniques were used, and subsequently refined, during my Ph.D. to assess microbial community abundances and characterize the microbial community. Techniques ranged throughout this thesis, and began with the well-established chloroform-fumigation extractions to determine microbial biomass C and N. More detailed and precise assessments of microbial communities were then included, and the focus shifted towards the use of qPCR as the main technique used. Within the realm of qPCR applications, the process of determining total abundances of various target genes was an important development. This process involves an additional calibration of standard curves for each target gene, and each individual qPCR run, all of which needed to be rebuilt in each of the university laboratories where qPCR samples were processed.

The use and adaptation of functional genetic markers was coupled with 15N isotope pool dilution experiments, which showed gross rates of production and consumption of nitrate and ammonium. Together, these methods allow for deeper insights into how quickly microbial communities may shift under different forest canopies. These paired datasets can also inform whether tree species effects on N transformation rates are mirrored in shifts in soil characteristics like pH or C:N ratios, or more specifically mirrored within general fungi:bacteria ratios or functional gene abundances tied to N cycling.

1.4 Research objectives and hypotheses

The research objectives of this thesis were fourfold:

1) Identify the magnitude and influence of four tree species on gross forest floor N transformation processes and abundance of functional genes across two sites in British Columbia with different N availabilities.
2) Examine the influence of six tree species on gross mineral soil N transformation processes and calculate the abundance of functional genes as they extend along a pH and soil fertility gradient of three sites in Denmark.

3) Determine whether rhizospheric soil microbial communities and whole tree C and N concentrations, and N stocks differ between four seedlings tree species grown in pure and mixed-species mini-forests in Bangor.
4) Document whether ecosystem process rates and mineral soil microbial communities differ among 3 tree species and their mixtures in a tree species diversity experiment in Bangor.

I hypothesized that:

1) Tree species identity would influence soil microbial gene abundances, which would differ among tree species planted on the same site.
2) Tree species identity would influence N transformation rates, and these would differ among sites with different land-use histories.
3) Rhizospheric soils would respond differentially to tree species when grown in pure or mixed species settings, possibly dependent on different functional traits possessed by those species.
4) Tree species mixtures would result in non-additive or synergistic effects on forest productivity (height, biomass, etc.), and soil microbial communities.

1.5 Thesis structure

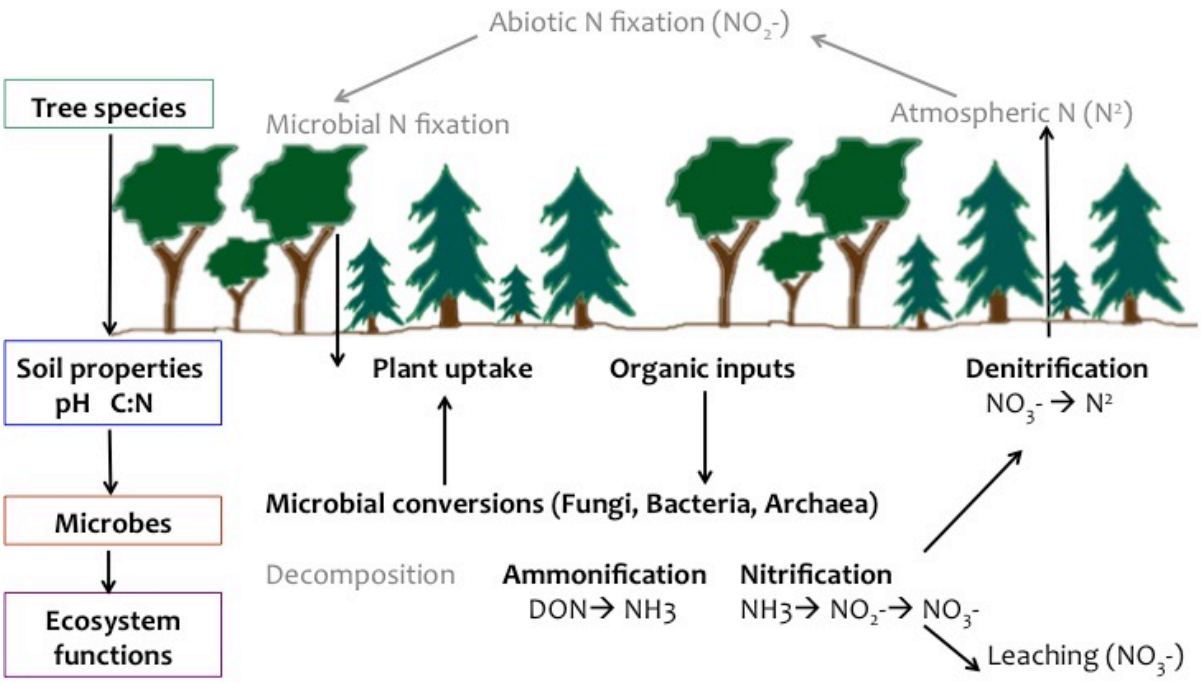

Figure 1. Conceptual diagram outlining the major research foci in coloured boxes, overlaid onto the major ecosystem process examined in this thesis- nitrogen cycling in contrasting forest species.

In this chapter, the importance of tree species on nitrogen cycling, and the use of molecular tools like functional genetics for the exploration of ecosystem processes including nitrogen cycling, and general characterization of soil microbial communities is introduced. This thesis aims to explore the relationships between tree species, soil properties, microbial communities, and ecosystem functions (Figure 1). This dissertation is arranged into four data chapters, each of which is intended for publication as its own paper. This introduction is followed by **Chapter 2** which is excerpted from a book volume co-authored on soil biological communities and ecosystem resilience, in press for publication in 2017. This chapter serves as a brief literature review and methodological overview, and functions as a prelude to the first of the data chapters (Chapter 3). The main data chapters are then presented, and are arranged chronologically with respect to my tenure as a Ph.D. candidate.

The first summer's fieldwork in 2014 was spent at the University of British Columbia-Vancouver, where work was undertaken as a member of the belowground ecosystem group led by Dr. Cindy Prescott and Dr. Sue Grayston. An experiment was designed to return to a long-term common garden forestry experiment Dr. Prescott had worked in extensively, called EP571. Previous work confirmed that some tree species have influenced soil N transformations rates and phospholipid fatty acid analysis (PLFA) which determined soil microbial biomarkers (Prescott and Grayston, 2005). The question remains whether differences in N cycling rates are mirrored by the abundance of relevant functional genes. This was the primary objective of **Chapter 3**, which was addressed using the ^{15}N isotope pool-dilution method, and targeted qPCR runs to examine total fungal and bacterial gene abundances as well as a suite of four functional genes**.** During this time at the University of British Columbia-Vancouver, the fundamentals of the ^{15}N pool-dilution method and microbiological techniques were tested, which served as a stepping-stone for further isotope- and genetics-based research efforts in Denmark and the United Kingdom. The British Columbia soil samples which originated from the thick matted forest floor material found under the four coniferous tree species: Douglas-fir, Sitka spruce, western hemlock (*Tsuga heterphylla*), and western red cedar (*Thuja plicata*). This chapter has subsequently been published in 2016 in the journal Soil Biology and Biochemistry.

Chapter 4 was intended to expand upon the scope of tree species examined in the EP571 experiment, to include 5 broadleaf trees (European beech, pedunculate oak (*Quercus robur* L.), lime (*Tilia cordata* L), sycamore maple (*Acer pseudoplatanus* L), and ash (*Fraxinus excelsior* L) and one coniferous temperate tree species (Norway spruce (*Picea abies* (L) Karst.)), again grown in a long-term common garden forest experiment in Denmark. The chief research objective of this chapter was to explore whether tree species effects exist for these broadleaf species, in terms of their effects on gross N transformation rates. An Additional aim was to determine whether or not these effects were consistent across sites along a pH gradient and differing land use histories. The final aim was to link N transformation rates and tree species influences on mineral soil microbial communities together. During this time period the standard 15N pool-dilution protocols were refined to accommodate to the soil material used and research equipment available at the University of Copenhagen. Some of the techniques and approaches used for Chapter 3 were also modified to stay current with recent advances in microbiology research, such as the use of new primer pairs for qPCR runs. The statistical approaches in this study were further refined to accommodate the different experimental design of this common garden vs. the EP571 project. Chapter 4 is in preparation for peer-review at New Phytologist.

Chapter 5 reports the experiments from the Bangor University Rhizotron. These experiments were planted with four contrasting tree species: Douglas-fir, red alder, common oak, and sycamore maple. These species were selected due to their differing functional traits, from broad biological characteristics like coniferous (Douglas-fir) vs. broadleaf, to differences in mycorrhizal associates (arbuscular vs. ectomycorrhizae in the sycamore maple and the oak) and symbiotic relationships with nitrogen fixing bacteria (found only on the alder). Each of these trees characteristically possesses differences in leaf traits as well, including decomposability and C and N concentrations. Trees were planted in single-species settings, and naturally occurring two-species mixtures (red alder paired with Douglas-fir; sycamore maple paired with common oak). We aimed to assess the development of pure and mixed species tree seedlings and their resulting influences on soil development, nutrient cycling, and rhizospheric soil microbial communities. After 2.5 years of growth the experiment was harvested and subsets of plant tissues and rhizospheric soils were used to assess C and N concentrations, and determine potential additive or mixture effects from the tree species treatments. Rhizospheric soil microbial

communities were assessed using the fungal *ITS*, bacterial *16S*, and nitrogen cycling functional genes (*nirK, nirS, amoA* AOA, and *amoA* AOB). Chapter 5 is in preparation for peer-review submission to the Journal of Ecology.

The final data chapter, **Chapter 6,** further expands on the question of how forest biodiversity influences forest ecosystem functioning. This project was designed to focus on the Bangor Diverse experiment, and uses this framework to explore the effects of tree species mixtures using a fully factorial design. The Bangor Diverse experiment was established just over a decade prior, and is a good mid-point for forest development between the establishment phase focus of the Rhizotron, and the long-term 60 and 40 year old experiments of EP571 and the Danish tree species trials, respectively. For this experiment the mineral soils of pure alder, birch, and beech stands, as well as the mixtures of each of these paired species and a full mixture of all three species were sampled. These soils were used to experimentally test for differences in N transformation rates using a different suite of protocols from previous experiments, which were conducted by my collaborator- Anna Gunina. qPCR analyses were completed to determine differences in mineral soil microbial community abundances and functions, using the general fungal *ITS* and bacterial *16S* markers and the same 4 target functional genes as in my previous works (*nirK, nirS, amoA* AOA, and *amoA* AOB). Chapter 6 is in preparation for a short communication in the journal Plant and Soil Fertility.

This thesis concludes with **Chapter 7**, which contains a discussion of the major themes across the research completed over the past three years. Firstly, tree species effects on soil microbial communities as examined through the use of qPCR. Secondly, tree species effects on nitrogen transformation rates, and the modulating factors of soil properties like pH and site nitrogen status. Thirdly, the effects, or lack thereof, of tree species identity on soil microbial communities broadly using bacterial and fungal genetic markers were then discussed. After which, the use of functional genetic markers tied to nitrogen cycling, such as the nitrifying and denitrifying microbes, and their response to different forest cover types was explored. This thesis closes with a discussion of the contributions of this body of work to the collective knowledge about forest ecosystem functioning, and knowledge gaps filled and discovered throughout this Ph.D.

1.6 Authorship

All chapters in this thesis have been prepared as manuscripts for peer review journals, many of which have co-authors. Throughout this thesis I use the pronoun 'we' rather than 'I' as a result of the manuscript preparation process. However, the input from my co-authors was limited to supervisory support and advice, and commenting on drafts. At the time of submission, Chapter 3 has been published, Chapter 2 has been accepted for publication. Chapters 4, 5, and 6 are each in preparation for publication. Below the contribution of the co-authors to each chapter is outlined.

Chapter 2: this chapter is an excerpt form a Book chapter accepted for publication as: Ribbons, R.R., McDonald, M.A., and Vesterdal, L. (In press) "Microbial communities, functional genes, and nitrogen cycling processes as affected by tree species" in Soil biological communities and ecosystem resilience. Ed. M. Lukac, P. Grenni, and M. Gamboni. I wrote this chapter with advice from Lars Vesterdal and Morag McDonald. A case study used in this chapter briefly summarizes work published as Chapter 3 (see below for further details).

Chapter 3: this chapter has been published as: Ribbons, R.R., Levy-Booth, D., Masse, J., Grayston, S.J., McDonald, M.A., Vesterdal, L., and Prescott, C.E. (2016) Linking microbial communities, functional genes and nitrogen-cycling processes in forest floors under four tree species. *Soil Biology and Biochemistry*.

I conceived the study design with Cindy Prescott, Lars Vesterdal, and Morag McDonald. I collected and analysed the data, with the guidance of David Levy-Booth. I wrote the paper with advice form Cindy Prescott, Lars Vesterdal, and Morag McDonald, and all co-authors commented on the draft of this paper.

Chapter 4: This chapter will be submitted to peer-review as: Ribbons, R.R., Kosawang, C., Ambus, P., McDonald, M.A., Grayston, S., Prescott, C.E., and Vesterdal, L. Broadleaf tree species effects on nitrogen cycling and soil microbial communities.

I conceived the study design with Lars Vesterdal, Morag McDonald, and Cindy Prescott. I collected and analysed the data. I wrote the paper with advice form Lars Vesterdal, Morag McDonald, and Cindy Prescott, and all co-authors commented on the draft of this paper.

Chapter 5: This chapter will be submitted to peer-review as: Ribbons, R.R., Del Toro, I., Smith, A.R., Healey, J.R., Vesterdal, L., and McDonald, M.A. A serenade for the role of humble roots: experimentally testing tree species effects on rhizosphere soil microbial communities.
I conceived the study design with Israel Del Toro, Lars Vesterdal, Andy Smith, John Healey, and Morag McDonald. I collected and analysed the data with assistance from Israel Del Toro. I wrote the paper with advice form Lars Vesterdal, John Healey, Andy Smith, and Morag McDonald, and all co-authors commented on the draft of this paper.

Chapter 6: This chapter will be submitted to peer-review as: Ribbons, R.R., Gunina, A., Smith, A.R., Vesterdal, L., and McDonald, M., Kuzyakov,Y., and Jones, D. Soil microbial communities are slow to respond one decade after afforestation in Bangor Diverse experiment.

This study was designed along with Anna Gunina and Davey Jones. Data from experiments were collected by myself and Anna Gunina, and analysed by myself. The paper was written with advice from all co-authors.

Chapter II. Microbial communities, functional genes, and nitrogen cycling processes as affected by tree species

1. Introduction

Global changes, such as increased temperature and the spread of invasive species, are leading to large transitions in both the structure and function of ecosystems (Vitousek et al. 1997, Chapin et al. 2000, Ribbons 2014). A major question in ecology is: how will global change influence community structure and ecosystem function? Forests serve as important buffers for climate change through processes such as carbon sequestration, are significant sources of primary productivity, and provide a variety of major ecosystem services. There is uncertainty in how global change will influence forests, which could lead to declines in primary productivity, and reductions in ecosystem function. How forests will respond to global change is important to study at both a community and ecosystem level, because changes in forest structure and composition have large influences on ecosystem functions. This chapter aimed to address the following three questions: 1) How do soil microbial communities differ between tree species grown on the same forest sites? 2) How do soil microbial communities and functional genes for nitrogen cycling differ between tree species? 3) How do gross rates of nitrogen cycling vary under different tree species grown on the same sites, and do these effects differ when trees are grown in nitrogen-rich and nitrogen-poor environments?

Plant soil-feedbacks play an important role in regulating nutrient cycling and ecosystem processes (Bardgett 2011), with important implications for ecosystem responses to climate change and mitigation. Diversity within a community has been widely recognized as a factor that renders ecosystems resilient to perturbation (Loreau et al. 2001), but the mechanisms of how these processes work are less well understood, especially when scaling to soil and ecosystem nutrient cycling (Van der Putten et al. 2010). Understanding how forests are responding to shifts in species composition, and resulting shifts in ecosystem processes, is crucial to planning global climate change mitigation strategies.

A foundational concept within ecology is the role of species diversity and richness on community dynamics and ecosystem function (Huston 1979; McCann 2000). More recently, the field of functional trait diversity has blossomed, such that large databases on functional traits and characteristics at the species-level are now commonplace (see TRY database, Kattge et al. 2011). Functional traits are often used to categorize plants based on the characteristics that are considered relevant to their response to the environment and/or its effects on ecosystem functioning (Diaz and Cabido 2001). Recently, research discussing plant functional traits has increased in the ecological literature (Diaz and Cabido 2001, Tilman et al. 1998), due to their applicability in management and climate change mitigation. For example, plants which naturally form symbiotic relationships with root-nodules are able to fix atmospheric nitrogen, and are thus categorized as having the functional trait of nitrogen-fixation. Other examples include physical features such as leaf size, canopy height, and ability to re-sprout. The diversity-stability theory in ecology states that increased diversity leads to increased ecosystem stability (Elton 1958, Tilman et al. 1998) in terms of maintaining ecosystem functions. However, using the litter of 32 species of contrasting litter quality, and categorized into four functional groups, Wardle et al. (1997) found that increasing species richness had no effect on an ecosystem function such as the rate of decomposition.

Functional diversity within an ecosystem can also increase ecosystem stability by providing functional redundancy (e.g. several plants with identical functional traits). Functional diversity may play a more important role than species richness in maintaining ecosystem stability. For example, Isbell et al. (2011) found that high plant diversity maintained ecosystem function over time, and that functional redundancy is required within systems to adapt to a changing planet. Tree species have different functional traits and occupy different functional niches, suggesting that certain combinations of species can enhance specific ecosystem functions, and increase ecosystem resilience to changing climatic conditions. The capacity for different tree species to foster functionally distinct soil microbial communities requires further exploration, but a wealth of evidence and data support the role of plant-soil feedbacks in shaping community structure and ecosystem function.

1.1 Plant-Soil Feedbacks

Plant-soil feedbacks, such as biotic interactions, play a large role in how combinations of plants will function. Some species have well-documented negative plant-soil feedbacks, such as *Prunus serotina* (black cherry), which creates a soil microbial community containing pathogens that specifically inhibits the growth of its own seedlings in the direct vicinity of the parent tree (Reinhart et al. 2003, Reinhart et al. 2010), an example of the Janzen-Connell effect (Janzen 1970; Connell 1971). Other species have positive plant-soil feedbacks, which is especially important when the species in question is an invasive species (Levine et al. 2006), such as *Ailanthus altissima* (Tree of Heaven) which alters soil communities resulting in the suppression of competitor species (Felker-Quinn et al. 2011). By combining specific life history traits, functional traits, and known feedbacks we can better predict how combinations of tree species may persist and maintain high levels of forest productivity over time, thus yielding species of interest for influencing soil nutrient dynamics by fixing abiotic N or increasing soil N availability.

It has long been known that sites with different soils will support plant communities differing in species composition with species selected based on differences in environmental conditions (Binkley and Giardina 1998). Research suggests that there are tight couplings between species and the soils they are grown on, whereby a species may achieve different growth forms, biomass, and nutrient cycling when grown under a range of environmental conditions, such as in monoculture or polyculture (Bardgett 2010, Harrison and Bardgett 2010, Orwin et al. 2010). There is growing evidence that plant species can alter the soil through their litter chemistry, and exudation or turnover of root and mycorrhizal symbionts (Bardgett 2010), with recent experiments exploring additional soil properties (Prescott and Vesterdal 2013). Soil microbial communities (bacteria and fungi) play critical roles in cycling nutrients and decomposing litter. For example, mycorrhizal infection can have a positive effect on tree growth, as hyphae improve nutrient acquisition of tree roots by enhancing root surface area in exchange for carbohydrates. The next step is to consider how sites with different soils are coupled with plant-mediated alterations in these soil communities. How will this coupled system respond to changes in species composition (such as the removal or addition of species) due to climatic change or forest management practices?

Interactions between plants and soils are widely recognized as important for ecosystem functions. However, species-specific interactions and their effects on ecosystem functions such as nutrient cycling and decomposition remain poorly understood, and results are mixed. Plant diversity positively influenced soil carbon storage in grasslands (Steinbeiss et al. 2008, De Deyn et al. 2011), peatlands (Ward et al. 2009), forests (De Deyn et al. 2008), and drylands around the globe (Maestre et al. 2012); while plant diversity did not influence carbon storage in New Zealand forests altered by mammal browsing (Wardle et al. 2001), had a modest positive effects in deeper soil layers of temperate forests of Poland (Dawud et al. 2016), and increased diversity had a negative effect on soil carbon storage in forests invaded by lianas (Phillips et al. 2002). Further research is needed to address how species diversity across trophic levels in conjunction with abiotic factors influence ecosystem functions (Midgley 2012). Plant functional composition (e.g. conifer/broadleaves) can play an important role in determining how ecosystems cycle nutrients, as has been demonstrated for carbon and nitrogen accumulation in grasslands (Fornara and Tilman 2008) and forests (Dawud et al. 2016).

Research on plant effects on soil properties has increased recently, due to an increased interest in determining the role of plants in regulating carbon to mitigate climatic change, for example by sequestering atmospheric carbon dioxide. Much of this research on specific examples of above- and belowground linkages in forest and grassland ecosystems has been thoroughly examined and synthesized in Bardgett and Wardle (2010). In addition, a plethora of studies have examined the role of plant species diversity on forest soil properties (Laakso and Setala 1999, Small and McCarthy 2005, Blaško et al. 2015). Understanding the linkages between above- and belowground components within natural systems is an important challenge for predicting climate change effects on ecosystems (Bardgett and Wardle 2010). Examining multiple taxa and functional groups in both the above- and belowground communities will provide insights into how species may respond to climate change and yield practical and applied information.

Exploring belowground responses of soil communities or tree root growth to inter- and intra-specific competition between trees planted in forests has implications for climate change mitigation and carbon sequestration. Soil communities have been found to develop tight relationships with the litter they decompose, across many biomes (Keiser et al. 2011, Makkonen

et al. 2012). Soil communities often preferentially decompose the litter of plants that have been growing in that soil compared with foreign litter (from plants grown at other locations), suggesting a soil legacy which influences carbon and nitrogen dynamics (Carrillo et al. 2012), commonly referred to as the "home-field advantage" (Vivanco and Austin, 2008; Veen et al. 2014), although its generality is still debated. Alternatively, plants can be exerting top-down control over the soil community, effectively encouraging a community that is successful at decomposing litter of a certain species. Plant-soil feedbacks can play an important role in the cycling of nitrogen and carbon. In this review we aim to shed some light on the question of how will changes in the forest species composition and soil type influence ecosystem functions such as nitrogen cycling in the future.

2. Tree Species Common Garden Experiments

Trees tend to grow on different sites preferentially. For example, pines can generally tolerate sandy nutrient-poor soils and water-limited conditions, whereas ash or maple are more generally suited to richer soil substrates and less water-stressed conditions (Binkley and Fisher 2013). This seriously hampers separation of tree species and site-related factors on soil and ecosystem functioning (Vesterdal et al. 2013). However, experimental forests can provide an excellent research framework for examining tree species identity effects. Common garden experiments are especially pertinent for examining tree species identity or diversity effects while reducing the influence of confounding factors (such as topography, soil characteristics, hydrology) to a minimum.

An additional series of new common garden experiments have recently been established across Europe to address effects of species diversity (Scherer-Lorenzen et al., 2007), but studies of soil functioning will remain for the future when these experiments have matured. Instead, a targeted well-selected exploratory platform has recently been established and used to study effects of tree species diversity in mature forests (see Baeten et al. 2013). In this review we will focus on single-tree species effects on soil microbial communities and ecosystem functioning within a common garden experiment on Vancouver Island.

The University of British Columbia has conducted several studies in the species trial Experimental Project no. 571 (hereafter referred to as EP571) established by the B.C. Ministry of Forests, Research Branch. The trial is located on Vancouver Island in British Columbia, Canada, and includes four tree species: western redcedar (*Thuja plicata*), western hemlock (*Tsuga heterophylla)*, Douglas-fir (*Pseudotsuga menziesii),* and Sitka spruce (*Picea sitchensis)* established in 1961 (Klinka et al. 1996, Prescott et al. 2000)*,* at four sites with contrasting N status, two of which were used for the recent work by Ribbons et al. (2016). These two sites include Fairy Lake which has low N and high C:N ratio in the forest floors, and San Juan which has high N and low C:N ratio in the forest floors. Rates of net nitrogen mineralization in laboratory incubations have previously been assessed at the EP571 experimental forest (Prescott et al. 2000), in addition to PLFA analyses on microbial communities (Grayston and Prescott 2005). In contrast, gross quantification of nitrogen mineralization and nitrification rates was only recently explored in addition to the quantification of forest floor microbial communities and functional genes responsible for primary cycling of nitrogen within these forests (Ribbons et al. 2016). The following case study, presented in the next chapter, illustrates how multiple mechanism-based experiments can yield new insights into ecosystem function.

3. Experimental Considerations

Isotope-based methods, such as tracer experiments, as well as natural abundance, and pool-dilution experiments are examples of tools best applied to research questions that require very precise datasets due to the specialized training for implementing and high cost of analyzing those samples (see Murphy et al. (1999) review for a further discussion on these experiments and Booth et al. 2005). With the EP571 project (see Chapter II) we designed our study to use ^{15}N pool-dilution experiments to acquire data on the real-time production and consumption of nitrogen in the form of either ammonium of nitrate. This allows us to determine gross process rates and associated information that is typically aggregated in traditional net N mineralization incubations. To collect meaningful data, sample replicates within the experiment, and technical replicates to ensure analytical quality are needed. Furthermore, alternatives such as natural isotopic abundance studies may be considered before enriching samples with isotopes. Would natural ^{15}N abundance be useful for answering the research question, or does it require additional fertilization of an isotope, such as 99% ^{15}N enrichment studies? With an ecosystem-scale N

cycling experiment natural abundance would be useful for quantifying ecosystem N inputs and outputs. A ^{15}N enrichment approach would be useful for exploring gross rates of N mineralization under contrasting agricultural fertilization experiments.

The application of ^{15}N enrichment studies is wide-ranging (Murphy et al. 1999) including the fields of agriculture, environmental conservation, ecosystem ecology, and soil biology. The use of isotope-based methods enables us to track real-time production and consumption of isotope-labelled components thus allowing us to calculate gross rates of nitrogen mineralization and nitrification. Non-isotope techniques, such as a standard 28-day incubation of net mineralization and nitrification, can be informative for getting a general picture of how nitrogen is available within soils. In contrast, tracing which forms of nitrogen are being produced and consumed by microbial counterparts is possible using ^{15}N enrichment and gross nitrogen incubations (Booth et al. 2005).

Molecular techniques including DNA and RNA analyses can provide excellent data on the composition, structure, and function of microbial communities. In comparison there is a technique for phospholipid fatty acid analysis (PLFA) which is aimed at discerning general patterns in fatty acids and using those as biomarkers for estimating bacterial and fungal biomass within soil communities (Frostegård and Bååth 1993). While PLFAs have been used with success (Frostegård et al. 1996, Grayston and Prescott 2005), this method does not isolate specific gene-encoding enzymes, which would allow us to track changes in nitrifying bacterial communities, for example. Recent methods have now been refined such that PLFA is growing less common in comparison with DNA or RNA based analyses. Historically, microbial biomass chloroform-fumigation extractions (CFE), or variations on chloroform incubations, have been used to quantify microbial activity levels. CFE relies upon CO_2 production as a proxy for microbial consumption after exposure to chloroform (Brookes et al. 1985), but this is a coarse metric and does not allow us to partition components of the microbial community at a finer resolution.

Recent molecular approaches vary in complexity and associated lab-requirements, but also tend to be a more expensive option, especially if next generation sequencing such as illumina is to be

performed, which can be used for whole genome or transcriptome studies. The use of genetic markers and DNA-based technologies to determine abundance of microbial communities has some distinct advantages over alternative methods, such as chloroform-fumigation extraction microbial biomass assays (Philippot et al. 2012). DNA-based methods such as PCR and qPCR enable us to determine not just the abundance of total microbial biomass, but can be used to separate broad functional groups (bacterial vs. fungal) or determine specific functional gene markers (e.g. nitrifying bacteria). Chloroform fumigation extraction, on the other hand, is a proxy for microbial biomass and not a direct measure, as it is calculated from CO_2 production after exposure to chloroform and combined with a study-system-specific constant value (often not adjusted and adds to the lack of reliability with this data). In this example, qPCR can be used to provide a more reliable dataset than chloroform fumigation extraction.

However, DNA-based methods only provide information about the genetic potential for the respective functioning of the microbes. For instance, DNA may persist within the soil long-after a microbe has perished or microbes may be inactive as a result of adverse environmental conditions in the field. More specific information is provided by enzymatic assays which indicate the production of enzymes required in a given sample, RNA-based methods which indicate the active replication of DNA in a given sample, or proteomics-based methods, which can provide real-time information about proteins within cells. Each of these methods provides higher resolution data on the level of activity of specific genes identified via qPCR (see Gotelli et al. 2012, and Ogunseitan 2006). There are drawbacks to these active methods though, including the challenges in accounting for temporal and spatial variability within these datasets, and the time and labor-intensive nature of the data collection required for sufficient sample sizes for statistics. We suggest careful consideration of pairing research methods required with primary research questions of interest and the required replication and power to be able to make meaningful conclusions from the data.

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Chapter III. Linking microbial communities, functional genes and nitrogen-cycling processes in forest floors under four tree species

1. Abstract

Tree species can influence rates of soil N transformations, but the question remains whether differences in N cycling rates are mirrored by the abundance of relevant functional genes. We studied whether the influence of tree species on soil N transformation processes and abundance of functional genes exist across two sites in British Columbia with different N availability. We used the ^{15}N pool-dilution method to estimate gross rates of ammonification and nitrification in forest floors of four conifers in a common garden experiment. The abundances of bacteria, fungi, nitrification (AOA *amoA*, AOB *amoA*) and denitrification (*nirS*, *nirK*) genes were determined by qPCR. Western red cedar (*Thuja plicata)* had the highest rates of gross ammonification and NH_4^+ consumption, followed by Sitka spruce (*Picea sitchensis)*, hemlock (*Tsuga heterophylla)*, and Douglas-fir (*Pseudotsuga menziesii)*; all species showed net nitrate immobilization. Western red cedar forest floors had the greatest abundance of bacterial *16S* genes and ammonia-oxidizing archaea *amoA* genes. This suggests that tree species foster different abundances of ammonification and denitrification functional groups. Differences in N transformation rates between the sites were related to site N status, as reflected in C:N ratios of the forest floor and microbial biomass, and were more closely tied to rates of N consumption rather than gross mineralization. Rates of most N transformation processes were related to microbial C:N ratio, indicating that the N status of microbes rather than their biomass or activity level determined the rates of N cycling. Ammonification rates were associated with forest floor and microbial biomass C:N ratio as well as bacterial and fungal abundances. Nitrification rates and denitrification gene abundance were associated with microbial biomass C:N ratios and AOA *amoA* gene abundance. The forest floor's genetic potential for denitrification was positively correlated with its nitrification potential as indicated by ammonia-oxidizer abundance. We conclude that tree species influenced forest floor N cycling and soil microbial gene abundances, and that functional genetics can be useful for exploring mechanistic links between tree species and nitrogen cycling processes.

2. Introduction

Nitrogen (N) availability is often the primary limitation on ecosystem productivity in conifer forests (Vitousek et al., 1997, 2002; Lauber et al., 2008), and factors that regulate N availability have far-reaching consequences for ecosystem properties such as microbial activity, biogeochemical cycling, carbon (C) sequestration, aboveground biomass production, and greenhouse gas (GHG) fluxes (Binkley and Giardina, 1998). Soil N transformation processes (e.g., mineralization of organic N to NH_4^+ and nitrification of NH_4^+ to NO_3^-) are largely mediated by interactions between functional communities of soil microorganisms and their environment (Bengtsson et al., 2003; Balser and Firestone, 2005; Högberg et al., 2013). The balance between gross and net rates of mineralization and nitrification can indicate turnover and immobilization rates of $NH_4^+$and NO_3^-, respectively, which can be rapid in forest soil (Davidson et al., 1992; Stark and Hart, 1997). Rates of mineralization and nitrification have further implications for N loss through leaching of NO_3^- and denitrification to N_2 (Bengtsson et al., 2003; Szukics et al., 2010).

Quantification of marker genes for biogeochemical functions can be used to characterize N cycling processes and communities (Levy-Booth et al., 2014). Ammonia-oxidizing archaea (AOA) and bacteria (AOB) carry out the first, and potentially rate-limiting, step of nitrification. AOA and AOB abundance can be quantified using the ammonia-monooxygenase (*amoA*) gene (Rotthauwe et al., 1997; Francis et al., 2005; Leininger et al., 2006; Szukics et al., 2010). While AOB have long been thought to dominate ammonia-oxidation, the quantitative importance of AOA in soil has recently been recognized (Leininger et al., 2006; Petersen et al., 2012), and AOA are generally more abundant than AOB in acidic forest soil (Petersen et al., 2012). The relative contributions of AOA and AOB to gross and net nitrification in forests with different tree species remain to be seen. Denitrification is a biological pathway through which N returns to the atmosphere from soil or water by the reduction of nitrate to nitrogen gas (Henry et al., 2004) via the nitrite reductase enzyme genes *nirS* and *nirK*. Genes that encode enzymes involved in biogeochemical cycling can be used to compare measured N mineralization and nitrification rates with bacterial, fungal, nitrifier and denitrifier population sizes (inferred from gene abundances) and in so doing, link N-cycling pathways to functional microbial groups.

Tree species have been shown to influence chemical and biological properties of soil, particularly forest floor layers, and rates of processes therein. Tree species influence soils directly via leaf litter inputs and formation of forest floors (Hobbie et al., 2006; Vesterdal et al., 2012), and via root litter inputs and alteration of soil structure. Tree species also influence rates of litter decomposition, nutrient release, C turnover and soil respiration through differences in foliar N, Ca, Mg and lignin concentrations (Hobbie et al., 2006, Vesterdal et al., 2012; Vesterdal et al., 2013). Several studies have reported differences in rates of N-cycling processes in soils under different tree species (e.g., Ste-Marie and Paré, 1999; Malchair and Carnol, 2009), and distinct microbial communities have also been reported in soils and forest floors under different tree species (Leckie et al., 2004; Prescott and Grayston, 2013). However, despite decades of research on this question, categorization of species according to their influence of soil properties has proven elusive, as tree species effects have been inconsistent among studies, and even at different sites within a single study. For example, in a common garden experiment on Vancouver Island, the indirect influence of site factors (particularly slope position) on rates of net N mineralization and nitrification in the forest floors appeared to overwhelm the influence of tree species, with appreciable net rates detected only at the valley bottom sites where the forest floor C:N ratio was less than 35 (Prescott et al., 2000a). Likewise, Gurmesa et al. (2013) found an influence of broadleaved tree species on soil carbon only at relatively rich sites, which they attributed to the lack of earthworms at the infertile sites, regardless of tree species. These observations prompted Prescott and Vesterdal (2013) to propose that the expression of tree species influence on soils is context-dependent, and more likely to be detectable on rich or intermediate sites.

In the common garden experiment on Vancouver Island, previous studies of the forest floors discerned some distinct characteristics of the forest floors under four coniferous tree species, which have been summarized by Prescott and Vesterdal (2005). Western red cedar forest floors had the lowest rates of litter decomposition and net N mineralization, but the highest proportion of nitrate and the highest bacterial:fungal ratio of the four species. Western hemlock forest floors had low pH and low Ca concentrations, low bacterial:fungal ratios, and were dominated by NH_4^+ rather than nitrate. Sitka spruce forest floors had intermediate to high concentrations of N, P, Ca, and K, low bacterial:fungal ratios, and moderate rates of net N mineralization. Finally,

Douglas-fir forest floors had intermediate pH and Ca, high bacterial:fungal ratios, high N concentrations and rates of net N mineralization and nitrification.

In this study, we revisit the common garden experiment on Vancouver Island and compare the forest floors that have developed under four tree species at one nutrient-rich, valley-bottom site and one nutrient-poor, mid-slope site. We examine differences in forest floors among tree species and the extent to which these differ according to the site. We measure rates of N mineralization and nitrification using ^{15}N pool-dilution and apply quantitative PCR of microbial genes involved in N-cycling processes to explore interactions among microbial communities (nitrifiers and denitrifiers) and rates of N-cycling processes.

3. Materials and methods

3.1. Study location

We sampled two sites, as case studies rather than replicates, from the EP571 common garden experiment on Vancouver Island: San Juan (48°35′N, 124°12′W) and Fairy Lake (48°35′N, 124°19′W), both located near Port Renfrew. The two sites were comparable in terms of elevation (65–85 and 75–85 m, respectively), and were within the Sub-montane Very Wet Maritime Coastal Western Hemlock variant (Prescott et al., 2000a). San Juan is a valley-bottom site with understory largely composed of *Rubus spectabilis* Pursh (salmonberry) and *Polystichum munitum* (Kaulf.) Presl. (swordfern), while Fairy Lake is a mid-slope site with understory dominated by *Gaultheria shallon* Pursh (salal) and *Vaccinium parvifolium* Smith (red huckleberry). Significantly higher N mineralization and nitrification rates and concentrations of P and K were measured in forest floors at the San Juan site compared with the Fairy Lake site (Prescott et al., 2000a), which were related to the differences in slope position and understory vegetation. We selected these two study sites because of their contrast in terms of N cycling, to enable us to test whether species effects on gross N cycling and functional genes would be consistent across contrasting sites.

Replicate plots of each species (western red cedar, western hemlock, Douglas-fir, and Sitka spruce) were planted in 1961, as a part of Experimental Project No. 571. The previous forest cover of western hemlock, western red cedar, amabilis fir, and Sitka spruce had been clear-cut

and slash burned. The full experimental design contained 24 0.07-ha plots at each site, with 81 tree seedlings planted in three densities (2.7, 3.7, and 4.7 m); for the current study we sampled the two densest (2.7-m spacing) plots of each species. We used this pre-existing experimental design to determine if tree species differ in their dominant nutrient cycling characteristics between N-rich and N-poor sites.

3.2. Soil sampling and soil physical and chemical analyses

We focused on the F-layer of the forest floor. This is the layer in which the greatest differences in soil microbial communities of these tree species have previously been found (Grayston and Prescott, 2005), and where soil fungi and fauna are expected to be most abundant and active (Kurbatova et al., 2009). It also ensured that we were comparing the influence of tree species on microbial communities at the same stage of decay, as recommended by Prescott and Grayston (2013).

Composite samples were collected from nine randomly selected 450 cm^2 samples of the F-layer in each plot in June of 2014. This layer consisted of partially decomposed material and the humus-layer, but no fresh needles or mineral soil were collected. Brockett et al. (2012) showed that composite samples provide similar results to individually analyzed samples for plot-level values, albeit at a loss of information regarding spatial heterogeneity. Each composite forest floor sample was passed through a 2-mm mesh sieve and one 15-g subsample was immediately removed for the ^{15}N pool-dilution analysis. The remaining bulk sample was stored at −20 °C for DNA extraction. For pH analysis, a 5-g field-moist subsample of forest floor was added to a small jar with 20 mL distilled water, shaken for 30 min then measured with a pH meter. A subsample of field-moist forest floor was also removed for C and N analysis. These samples were oven dried to 70 °C for 48 h, ground using a mortar and pestle, and 5 mg weighed into tin capsules and analyzed for C and N by high-temperature flash combustion using a Vario EL Cube elemental analyzer (Elementar Americas Inc., Mount Laurel, NJ).

3.3. Gross rates of ammonification and nitrification

The ^{15}N pool-dilution method (modified from Drury et al., 2008) was used to determine gross rates of ammonification and nitrification, with samples analyzed in triplicate. Six 15 g

subsamples from each plot were passed through a 2-mm mesh sieve and transferred to 500 mL glass Mason jars and sealed with parafilm (n = 3 subsamples for both $^{15}NH_4^+$ and $^{15}NO_3^-$). The parafilm seal was punctured to enable gas exchange and maintain aerobic conditions. Samples were incubated in the dark at room temperature for 24 h prior to initial ^{15}N treatments. These treatments consisted of either: 4 mL of $^{15}NH_4Cl$ solution (99 atom%; Cambridge Isotope Laboratories) or 4 mL of $K^{15}NO_3$ (99 atom%; Cambridge Isotope Laboratories) added to the forest floor samples in each respective jar, which was an equivalent application rate of 12 μg N g^{-1} forest floor. Labeled N was injected into the samples in 1 mL intervals four times over 1 min, and gently homogenized to ensure isotopic labeled N was applied uniformly throughout the forest floor sample, and the parafilm seal was replaced.

i) Immediately after ^{15}N addition and ii) 24 h after ^{15}N addition, a 5-g forest floor subsample was removed from each Mason jar, this subsample was added to 100 mL of 2.0 M KCl, shaken for 1 h, and filtered through glass fiber filter paper (Fisher Inc.). 10 mL of the KCl extract were analyzed for NH_4N and NO_3N using a flow injection analyzer (Lachat; Quickchem 8000) at the Environmental Engineering Department at the University of British Columbia. The remainders of the extracts were used for microdiffusion of $^{15}NH_4N$ and $^{15}NO_3N$ with the use of acid traps, and the sequential addition of MgO and Devarda's alloy according to the protocol developed by the International Atomic Energy Agency (IAEA, 2001). Acid traps were dried, packaged in tin cups, and sent to the University of Saskatchewan Isotope Laboratory for stable isotope ratio analysis using a Costech ECS4010 elemental analyzer coupled to a Delta V mass spectrometer with a Conflo IV interface. Net rates of ammonification were calculated as the difference in NH_4^+ between the incubated samples and the initial soil extractions at the start of the incubation. Net nitrification was calculated as the difference in NO_3^- between the initial and incubated samples. The gross rates of ammonification, nitrification and microbial consumption were calculated following Hart et al. (1994).

3.4. Microbial biomass determination

Microbial biomass nitrogen (MBN) and microbial biomass carbon (MBC) were determined using a modified chloroform-fumigation extraction (Brookes et al., 1985). Briefly, 100 mL of 2 M KCl was used in lieu of 40 mL of 0.5 M K_2SO_4 for extractions (Verchot et al., 2001). 20 mL

extracts were analyzed for total organic C (TOC) and total N (TN) on a Shimadzu 5000A TOC analyzer at the Analytical Services Laboratory of the University of Alberta. The additional remaining extractant was used for acid diffusion traps, identical to the above protocol for the ^{15}N pool-dilution method. To determine if microbial communities were immobilizing N during the course of the incubation we assessed MBN and MBC in $^{15}NNHj_4$ and $^{15}NNO_3$ added samples at the end of the pool-dilution experiment, as well as from unfertilized soil samples.

3.5. DNA isolation and quantitative PCR

DNA was extracted from 0.1 g of field-moist forest floor using the MoBio Power Soil DNA isolation kit (MoBio Laboratories, Inc., Carlsbad, CA, USA). DNA quality and concentration was measured using a nanodrop spectrophotometer (Thermo Fisher Scientific Inc., Wilmington, DE, USA) and electrophoresis in agarose gels (1% w/v in TAE), then stored at −20 °C prior to amplification.

Quantitative PCR (qPCR) was used to quantify gene copy numbers. Reactions were carried out with an Applied Biosystems® StepOnePlus™ real-time PCR system. Each 20 µl reaction contained 10 µl of SybrGreen (2×) PCR Master Mix (Life Technologies Corp., Carlsbad, CA, USA), 0.5 µl of each primer, 250 ng μl^{-1} bovine serum albumin (BSA), and 1 µl of DNA template. Table 1 shows primer sequences for qPCR assays. PCR conditions for AOA *amoA*, AOB *amoA* were 10 min at 95 °C, followed by 40 cycles of 95 °C for 30 s, 30 s at 57 °C, and 72 °C for 1 min, and 10 s at 80 °C. Triplicate 10× standard curves ranged from 10^2 to 10^7 copy numbers of AOA and AOB *amoA* in linearized plasmids. PCR conditions for *nirK* and *nirS* were 10 min at 95 °C and 40 cycles of 95 °C for 1 min, 60 °C for 1 min and 72 °C for 1 min, with fluorescence quantified at extension (Levy-Booth and Winder, 2010). The standard curves for *nirS* and *nirK* used a triplicate 10-fold serial dilutions of 10^1 to 10^7 gene copies from *Pseudomonas aeruginosa* (ATCC 47085) and *Pseudomonas chlororaphis* (ATCC 13985) genomic DNA, respectively. Bacterial 16S rRNA PCR conditions were 5 min at 95 °C, followed by 40 cycles of 95 °C for 30 s, 30 s at 57 °C, and 72 °C for 1 min, and 10 s at 80 °C. Florescence quantification occurred during annealing. Triplicate standard curves were run using a 10× dilution of 10^2 to 10^7 amplified 16S rRNA in linearized plasmids. PCR conditions for fungal ITS were 10 min at 95 °C, followed by 40 cycles of 95 °C for 1 min, 30 s at 53 °C, 50 s at 72 °C and 10 s at 80 °C. Fluorescence was read at 80 °C to reduce the formation of non-target and primer

self-complementation structures. Triplicate standard curves for fungal ITS quantification were constructed using 10× dilutions between 10^3 to 10^9 ITS copies amplified from soil and *Aspergillus citrisporus* genomic DNA. Standard curve ranges are indicative of lower and upper limits of detection, respectively. All qPCR analyses were run in duplicate.

Table 1.

Group-specific primers for qPCR gene quantification assays.

Primer target	Primer name	Primer sequence (5′–3′)	Reference
All bacteria (16S rRNA)	519F	CAG CMG CCG CGG TAA NWC	Baker et al. (2003)
	907R	CCG TCA ATT CMT TTR AGTT	Muyzer et al. (1995)
All fungi (ITS)	ITS-1F	TCC GTA GGT GAA CCT GCG G	Gardes and Bruns (1993)
	5.8s	CGC TGC GTT CTT CAT CG	Vilgalys and Hester (1990)
AOA[a] *(amoA)*	*amoA*-23F	ATG GTC TGG CTW AGA CG	Francis et al. (2005)
	amoA-616R	GCC ATC CAT CTG TAT GTC CA	
AOB[b] *(amoA)*	*amoA*-1F	GGG GTT TCT ACT GGT GGT	Rotthauwe et al. (1997)
	amoA-2R	CCC CTC KGS AAA GCC TTC TTC	
Cd-nitrite reductase (*nirS*)	*nirS*-1F	CCT AYT GGC CGG CRC ART	Braker et al. (1998)
	nirS-3R	GCC GCC GTC RTG VAG GAA	
Cu-nitrite reductase (*nirK*)	*nirK-1F*	GGG CAT GAA CGG CGC GCT CAT GGT G	Braker et al. (1998)
	nirK-1R	CGG GTT GGC GAA CTT GCC GGT GGT C	

b Ammonia Oxidizing Archaea. B Ammonia Oxidizing Bacteria.

3.6. Statistical analyses

At each site one composite forest floor sample from the two replicate plots for each tree species was sampled (n = 2). The influences of tree species and sites on forest floor chemistry, microbial biomass C and N, process rates and gene abundances were evaluated using two-way permutational analysis of variance (with 999 permutations). Permutational analysis of variance

was used to address the low sample sizes in this study (Andersen and Legendre, 1999) with tree species and site as fixed effects. Microbial gene abundances were $\log_{10}$ transformed prior to two-way permutational analysis of variance (with 999 permutations). We used multiple linear regressions to determine which environmental variables were the best predictors of N transformations on standardized data (*decostand* function in the *vegan* package in R (Oksanen et al., 2013)). Rates of N transformations, including gross and net ammonification and nitrification, were related to all potentially regulating parameters using multiple linear regressions, following the removal of collinear variables. Model selection was based on stepwise variable selection with Akaike's Information Criterion (AIC), whereby the lowest value indicates the model with the highest explanatory power.

Redundancy analysis (RDA), a form of constrained ordination that determines how much of the variation from one set of variables explains the variation in another set of variables, was performed to test relationships between site and forest floor parameters and gene abundances (*vegan* package in R). Forward selection of explanatory variables for RDA was carried out using the *packfor* function using the method recommended by Blanchet et al. (2008). The significance of the RDA model and its individual terms were calculated using Monte-Carlo tests with 10,000 permutations. All analyses were conducted with R v. 2.15.3 (R Core Team, 2015).

4. Results

4.1. Forest floor pH, C and N

There were no significant differences in pH, although Sitka spruce and western red cedar had the highest average pH values (Table 2). Concentrations of total C and N also did not significantly differ between forest floors under the four tree species or at the two sites (Table 3). However, forest floor C:N ratio was significantly lower ($p = 0.02$) at the San Juan (nutrient-rich) site (36.5 ± 1.1) than at Fairy Lake (nutrient-poor) (42.2 ± 1.5). C:N ratio also differed among tree species, with hemlock forest floors having significantly higher C:N ratio (43.3 ± 2.3) than Sitka spruce (36.4 ± 2.1; $p = 0.018$) and Douglas-fir (36.6 ± 1.2; $p = 0.015$). There were no significant species-by-site interaction effects, indicating consistent tree species effects on C:N ratios across sites.

4.2. Microbial biomass C and N

Microbial biomass C (MBC) differed among tree species across both sites and was significantly lower in forest floors at San Juan than Fairy Lake (Fig. 1, Table 3). Significant species-by-site interaction indicated that influences of tree species on MBC differed between the two sites ($p = 0.03$). At Fairy Lake, Sitka spruce had lower MBC than other species whereas cedar had lower MBC than other tree species at San Juan (Fig. 1). Microbial biomass N (MBN) did not differ significantly between sites ($p = 0.89$) or among tree species across sites (Table 3, $p = 0.39$), but at San Juan, Douglas-fir had higher MBN than other species (Fig. 1). Microbial C:N ratios did not differ significantly between sites or tree species, although there was a tendency for lower microbial C:N at San Juan (Fig. 1, $p = 0.10$).

Table 2.

Forest floor chemistry: pH, total soil C (mg/g), N (mg/g), and C:N ratios of the four tree species at the two sites. Mean ± SE.

Site	Tree species	pH	Total C (mg g^{-1})	Total N (mg g^{-1})	C:N ratio
San Juan	WRC	4.01 ± 0.18	436 ± 31	110 ± 0	38.5 ± 3.1
	DF	3.95 ± 0.02	466 ± 9	135 ± 1	34.6 ± 0.9
	WH	3.98 ± 0.21	449 ± 17	114 ± 1	39.4 ± 1.0
	SS	4.63 ± 0.45	369 ± 85	112 ± 26	33.6 ± 0.9
Fairy Lake	WRC	4.31 ± 0.30	485 ± 8	11.0 ± 0.1	44.0 ± 0.6
	DF	4.05 ± 0.01	430 ± 52	11.1 ± 1.1	38.5 ± 0.9
	WH	3.75 ± 0.03	498 ± 4	10.5 ± 0.2	47.2 ± 0.7
	SS	4.29 ± 0.05	460 ± 18	11.8 ± 0.5	39.2 ± 3.1

WRC = western red cedar; DF = Douglas-fir; WH = western hemlock; SS=Sitka spruce.

Table 3.
F-statistics following permutation ANOVA testing of tree species (western red cedar, Douglas-fir, western hemlock, Sitka spruce, df = 3), site (Fairy Lake, San Juan, df = 1), and interaction (T × S, df = 3) effects on forest floor chemistry (pH, total C, total N, C:N ratio), microbial biomass (C, N and C:N ratios), N transformations (gross and net nitrogen ammonification and nitrification, and NH_4^+ and NO_3^- consumption) and microbial gene abundances.

Forest floor chemistry	Tree (T)		Site (S)		Tree × site (T × S)	
	F	p	F	p	F	p
pH	2.93	0.10	0.08	0.79	0.90	0.48
C	0.89	0.49	2.03	0.20	0.97	0.43
N	0.58	0.64	0.87	0.40	0.71	0.59
C:N	8.49	0.01	23.77	<0.01	0.45	0.71
Microbial biomass						
MBC	6.77	0.02	33.16	<0.01	5.25	0.03
MBN	2.26	0.18	0.02	0.90	1.11	0.39
MBC:N	1.62	0.28	3.90	0.10	1.67	0.29
N transformation rates						
Gross ammonification	18.43	<0.01	64.57	<0.01	1.08	0.40
NH_4^+ consumption	6.75	0.01	7.31	0.03	0.50	0.73
Net ammonification	4.43	0.05	18.27	0.01	0.73	0.58
Gross nitrification	0.74	0.58	20.81	<0.01	1.95	0.18
NO_3^- consumption	1.19	0.38	132.72	<0.01	1.68	0.25
Net nitrification	1.41	0.34	67.09	<0.01	5.84	0.02
Microbial gene abundances						
Bacteria *16S*	3.88	0.05	10.20	0.01	1.24	0.32
Fungal *ITS*	0.20	0.90	0.38	0.55	0.99	0.45
AOA *amoA*	0.76	0.51	18.62	0.01	0.02	1.00
AOB *amoA*	1.06	0.46	4.02	0.07	1.42	0.33
nirK	2.73	0.13	1.94	0.20	0.97	0.45
nirS	1.47	0.27	8.54	0.02	1.73	0.22

C = carbon; N = nitrogen; MB = microbial biomass.

4.3. Microbial gene abundance

Bacteria *16S* rRNA gene abundance was significantly greater in forest floors from San Juan than from Fairy Lake (Table 3, $p = 0.01$), and differed significantly among tree species (Table 3, $p = 0.05$) with no species-by-site interaction. Bacterial genes were more abundant in

forest floors of western red cedar at both sites, with hemlock or spruce having the lowest abundance depending on the site (Fig. 2). Fungal *ITS* abundance did not differ between sites ($p = 0.55$) or tree species ($p = 0.89$). AOA *amoA* had the most pronounced site effect of any of the microbial genes quantified in this study, with San Juan having several orders of magnitude more AOA *amoA* genes than Fairy Lake ($p = 0.01$), but no species effects. AOB *amoA* genes were more abundant at San Juan than at Fairy Lake, but within the same order of magnitude ($p = 0.10$). The abundance of the Cu-nitrite reductase *(nirK)* gene did not differ by site ($p = 0.19$), or tree species ($p = 0.13$), although hemlock tended to have the lowest gene abundance. In contrast, Cd-nitrite reductase (*nirS*) genes were significantly more abundant in forest floor at San Juan than at Fairy Lake ($p = 0.02$), but did not differ by tree species ($p = 0.27$).

3.4. Gross and net N ammonification and nitrification rates

Gross ammonification rates were significantly higher in forest floors at San Juan than at Fairy Lake (Fig. 3a), with mean rates of 32.9 and 19.4 mg N g^{-1} soil (dw) day^{-1}, respectively. A highly significant trees species effect on gross ammonification was observed with gross ammonification under western red cedar being significantly greater than under Douglas-fir ($p = 0.03$), hemlock ($p = 0.006$) and Sitka spruce ($p = 0.03$). Gross ammonium (NH_4^+) consumption was significantly higher in forest floors from San Juan than Fairy Lake ($p = 0.002$), and was significantly greater in forest floors under cedar than the other species (Fig. 3b). Net ammonification rates were mostly negative, indicating net immobilization of NH_4^+, with occasional positive mineralization occurring only in Sitka spruce at San Juan (Fig. 3c). San Juan forest floors had higher (i.e. less negative) rates of net ammonification than Fairy Lake forest floors ($p = 0.01$), and net ammonification rates differed among tree species ($p = 0.05$).

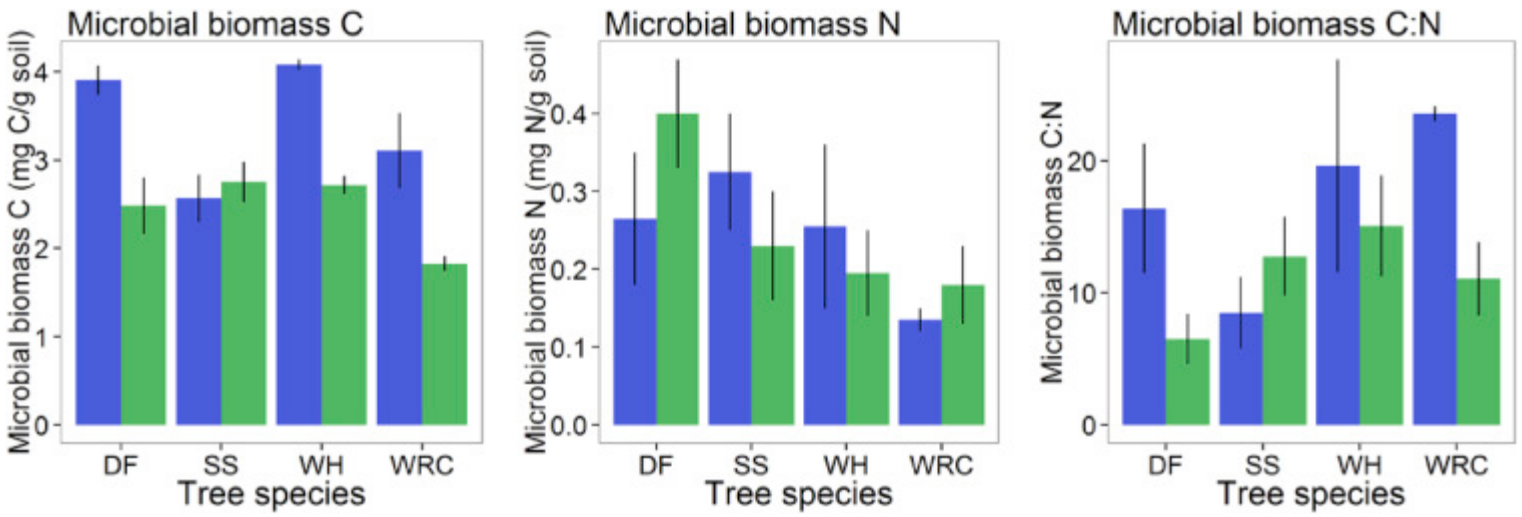

Figure 1.
Microbial biomass C, N and C:N ratio in forest floors of four tree species at the two sites (blue bars = Fairy Lake; green bars = San Juan; DF = Douglas-fir; SS=Sitka spruce; WH = western hemlock; WRC = western red cedar).

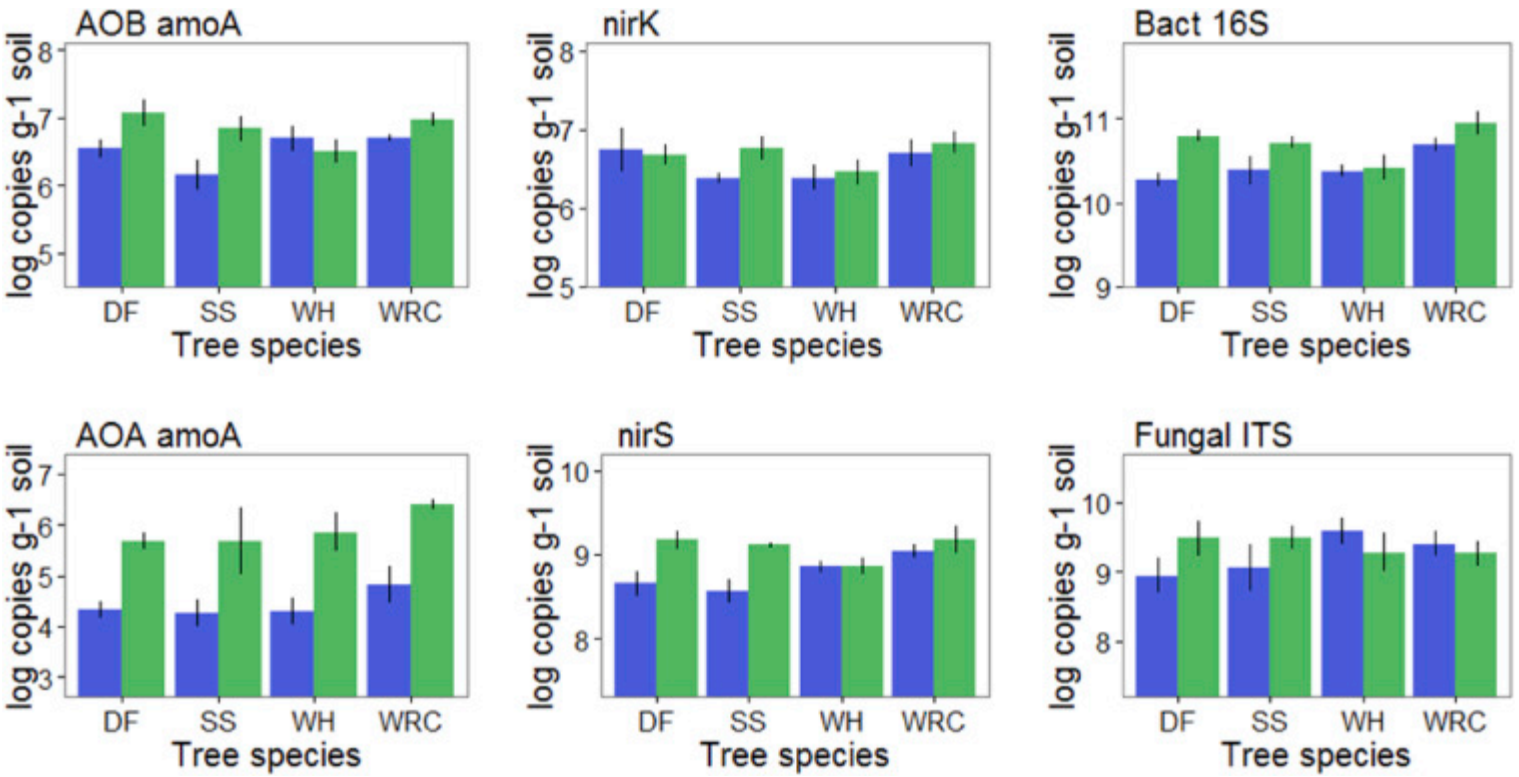

Figure 2.
Mean (±SE) gene abundance (log of gene copies/g soil) of total ammonia oxidizers (AOA *amoA* and AOB *amoA)* and total denitrifiers *(nirK* and *nirS,* bacteria (*16S)*, and fungi (*ITS)* in the forest floors of four tree species at the two sites. Blue bars = Fairy Lake; green bars = San Juan; DF = Douglas-fir; SS=Sitka spruce; WH = western hemlock; WRC = western red cedar.

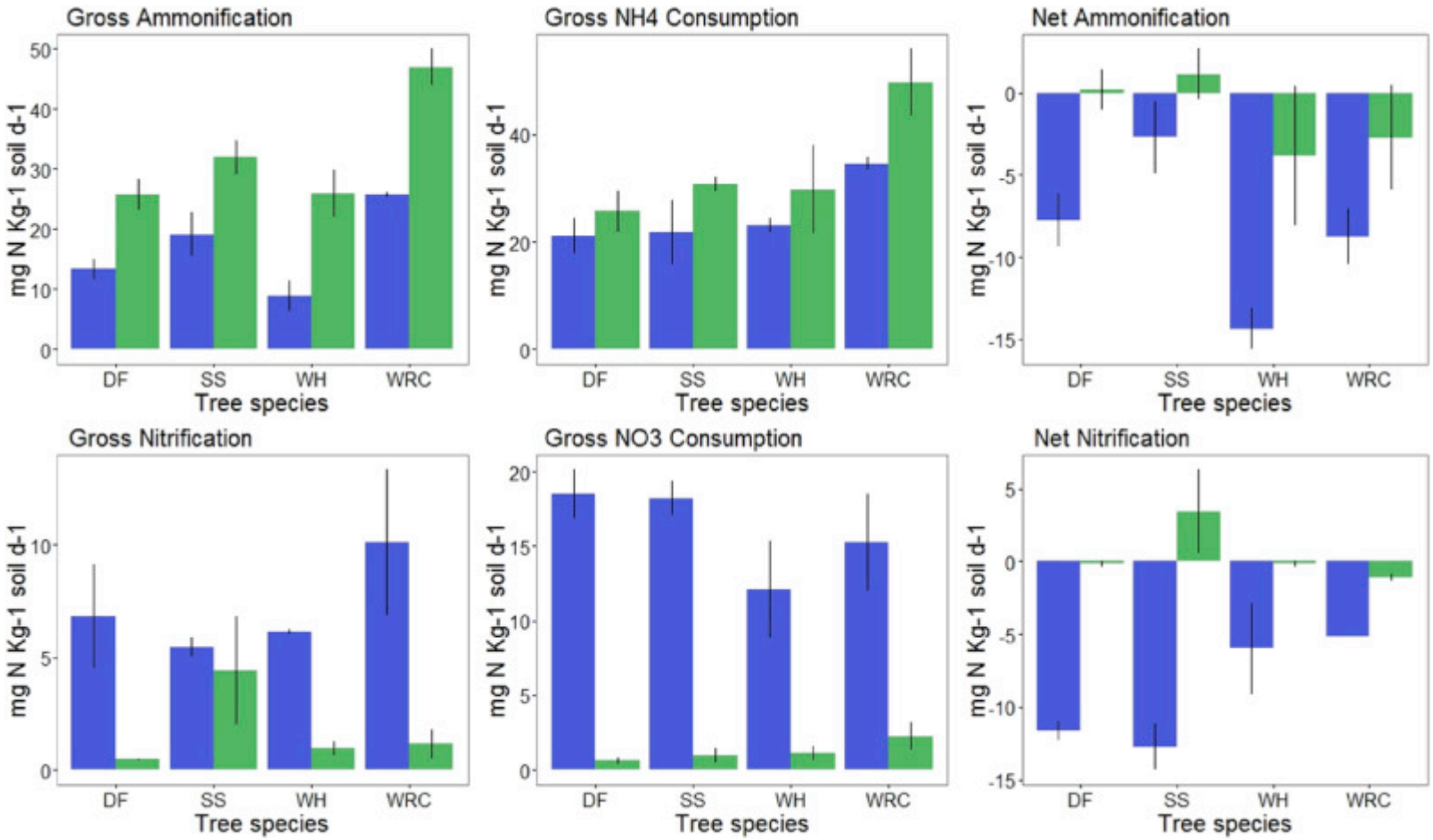

Figure 3.

Rates of nitrogen transformations in the forest floors of four tree species at the two sites; (mean ± SE). Blue bars = Fairy Lake; green bars = San Juan; DF = Douglas-fir; SS=Sitka spruce; WH = western hemlock; WRC = western red cedar.

Rates of gross nitrification, nitrate consumption and net nitrification did not differ between species, but did differ between sites with slightly more N transformed at Fairy Lake than San Juan ($p < 0.01$). At Fairy Lake, western red cedar had the highest rates of gross nitrification, which significantly differed from hemlock and Sitka spruce, but not from Douglas-fir. Douglas-fir and Sitka spruce had the highest and similar rates of nitrate consumption, followed by western red cedar, spruce, and hemlock at Fairy Lake. At San Juan, Sitka spruce had the highest rates of gross nitrification. Douglas-fir, western red cedar, and hemlock all had negligible rates of gross nitrification and nitrate consumption. Net nitrification rates were negative, indicating net nitrate immobilization in all plots except Sitka spruce at San Juan (Fig. 3f).

4.5. Relationships between N ammonification and nitrification rates and microbial parameters

In general, we had greater explanatory power for ammonification rates than nitrification rates (Table 4). Gross ammonification was best explained by the total number of bacterial *16S* rRNA and fungal *ITS* genes. Gross NH_4^+ consumption was best explained by gross ammonification, pH, forest floor C:N ratio, and microbial biomass C:N ratio. Net ammonification was best explained by forest floor pH and C:N ratios and microbial biomass C:N ratios. Gross nitrification was best explained by microbial biomass C:N ratios and AOA *amoA* gene. Gross NO_3^- consumption was best explained by net ammonification, gross nitrification, microbial biomass C:N ratios, bacterial 16S and AOA and AOB *amoA* genes. Net nitrification rates were best explained by net ammonification, microbial biomass C:N ratios, bacterial 16S, and AOA and AOB *amoA* genes. The sum of AOA and AOB gene abundances showed a strong, positive relationship with the sum of the abundance of nitrite reductase genes (*nirK* and *nirS*) (Fig. 4). Redundancy analysis showed clear separation of the two sites largely due to differences in forest floor C:N ratio and pH, but no clear grouping of microbial abundance patterns according to tree species (Fig. 5). Fairy Lake was associated with high C:N ratio, and San Juan with higher microbial gene abundance for AOA and AOB *amoA, nirK, nirS,* and *16S*.

Table 4.
Multiple linear regressions of gross and net N ammonification and nitrification rates, and consumption rates with adjusted R^2 values, with best models selected using the lowest AIC values.

Variable	Coefficient	t value	p-value
Gross ammonification = bacterial *16S* gene copies−fungal *ITS* gene copies			
bacteria *16S* gene copies	1.057	6.253	2.96E-05
fungal *ITS* gene copies	−0.537	−3.179	0.007
F-statistics: 19.68 on 2 and 13 degrees of freedom; Adj. R2: 0.71; p-value: <0.0001			
Gross NH4 + consumption = gross ammonification + C:N ratio + MBC:N ratio			
Gross ammonification	1.055	13.084	1.84E-08
Forest Floor C:N ratio	0.243	2.680	0.020
Microbial Biomass C:N ratio	0.262	3.178	0.008
F-statistics: 61.5 on 3 and 12 degrees of freedom; Adj. R2: 0.92; p-value: <0.001			
Net ammonification = −C:N ratio−MBC:N ratio			
C:N ratio	−0.526	0.153	0.005
Microbial biomass C:N ratio	−0.486	0.153	0.008
F-statistics: 21.73 on 2 and 13 degrees of freedom; Adj. R2: 0.73; p-value: <0.001			
Gross nitrification = MB C:N ratio−*amoA* AOA gene copies			
Microbial biomass C:N ratio	0.340	1.770	0.100
amoA AOA gene copies	−0.599	−3.179	0.008
F-statistics: 7.265 on 2 and 13 degrees of freedom; Adj. R2: 0.46; p-value: 0.008			
Gross NO3− consumption = gross nitrification			
gross nitrification	0.755	0.175	<0.001
F-statistics: 18.58 on 1 and 14 degrees of freedom; Adj. R2: 0.54; p-value: <0.001			
Net nitrification = *amoA* AOB gene copies			
amoA AOB gene copies	0.477	2.034	0.061
F-statistics: 4.135 on 1 and 14 degrees of freedom; Adj. R2: 0.1729; p-value: 0.061			

Ammonification rates: mg N kg^{-1} soil (dw) d^{-1}; nitrification rates: mg N g^{-1} soil (dw) d^{-1}; microbial C and N, mg N g^{-1} soil (dw); Bacterial *16S,* Fungal *ITS,* AOA and AOB *amoA*: genes g^{-1} soil (dw).

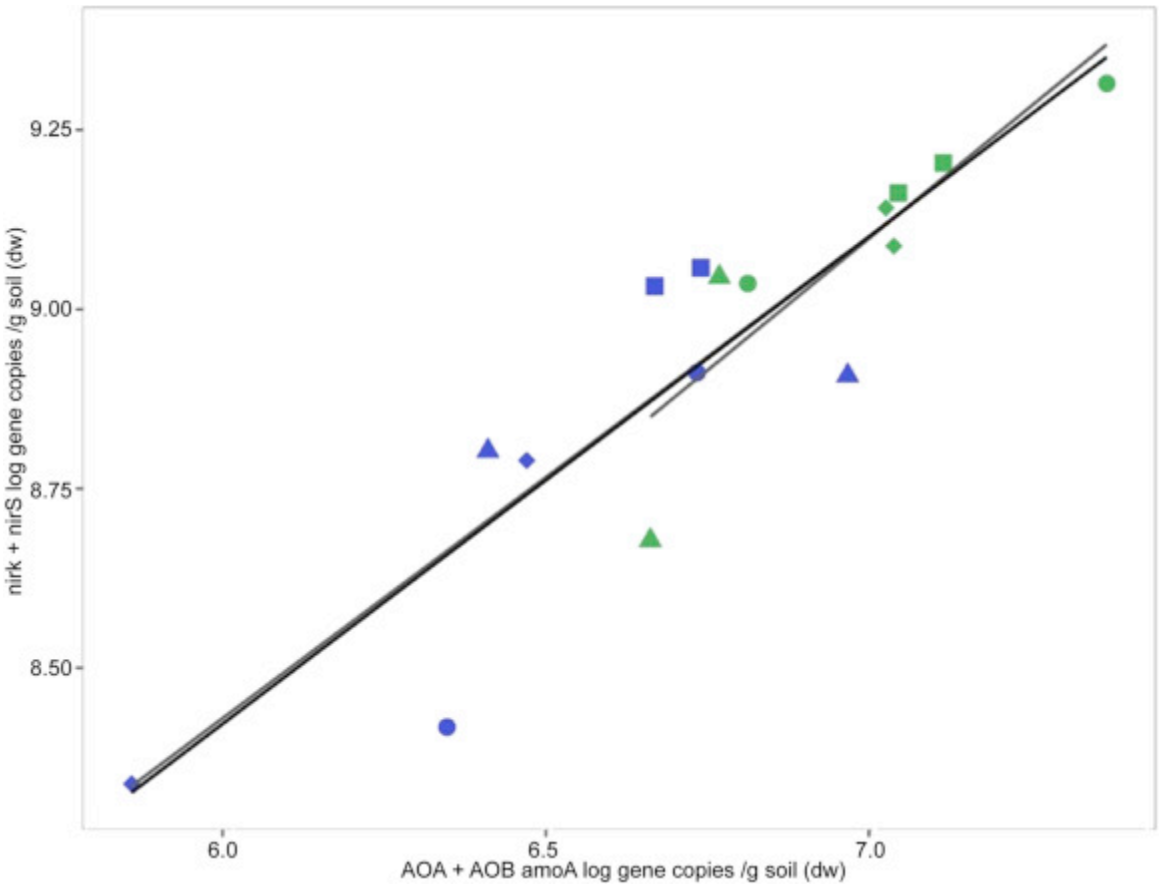

Figure 4.
Regression of the sum of denitrification (*nirK* and *nirS*) and ammonia-oxidation (AOA and AOB *amoA*) gene abundance by site ($p > 0.05$, *; $p > 0.01$, **, $p > 0.001$, ***). Sites are differentiated in the coordinate plot by colour (blue = Fairy Lake; green = San Juan) and tree species are differentiated by symbols (circle = hemlock; triangle = Douglas-fir; diamond = spruce; square = cedar).

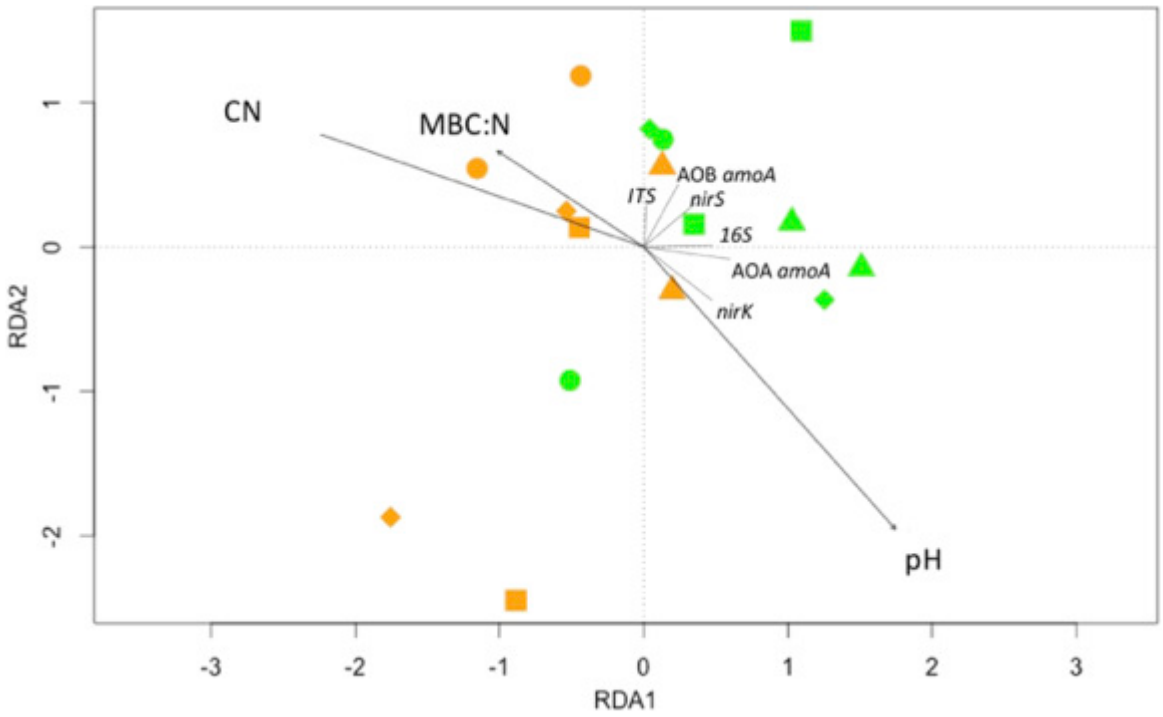

Figure 5.
Canonical redundancy analysis (RDA) and variation partitioning to determine the factors contributing to the abundance of functional gene abundance at Fairy Lake and San Juan sites. RDA was used to ordinate gene abundance measurements for total bacteria (*16S*), total fungi (ITS), AOA (*amoA*), AOB (*amoA*) and nitrite reducers (*nirK* and *nirS*) against forest floor physico-chemical factors (pH, total C, total N and C:N ratio). Sites are differentiated in the coordinate plot by colour (orange = Fairy Lake; green = San Juan) and tree species are differentiated by symbols (circle = hemlock; triangle = Douglas-fir; diamond = spruce; square = cedar).

5. Discussion

The relationships between rates of production and consumption of N and microbial parameters uncovered in this study provide insights into the mechanisms underlying differences in N cycling and availability in forest floors. The prominence of forest floor C:N ratio in the relationships indicate that site N status exerts a dominant influence on N cycling. The proportion of mineralized N taken up by microbial biomass, rather than the gross mineralization rate, appeared to be the primary driver of N release, consistent with conclusions of Mooshammer et al. (2014). While gross ammonification was influenced by the abundance of bacteria and fungi (i.e. overall saprotrophic activity), consumption of the mineralized NH_4^+ depended on microbial demand for N, as indicated by microbial C:N ratio. This is consistent with forest floors at these sites having different rates of N mineralization and nitrification despite similar rates of litter decomposition and C mineralization (Prescott et al., 2000b; Prescott et al., 2000c). The strong relationships between rates of most N transformation processes and microbial C:N ratio indicate that the N

status of microbes, *i.e.* the degree to which N is available excess to their needs, rather than their biomass or activity level, determined the amount of NH_4^+ that remained available in the forest floor. High microbial N status (*i.e.* low microbial C:N ratio) led to a smaller proportion of the NH_4^+ being consumed by microbes, and the resulting 'excess' NH_4^+ stimulated nitrifying organisms (as indicated by the relationships between microbial biomass C:N ratios and *amoA* gene abundances). Nitrate production then creates conditions conducive to denitrifying organisms as indicated by the relationship between AOA and AOB gene abundances and abundance of nitrite reductase genes (*nirK* and *nirS*).

The manner in which these interrelationships between site conditions, soil microorganisms and N cycling processes play out at the two study sites is illustrated in Fig. 6. Despite similar rates of decomposition and C mineralization between sites, the high C:N ratio in organic matter and microbes at Fairy Lake causes all of the mineralized NH_4^+ to be consumed by microbes and plants. In contrast, at San Juan, the low C:N ratio of the organic matter causes more N to be mineralized per unit C mineralized, and only a portion of this is consumed by microbes and vegetation. The resulting accumulation of NH_4^+ stimulates ammonia-oxidizers (as evident in the higher microbial gene abundance for AOA and AOB *amoA),* which liberate N in the form of nitrate. Some of this nitrate is consumed by microbes and plants, and some is used by denitrifiers, as evident in the detection of higher microbial gene abundance for *nirK,* and *nirS* in San Juan forest floors.

For many of the variables in this study, the influence of site N status on N cycling processes was greater than the influence of tree species. Observed gross ammonification rates support earlier findings of greater net N mineralization rates and higher bacterial:fungal ratio in forest floors at nutrient-rich, valley-bottom sites (including San Juan) than at nutrient-poor, mid-slope sites (including Fairy Lake) (Prescott et al., 2000a; Grayston and Prescott, 2005). Microbial gene abundances were also strongly affected by site, with forest floors from the San Juan site have a higher potential for nitrification and denitrification than those from Fairy Lake.

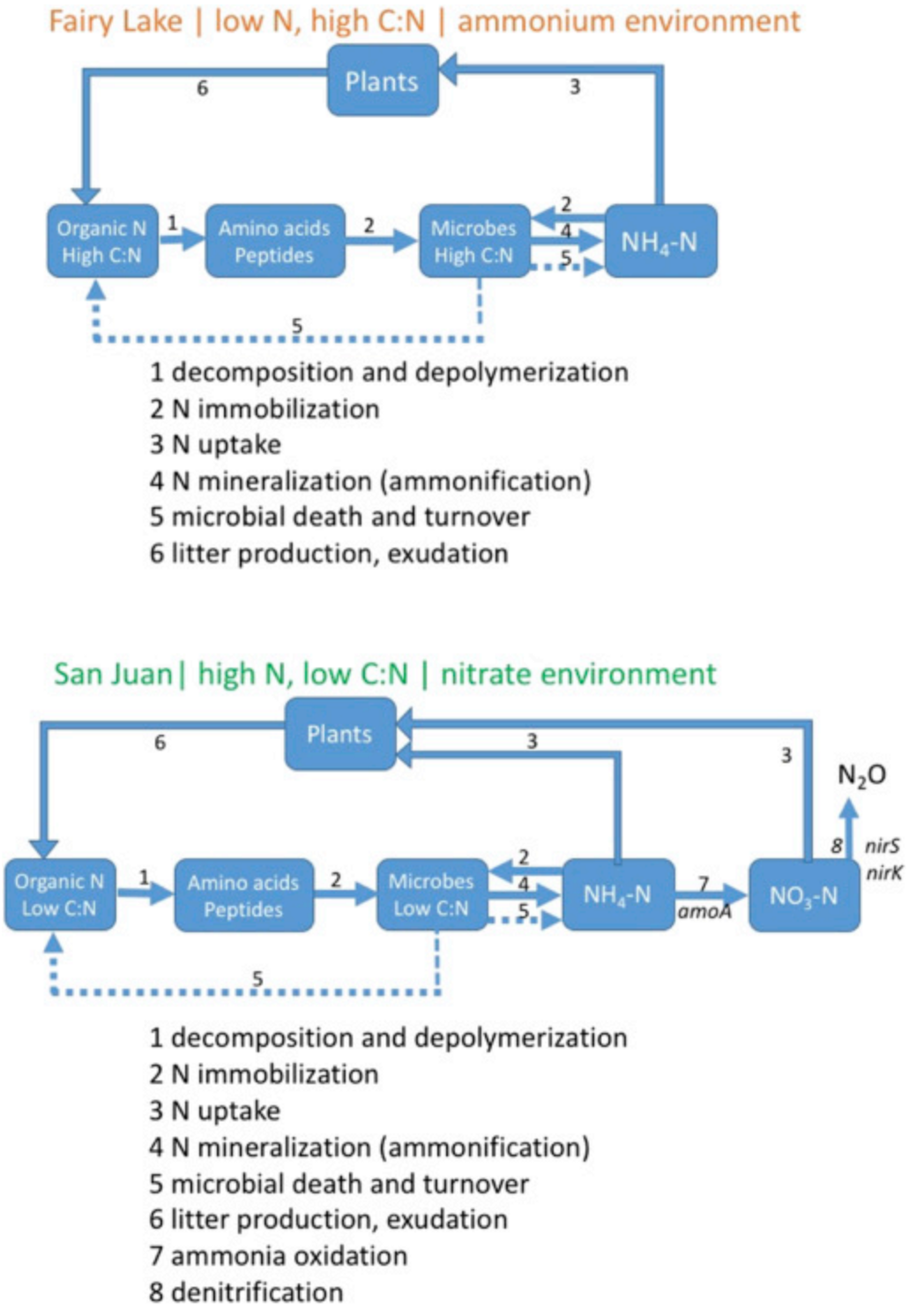

Figure 6.
Conceptual model of N cycling processes at the two study sites with differing N status – Fairy Lake and San Juan.

Forest floor C:N ratios were the primary feature of site influence on microbial biomass, gene abundance and N transformations. This is consistent with earlier findings that rates of N mineralization in forest floors along a site fertility gradient in Douglas-fir stands were most closely related to forest floor C:N ratios, with net mineralization being appreciable only at sites where the forest floor C:N ratio was less than 35. Similarly, Högberg et al., 2007a; Högberg et al., 2007b found forest floor C:N ratios to be as important as pH and base cations in predicting microbial community composition in forest floors in a boreal Fennoscandian forest ecosystem, while Chen and Högberg et al., 2007a; Högberg et al., 2007b found negative correlations between fungal:bacteria ratios, forest floor C:N ratios and gross mineralization rates in forest floors. Bates et al. (2011) found soil C:N ratio to be the best predictor for archaeal relative abundances, with higher C:N ratios leading to higher archaeal relative abundances.

Tree species effects were smaller than site effects, and were more pronounced for NH_4^+transformations than for NO_3^- transformations. Western red cedar had consistently high abundance of *16S* and AOA *amoA* at both sites, and higher rates of gross ammonification and NH_4^+ consumption, which made it the most ecologically extreme of the four tree species. This is consistent with previous studies highlighting the different N transformation pattern and microbial community structure in western red cedar forest floors (Turner and Franz, 1985; Prescott et al., 2000a; Prescott and Grayston, 2013). Douglas-fir and Sitka spruce had similar abundances of all targeted genes, which did not significantly differ from each other. Some tree species effects appeared to depend on site N status. For example, Sitka spruce and western hemlock differed in rates of net ammonification, but only at the nutrient-poor site, Fairy Lake. The data from this study support both direct effects of tree species on soil N cycling and soil microbial communities, but also highlight the context-dependency of tree species effects (Prescott and Vesterdal, 2013).

The positive correlation between gross ammonification rate and abundance of bacterial 1*6S* rRNA genes suggests an important role of bacteria in ammonification, which aligns with current thinking about N-cycling processes (Laverman et al., 2001; Kowalchuk and Stephen, 2001; Wallenstein et al., 2006). The significantly positive correlations between the abundance of AOA *amoA* genes and rates of both gross and net nitrification indicate that nitrification in these

forest floors is modulated primarily by archaeal ammonia-oxidation, with AOB playing a negligible role. Although archaea are considered to contribute little to soil microbial biomass (Gattinger et al., 2002; Bardgett and Griffiths, 1997), archaea in forest soils can have a functional role in N cycling akin to a keystone species (Prosser and Nicol, 2008; Verhamme et al., 2011). AOA are generally more abundant than AOB in acidic forest soils (Petersen et al., 2012) and AOA can have higher ammonia-oxidation rates relative to AOB under similar N availabilities (Wertz et al., 2012). The strong, positive relationship between the abundance of nitrite reductase genes (*nirK*, *nirS*) and the sum of AOA and AOB indicates that the genetic potential for denitrification in these forest floors was strongly influenced by ammonia oxidizer abundance. We measured gene abundance rather than directly assessing activity associated with specific genes; in other studies, gene abundances have shown a high degree of correlation with substrate concentrations and process rates (Wertz et al., 2012; Penton et al., 2013; Levy-Booth et al., 2014). Recent studies have characterized the comammox *Nitrosospira* species which contain enzymes that catalyze complete nitrification (van Kessel et al., 2015; Dairns et al., 2015), but these organisms were not considered in this study.

Bacterial *16S* and *nirK* varied amongst tree species, which suggests that tree species foster different abundances of denitrifying bacteria, in addition to the elevated AOA *amoA* in western red cedar plots. AOA *amoA* can oxidize ammonia via an alternate pathway that requires less oxygen than the bacterial (AOB *amoA*) channel, which enables ammonia oxidation in anoxic soils (Schleper and Nicol, 2010; Levy-Booth et al., 2014). Similarly, organisms that contain the gene *nirS* often do not contain *nirK,* which suggests that tree species-specific soil microbial communities are associated with specific denitrifying bacteria (Levy-Booth et al., 2014). Consistent with previous studies of forest floor microbial communities associated with these tree species (Grayston and Prescott, 2005 ;Turner and Franz, 1985), we found cedar forest floors to be more bacteria-dominated (*16S*) while forest floors of all four tree species were similar in fungal abundance (fungal *ITS*).

The net N transformation rates presented here were derived from the 24-h gross-N incubations, not a standard 28-day incubation for rate of net N mineralization such as in the previous study of forest floor N dynamics in the common garden experiment (Prescott et al., 2000a). The short-

term incubation study found tree species differences in N ammonification rates, but these were overshadowed by site effects. This is consistent with results of the longer-term incubations (Prescott et al., 2000a), although the values should not be directly compared, and more studies are needed to confirm the patterns. Nevertheless, this study demonstrates that a) differences between tree species and sites are discernible with these methods, and b) insights into the linkages between forest floor physico-chemical parameters, microbial gene abundance and biogeochemical cycling can be gained using these methods.

6. Conclusions

Quantification of key microbial marker genes involved in biogeochemical transformations were used to explore mechanistic links between site factors, tree species and N cycling processes. Rates of N transformation and microbial gene abundances were higher at the San Juan site, which had higher forest floor C:N ratios, higher microbial gene abundances related to nitrification and denitrification, and higher gross N transformation rates. Differences between the sites were related to site N status, as reflected in C:N ratios of the forest floor, and were more closely tied to rates of N consumption rather than gross mineralization. The relative contributions of AOA and AOB to gross and net nitrification in forests were mainly influenced by site N status. Tree species influenced gross and net ammonification and NH_4^+ consumption. Western red cedar forest floors were the most distinct of the four tree species, with highest rates of NH_4^+ transformation, and the most distinctive forest floor microbial communities in terms of *16S* and *nirK* gene abundances. The coupling of techniques for assessing ecosystem process rates with molecular techniques, such as functional gene abundances, can provide a greater mechanistic understanding of links between tree species and N transformation processes, as demonstrated in this study.

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Chapter IV. Are soil nitrogen transformations linked with functional genes under six common European tree species in a common garden experiment?

1. Abstract

Above and belowground linkages in relation to nutrient cycling is an area of increasing scientific interest and exploration. This is concomitant with increasingly precise and targeted technologies that provide methods to address research questions focused on causal relationships and mechanistic links between aboveground and belowground communities. Large-scale ecosystem processes, such as terrestrial nitrogen cycling, are fundamental to linking aboveground and belowground communities. We here explored the mechanistic links between six single tree species, across three sites with varying land use legacies in Denmark, on gross nitrogen transformations, as measured through the 15N isotope pool-dilution method. We also assessed soil microbial community composition in the mineral soil below these trees, as measured through of qPCR of fungal *ITS*, bacterial*16S*, and nitrogen cycling function genes: nitrate reductase enzyme genes *nirK*, *nirS*, and ammonia-monooxygenase archaea and bacteria (*amoA* AOA and *amoA* AOB). We found significant tree species effects on soil microbial communities, and gross and net ammonification and nitrification rates. Spruce soils were the most distinct both in terms of N transformation rates, but also in relation to soil microbial gene abundances. Beech, oak, and ash were the next most distinct tree species, with maple and lime typically indistinguishable from one another. Fungal *ITS* was the only gene that did not differ among tree species, with *amoA* AOB being marginally significant, while *amoA* AOA, bacterial *16S*, *nirK*, and *nirS*, all differing significantly among tree species. Through multiple linear regressions, we found pH to be a consistently important predictor of N transformation rates. We conclude that land use legacies have lasting influences on soil properties, which mediate tree species effects on soil microbial communities and in turn influence N transformation rates and likely other ecosystem processes.

2. Introduction

Determining how community interactions influence nitrogen cycling microorganisms is a key step forward in understanding how a legacy of land use, vegetation type, and industrial N deposition influence microbial ecosystem processes (Nemergut 2013). Identification of genes tied to specific ecosystem processes like nitrogen cycling are a useful way to explore patterns in the functional role of soil microbes (Levy-Booth et al. 2014), and specifically how microbial communities respond to changes in vegetation types, or across larger biogeographical scales. The biogeographic distribution of ammonia-oxidizing bacterial (AOB) communities in soil has been explored. Fierer et al. (2009), found that the composition of AOB communities was highly variable, even within similar ecosystem types. Examining studies across the globe, Fierer et al. (2009) found the strongest correlation with AOB community composition was temperature, noting similar AOB communities in locations with similar mean annual temperatures. Several studies have explored broad patterns in microbial abundance (Finlay 2002; Martiny et al. 2006), with the key take-home message often being tied to the vegetation composition and underlying soil factors, and may extend to include site nutrient status and N deposition rates.

Tree species influence soils, but two major questions remain about: 1. the consistency of tree species effects vs. the context-dependency of these effects, and 2. the strength or effect size of tree species compared with site factors (Prescott and Vesterdal, 2013). An ideal experimental design for resolving these lingering questions is that of a common garden forest experiment, as it controls for underlying differences in site conditions including characteristic soil properties and parent material. A series of these common garden forest studies are located throughout Canada (Prescott et al. 2000; Thomas and Prescott, 2000), Ireland (Prescott et al. 1995), France (Mareschal et al. 2013), Poland (Hobbie et al. 2006; Muller et al. 2015), and Denmark (Vesterdal and Rauland-Rasmussen, 1998; Vesterdal et al. 2008, 2012).

Decomposition is a key process in the cycling or nutrients, and links the aboveground and belowground plant, soil, and microbial communities (Bardgett and Wardle, 2012). Decomposition rates can be useful for understanding general patterns in nutrient turnover, however there are many instances where decomposition rates are not intuitive in this regard (Prescott, 2005). Within European broadleaved and coniferous forests, data on decomposition

rates can be a useful indicator of how N may be cycling in these different forests (Vesterdal et al. 2008). Another common garden experiment found tree species effects on decomposition rates in Poland (Hobbie et al. 2006). Decomposition rates can be used as a proxy for microbial activity, although there is a need to explicitly test soil microbial community responses by other means than mass loss (Prescott 2010). As a result of these studies we might hypothesize that tree species with higher rates of decomposition would have higher abundance of functional genes tied to N cycling. We may also hypothesize that higher inputs of N into a forest would correspondingly increase N transformation rates within the forest soils and an increase in soil microbial community abundance.

Forest overstories commonly exert a dominant effect on biogeochemical cycles, including on C and N cycling (Binkley and Giardina, 1998; Priha and Smolander, 1999; Vesterdal et al. 2008; Mueller et al. 2012). Tree species identity plays an important role in determining nutrient cycling dynamics, and one such mechanism is through differences in functional traits those tree species possess (Kattge et al. 2011). Functional traits can be especially informative for understanding N cycling dynamics, for example plant litter nutrient concentrations informs how tree species influence nutrient release and mineralization (Prescott 2005). A global meta-analysis of nitrogen cycling in terrestrial ecosystems (Booth et al. 2005) sheds some light on global patterns of N dynamics, but is likely complicated by confounding site factors, including plant associations with specific soil types. Booth et al. found gross N mineralization to be positively correlated with microbial biomass, and soil C and N concentrations. Nitrification was a log-linear function of N mineralization, increasing rapidly at low N mineralization rates and only differing slightly at higher N mineralization rates.

Staelens et al. (2012) found gross N mineralization rates differed significantly between oak and pine forest soils grown in an experimental forest in North Belgium. They observed the rate of gross N mineralization in oak soils was twice that of pine soils, N immobilization was observed only for ammonium, and nitrate production via oxidation was three times faster in pine soils while ammonium oxidation was similar between pine and oak soils. Not all fractions of N may be equally informative for explaining N traits in relation to N cycling, as demonstrated by Hobbie et al.'s (2010) work, where she did not find any correlations between leaf litter N

concentrations and higher N concentrations in roots or wood. Previous work using the same focal tree species (Hobbie et al. 2007), found net N mineralization, and net nitrification rates were highest in maple, followed by spruce, beech, oak, with lime having the lowest rate. In that study, maple had the highest soil pH compared with the other tree species, suggesting pH also strongly influences N cycling rates. Hobbie et al. (2006) found rates of nitrification were often limited by ammonification rates, which is consistent with previous studies (Walters and Reich, 1997; Finzi et al. 1998; Lovett et al. 2004). Soil C:N ratios may also inversely correlate with net N mineralization and nitrification rates (Lovett et al. 2004; Hogberg et al. 2006).

Mueller et al. (2012) explored the different impacts hypothesis as a means for explaining the effects of plant functional types (evergreen gymnosperm vs. deciduous angiosperm) on forest soil net N mineralization, compiling data from 35 studies in temperate and boreal regions. They concluded that tree species and plant traits have strong impacts on soil N availability, but traits like leaf habit and mycorrhizal associations were poor predictors. They provide evidence that tree species effects on net N mineralization were not consistent across sites, which they attributed to the modulating effects on other environmental factors and stand properties such as age and soil type. They conclude by encouraging the use of common-garden experiments to better evaluate tree species effects on soil N mineralization.

Vesterdal et al. (2008, 2012) considered a series of common garden tree species trials, with pure plots of broadleaf and coniferous species originally established in 1973 (Bang, 1973). These common garden tree species trials have evidenced tree species effects on ecosystem processes and functions, including soil organic carbon accumulation (Vesterdal et al. 2008), leaf litter decomposition rates (Vesterdal et al. 2012), and nitrogen throughfall rates (Christiansen et al. 2010). Vesterdal et al. (2008) showed forest floor and mineral soil C stocks differed when assessed 30 years after planting. Christiansen et al. (2010) measured inorganic nitrogen leaching rates using a subset of these same study sites. They found beech to have the highest mean rates of nitrate leaching, compared with oak, spruce, lime, maple, and ash. Christiansen et al. (2010) also measured annual mean N throughfall flux, spruce had the highest values, followed by maple, beech, and oak. Together these studies provide evidence of tree species effects on N cycling, and set the stage for us to determine which are the driving factors behind these patterns.

Using tree species to foster different soil environments, it is possible to differentiate N transformations and how N cycles function.

This study aimed to address the following questions: 1) Do tree species differ in soil N transformation rates? 2) Do tree species foster specific soil microbial communities? 3) Does tree species identity influence functional gene abundances related to nitrogen cycling?

We hypothesized that these six tree species would form three distinct clusters in terms of nitrogen transformations, as suggested by pH or decomposition rates in Vesterdal et al. (2008): ash, maple and lime trees will have the highest N transformation rates; which differ from oak and beech who have similar N transformation rates; which differ from spruce with the lowest rates of N transformations. For mineral soil microbial communities, we hypothesized differences between tree species, with the largest differences observed between 3 clusters according to decomposition rates: spruce vs. beech & oak vs. ash, maple & lime. We specifically hypothesised that microbial communities in spruce mineral soils will have the highest fungi:bacteria ratio compared with ash, maple, and lime, which will have the lowest fungi:bacteria ratios, with oak and beech intermediate.

3. Methods

3.1 *Study site and sampling scheme*

Three sites were selected from the Danish tree species trials which were established in 1973 (Bang, 1973) as monoculture 0.25 ha stands of each tree species, across a pH and soil nutrient gradient. Mattrup had an agricultural land-use history, whereas both Vallø and Viemose were previously forested sites (beech forest). Six tree species were sampled at each site: beech (*Fagus sylvatica* L), pedunculate oak (*Quercus robur* L.), lime (*Tilia cordata* L), sycamore maple (*Acer pseudoplatanus* L), Norway spruce (*Picea abies* (L) Karst.) and ash (*Fraxinus excelsior* L, which was not replicated at Vallø as those trees died).

Four mineral soil cores taken from 0-10 cm depth were sampled in the cardinal points of three 5-m radius subplots. Soil samples were composited in the field yielding 3 replicate soil samples for each tree species per site (n=3). Forest floors with FH layers were only developed under Norway

spruce, so an additional 3 replicate forest floor samples were collected in Norway spruce by removing the L layer to expose the F-H layer. Soil samples were placed in coolers in the field and transported back to the University of Copenhagen for subsequent analyses. Soils were sieved through a 2-mm mesh sieve and one 15-g subsample was immediately removed for the ^{15}N pool-dilution analysis. A portion of each composite sample was stored at -20°C for DNA extraction.

3.2 *Gross rates of N ammonification and nitrification*

The ^{15}N pool-dilution method (modified from Drury, 2008) was used to determine gross rates of N ammonification and nitrification, with each soil sample analysed in triplicate. Six 15 g subsamples from each soil sample were transferred to 250 mL Nalgene jars and sealed with parafilm (n = 3 subsamples for both $^{15}NH_4^+$ and $^{15}NO_3^-$). The parafilm seal was punctured to enable gas exchange and maintain aerobic conditions throughout the experiment, and samples were then incubated at room temperature for 24 hours prior to initial ^{15}N fertilization treatments. 4 mL of $^{15}NH_4Cl$ solution (99 atom%; Sigma Aldrich) or 4 mL of $K^{15}NO_3$ (99 atom%; Sigma Aldrich) was added to the soil samples in each respective jar, with an equivalent application rate of 12 μg N g^{-1} soil. Labelled N was injected into the samples in 1 mL intervals four times, and gently homogenized to ensure isotopic labelled N was applied uniformly throughout the soil sample, and the parafilm seal was replaced.

A 5 g soil subsample was removed from each Nalgene jar and placed into a 50 mL falcon tube, immediately after ^{15}N fertilization, 24 hours after ^{15}N fertilization, and 48 hours after ^{15}N fertilization. 50 mL of 1.0 M KCl was added to each falcon tube, shaken for 1 hour, and filtered through glass fibre filters in a vacuum syringe filtration system. A 15 mL subsample of each extract was reserved for ammonium and nitrate concentration analyses which were determined colourimetrically using a flow injection analyser using the indophenol-blue and cadmium reduction methods for NH_4-N and NO_3-N, respectively. Due to high anticipated levels of N we prepared 10x dilutions of each KCl extract. The remainders of the KCl extracts were used for microdiffusion acid trapping of $^{15}NH_4$-N and $^{15}NO_3$-N, with the sequential addition of MgO and Devarda's alloy following the International Atomic Energy Agency protocol (IAEA, 2001). Acid traps were dried, packaged in tin cups, and analysed by elemental flash combustion analysis (EA 1110, Thermo Scientific, Bremen, Germany) in combination with stable isotope ratio mass

spectrometry (Delta PLUS, Thermo Scientific, Bremen, Germany) at the University of Copenhagen's Isotope Laboratory. Net rates of ammonification and nitrification were calculated as the difference in inorganic N between the incubated samples and the initial soil extractions.

The gross rates of ammonification, nitrification and microbial consumption were calculated following Hart et al. (1994), such that gross ammonification is equal to: ((Initial NH_4^+ concentration- Final NH_4^+ concentration)/extraction time) x [(log(APE ^{15}N Initial/APE ^{15}N Final)/log(Initial NH_4^+ concentration/Final NH_4^+ concentration)]. Gross consumption was calculated as: Gross ammonification – ((Final NH_4^+ concentration- Initial NH_4^+ concentration)/extraction time). Net ammonification was calculated as Gross ammonification – Gross consumption. Nitrification rates were similarly calculated.

3.3 *DNA extraction, amplification, and quantification*

DNA was extracted from 0.25 g of field-moist mineral soil or 0.1 g of field-moist forest floor using the MoBio Power Soil DNA isolation kit (MoBio Laboratories, Inc., Carlsbad, CA). DNA quality and concentration was measured using a nanodrop spectrophotometer (Thermo Fisher Scientific Inc., Wilmington, DE) and electrophoresis in agarose gels (1% w/v in TBE), then stored at -20^{o}C prior to amplification. All qPCR runs were completed in duplicate using 20 µl reactions consisting of: 10.0 µl of SYBRGreen (2x) PCR Master Mix (Life Technologies Corp., Carlsbad, CA, USA), 0.25 µl of each forward and reverse primer, 1 µl of DNA template, and 8.5 µl of nuclease-free water. All reactions were run on a Stratagene Mx3000P Real-Time PCR system (Stratagene, La Jolla CA, USA). The specificity of the PCR amplification was tested via the inspection of the melting curves that were prepared at the end of each PCR run. PCR products were also run on a gel to verify the presence of a single band of the correct size.

Table 1 contains information relating to the specific primer pairs and their respective references for all qPCR runs. qPCR conditions for fungal ITS were 95° C for 10 minutes followed by 40 cycles of: 95° C for 30 s, 55° C for 30 s, and 72° C for 30 s. Standard curves for fungal *ITS* were constructed using ten-fold serial dilutions of *Fusarium avenaceum* genomic DNA, which ranged from 10^3 to 10^9 gene copies. qPCR conditions for bacterial *16S* were 95° C for 10 minutes followed by 40 cycles of: 95° C for 15 s, 53° C for 30 s, and 72° C for 20 s, and we used ten-fold

serial dilutions of *Pseudomonas putida* genomic DNA, which ranged from 10^2 to 10^7 gene copies.

We quantified Cd-nitrite reductase (*nirS*) and Cu nitrite reductase (*nirK*) with qPCR conditions of 10 min at 95 ^{o}C and 40 cycles of: 95 ^{o}C for 1 min, 60 ^{o}C for 1 min and 72 ^{o}C for 1 min, with fluorescence quantified at extension (Levy-Booth and Winder, 2010). The standard curve for *nirS* and *nirK* used 10-fold serial dilutions of 10^1 to 10^7 gene copies from *Pseudomonas putida.* All gene copies were calculated using exact soil extraction weights, and are presented in analyses as log gene copies/ul/g of dry weight soil.

We quantified the number of *amoA* gene copies for bacterial ammonia oxidizers (*amoA* AOB) and archaeal ammonia oxidizers (*amoA* AOA). These primers generate amplification products of 491 bp for bacterial *amoA* and 440 bp for archaeal *amoA*, respectively. The amplification was performed at 95°C for 10 mins followed by 40 cycles of: 95°C for 30 s, 57°C/58°C for 30 s and 72°C for 60 s/45 s (AOA/AOB), and concluded with a high-resolution melting curve. As standards for AOB *amoA* quantification, we PCR-amplified the *amoA* gene of the *Nitrosomonas europaea* ATCC19718-derived lux-marker strain (pHLUX20) [29] using the primers amoA-1F and amoA-2R and cloned it into the E. coli pCR 2.1-TOPO vector (Invitrogen, Carlsbad, CA) (see Feld, 2015). We used the fosmid clone 54d9 as the standard for the AOA *amoA* (Feld, 2015). Standard curves were constructed from the extracted plasmids using tenfold dilutions of 10^1 to 10^7 gene copies AOB *amoA* copies per microliter, and 10^1 to 10^6 gene copies AOA *amoA* copies per microliter.

3.4 *Statistical analyses*

We used a mixed effects model and one-way analysis of variance (ANOVA) with site as a blocking factor, to determine differences between tree species and sites on nitrogen transformation rates (using replicate sample averages such that n=3 for all response variables, and n=3 for the number of sites). Since ash was missing at one of our sites, we used an unbalanced two-way ANOVA. Analyses had no interaction terms as there was no proper replication of tree species within sites (n=6 for Mattrup and Viemose, n=5 for Vallø; see Appendix 2 for R code). We used Tukey tests and least square means to make paired

comparisons between the 6 species. To determine which explanatory variables (e.g. environmental and gene copy data) most influenced rates of nitrogen transformation we used multiple linear regressions. We also explored relationships between site and soil properties, soil microbial gene copies, and N transformation rates through multiple linear regressions and principal component analyses. All analyses were completed in R version 3.3.1 (R Foundation for Statistical Computing, 2016) using the vegan (Oksanen et al., 2016), MASS (Venables et al., 2002) and lmer (Bates et al., 2016) packages for the mixed effects models and principal components analyses, the ggplot2 (Wickham, et al., 2017) package for all bar charts, lsmeans (Lenth et al., 2015) for Tukey's post hoc comparisons.

Table 1. Group-specific primers for qPCR gene quantification assays

Primer target	*Primers*	*Primer Sequence (5'-3')*	*Reference*
All Bacteria (16S rRNA)	Eub338 Eub518	ACT CCT ACG GGA GGC AGC AG ATT ACC GCG GCT GCT GG	Fierer et al. (2005)
All Fungi (ITS)	BITS B58S3	ACCTGCGGARGGATCA GAGATCCRTTGYTRAAAGTT	Bokulich and Mills (2013)
AOA[a] *(amoA)*	*amoA*-23F *amoA*- 616R	ATG GTC TGG CTW AGA CG GCC ATC CAT CTG TAT GTC CA	Francis et al. (2005)
AOB[b] *(amoA)*	*amoA*-1F *amoA*-2R	GGG GTT TCT ACT GGT GGT CCC CTC KGS AAA GCC TTC TTC	Rotthauwe et al. (1997)
Cd-nitrite reductase (*nirS*)	*nirS*-1F *nirS*-3R	CCT AYT GGC CGG CRC ART GCC GCC GTC RTG VAG GAA	Braker et al. (1998)
Cu-nitrite reductase (*nirK*)	*nirK-1F* *nirK-1R*	GGG CAT GAA CGG CGC GCT CAT GGT G CGG GTT GGC GAA CTT GCC GGT GGT C	Braker et al. (1998)

[a]Ammonia Oxidizing Archaea, [b]Ammonia Oxidizing Bacteria

4. Results

4.1 *Nitrogen concentrations and transformation rates under six tree species*

There were no significant differences in soil NH_4^+ concentrations among the tree species (Fig. 1 A). Net NH_4^+ ammonification (or immobilization in this case) was greatest in ash, with significantly different but negligible rates of immobilization observed in lime and spruce soils (net ammonification; Fig. 1C). All gross rates of NH_4^+ transformations differed significantly between tree species (Table 2). Gross ammonification rates were greatest in ash soils, which significantly differed from beech, maple, and lime, all of which in addition to oak, were significantly greater than in spruce (Fig. 1E, $p = 0.001$). Gross NH_4^+ consumption was also

greatest in ash, which differed significantly from lime and spruce (Fig. 1G, $p < 0.001$), and almost beech (see Supplementary file with data from paired contrasts tests, p=0.06).

There were no significant differences in NO_3^- concentrations among the tree species (Fig. 1 B). Net nitrification rates did not differ significantly between tree species (Fig. 1D, $p = 0.59$), and were generally lower than ammonification rates. All gross rates of NO_3^- transformations differed significantly between tree species (Table 2). Gross nitrification was highest in spruce and oak, which differed significantly from lime which had the lowest observed rates (Fig. 1F, $p < 0.001$). Gross NO_3^- consumption rates were again highest in oak and spruce soils, with lime soils differing significantly from oak and spruce while having the lowest rates of NO_3^- consumption (Fig. 1H, $p < 0.001$).

4.2 *Gene copy abundances under six tree species*

Spruce soils tended to have the lowest and most variable abundances of gene copies of the six tree species. There were no significant differences among the six tree species for fungal *ITS* gene copies (Fig. 2A). Spruce had the lowest abundances of bacterial *16S* gene copies, which differed significantly from ash, beech, lime, maple, and oak (Fig. 2B, $p = 0.004$). For denitrifying bacteria (*nirS,* and *nirK),* spruce was the most distinct with the lowest abundance which differed significantly from ash, beech, lime, and maple (Figs. 2C and 2D, $p < 0.001$ each, respectively). Ammonia-oxidizing archaea (AOA *amoA*) were lowest in abundance under spruce soils, which differed significantly from beech, lime, maple, and oak (Fig. 2E, $p < 0.001$). Ammonia-oxidizing bacteria were most abundant in maple, which differed from beech, but not ash, lime, maple or spruce (Fig. 2F, $p = 0.065$). The ratio of fungi to bacteria was greatest in spruce, which differed significantly from all other species (Fig. 3A, $p = 0.047$). Ammonia-oxidizing archaea to ammonia-oxidizing bacteria ratios were greatest in beech, which differed significantly from spruce (Fig. 3B, $p = 0.016$).

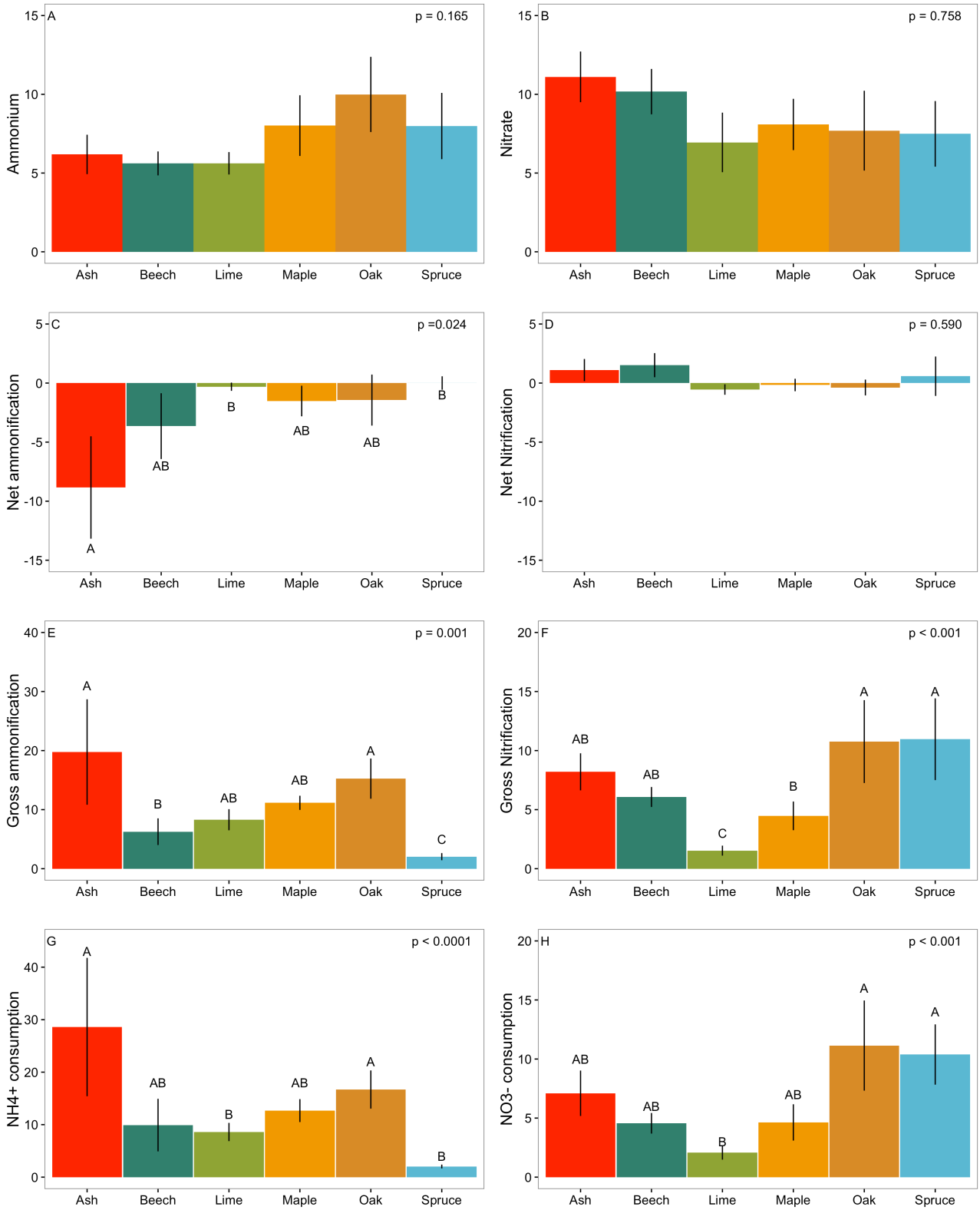


Fig. 1 Means ± standard errors of total ammonium (NH_4^+; A) and nitrate (NO_3^-; B) concentrations (mg N/kg soil dry weight), and net rates of ammonification (C) and nitrification (D), gross ammonification (E), ammonium consumption (F), nitrification (G), and nitrate consumption (H), averaged across sites.

Table 2. Mean values (followed by standard error) for mineral soil chemistry parameters among tree species: pH, total C, total N, C:N ratio. Letters indicate significant differences in least square means of tree species effects (ash, beech, lime, maple, oak, spruce, df=5) following Analysis of variance (Type III).

Species	***pH***	***C %***	***N%***	***C:N***
Tree species				
p*	< 0.001	< 0.001	< 0.001	< 0.001
Ash	4.29 (0.19)*a*	3.08 (0.14)*b*	0.26 (0.01)*b*	12.0 (0.1)*b*
Beech	3.49 (0.04)*bc*	3.38 (0.34)*b*	0.24 (0.02)*b*	14.2 (0.4)*b*
Lime	4.23 (0.12)*a*	3.65 (0.32)*b*	0.26 (0.02)*b*	13.8 (0.5)*b*
Maple	4.36 (0.09)*a*	3.10 (0.22)*b*	0.24 (0.01)*b*	13.0 (0.2)*b*
Oak	3.60 (0.05)*b*	4.87 (0.78)*b*	0.33 (0.04)*b*	14.2 (0.5)*b*
Spruce	3.32 (0.06)*c*	7.77 (0.05)*a*	0.42 (0.08)*a*	17.0 (1.0)*a*
Sites				
p*	0.003	< 0.001	< 0.001	< 0.001
Mattrup	4.15 (0.13)*a*	2.70 (0.11)*b*	0.21 (0.01)*b*	13.1 (0.2)*b*
Vallø	3.61 (0.11)*c*	7.22 (1.24)*a*	0.42 (0.05)*a*	16.3 (0.6)*a*
Viemose	3.76 (0.08)*b*	3.69 (0.09)*b*	0.28 (0.01)*b*	13.4 (0.3)*b*

4.3 Multiple linear regressions and principal components analysis

We used multiple linear regressions to determine the best predictors of our measured gross and net nitrogen transformation rates including soil characteristics and gene copy abundances (Table 3). Gross ammonification rates were positively correlated with soil C:N ratio, pH, *16S* gene copies, and negatively correlated with fungal *ITS* gene copies. Gross ammonium consumption was negatively correlated with fungal *ITS* gene copies and positively correlated with pH. Net ammonification was positively correlated with soil C:N ratio and fungal ITS gene copies. Gross nitrification was inversely related to both pH and soil C:N ratio. Gross nitrate consumption was negatively related to soil C:N ratio, pH, and fungal *ITS* gene copies. Net nitrification was negatively correlated to pH, soil C:N ratio, *amoA* AOB and positively correlated with *16S* gene copies.

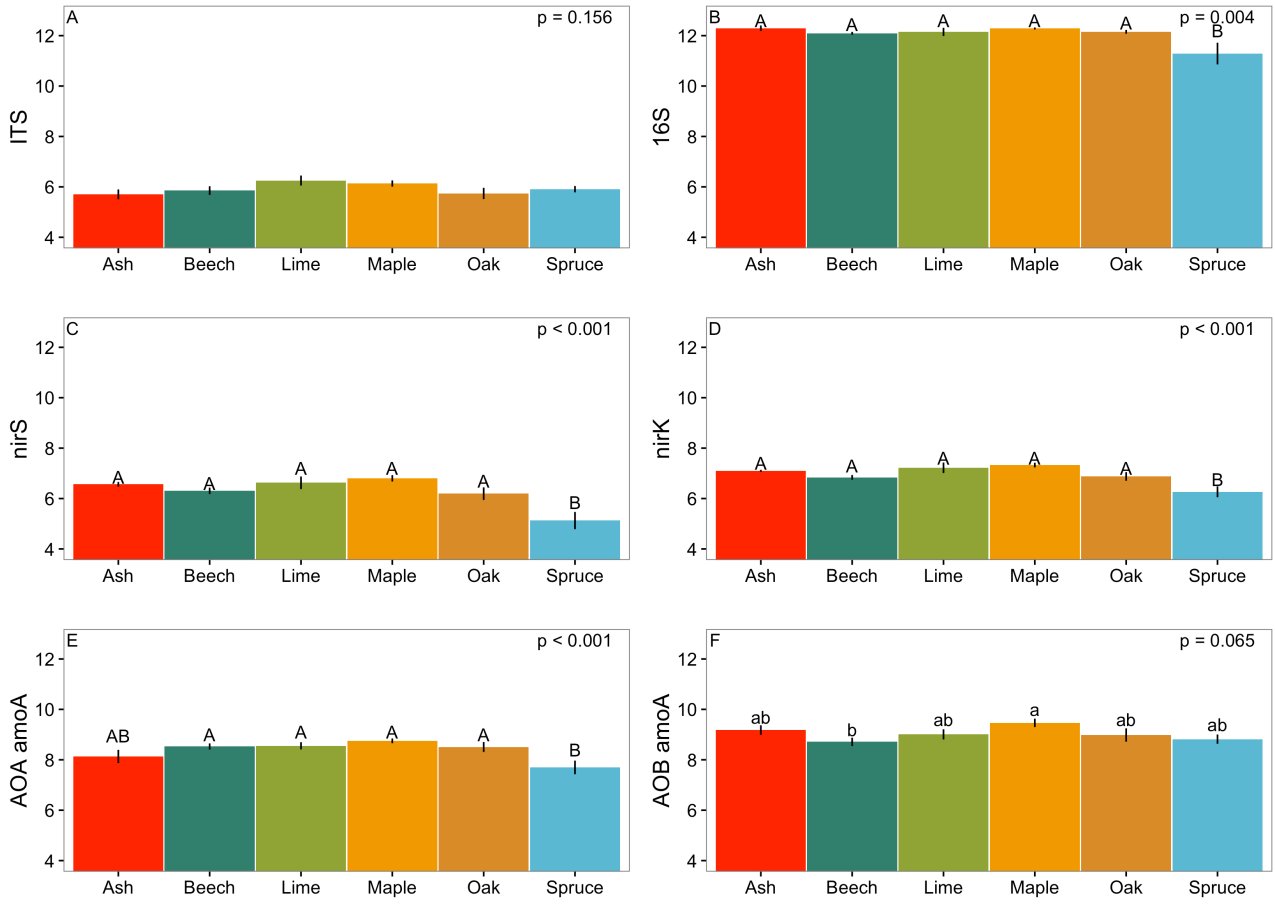


Fig. 2 Mean ± standard error of the log gene copies for fungal ITS, bacterial 16S, denitrifying bacteria nirk and nirS, and ammonia-oxidizing bacteria and archaea, averaged across sites.

Table 3. Multiple linear regressions of gross and net N ammonification, nitrification, and consumption rates with adjusted R^2 values, with best models selected using AIC values.

Gross ammonification = pH + C:N+ *16S* - *ITS*			
Variable	Coefficient	t value	p-value
pH	9.065	2.670	0.010
Soil C:N ratio	1.240	1.439	0.156
Bacterial *16S* gene copies	4.619	2.130	0.038
Fungal *ITS* gene copies	-8.044	-3.287	<0.001
F-statistic: 5.546 on 4 and 46 degrees of freedom; Adj. R2: 0.267; p-value: 0.0009			
Gross NH4+ consumption = pH - *ITS*			
Variable	Coefficient	t value	p-value
pH	11.723	3.232	0.002
Fungal *ITS* gene copies	-11.587	-3.365	0.001
F-statistic: 10.7 on 2 and 48 degrees of freedom; Adj. R2: 0.279; p-value: 0.0001			
Net ammonification = -C:N ratio + *ITS*			
Variable	Coefficient	t value	p-value
Soil C:N ratio	1.090	2.984	0.004
Fungal *ITS* gene copies	4.462	3.020	0.004
F-statistic: 8.683 on 2 and 48 degrees of freedom; Adj. R2: 0.235; p-value: 0.0006			
Gross nitrification = -pH -C:N ratio			
Variable	Coefficient	t value	p-value
pH	-6.794	-2.562	0.010
Soil C:N ratio	-1.617	-2.689	0.009
F-statistic: 4.286 on 2 and 48 degrees of freedom; Adj. R2: 0.12; p-value: 0.02			
Gross NO3- consumption = - pH- C:N - *ITS*			
Variable	Coefficient	t value	p-value
pH	-3.965	-1.535	0.132
Soil C:N ratio	-1.137	-1.876	0.067
Fungal *ITS* gene copies	-2.542	-1.385	0.173
F-statistic: 1.81 on 3 and 47 degrees of freedom; Adj. R2: 0.046; p-value: 0.158			
Net nitrification = pH – C:N - *amoA* AOB + *16S*			
Variable	Coefficient	t value	p-value
pH	-2.441	-2.400	0.021
Soil C:N ratio	-0.415	-1.655	0.105
Bacterial *16S* gene copies	1.115	1.762	0.085
amoA AOB gene copies	-1.064	-1.490	0.143
F-statistic: 3.275 on 4 and 46 degrees of freedom; Adj. R2: 0.15; p-value: 0.019			

Ammonification rates: mg N kg^{-1} soil (dw) d^{-1}; nitrification rates: mg N g^{-1} soil (dw) d^{-1}; microbial C and N, mg N g^{-1} soil (dw); Bacterial 16S, Fungal ITS, AOA and AOB amoA: genes g^{-1} soil (dw).

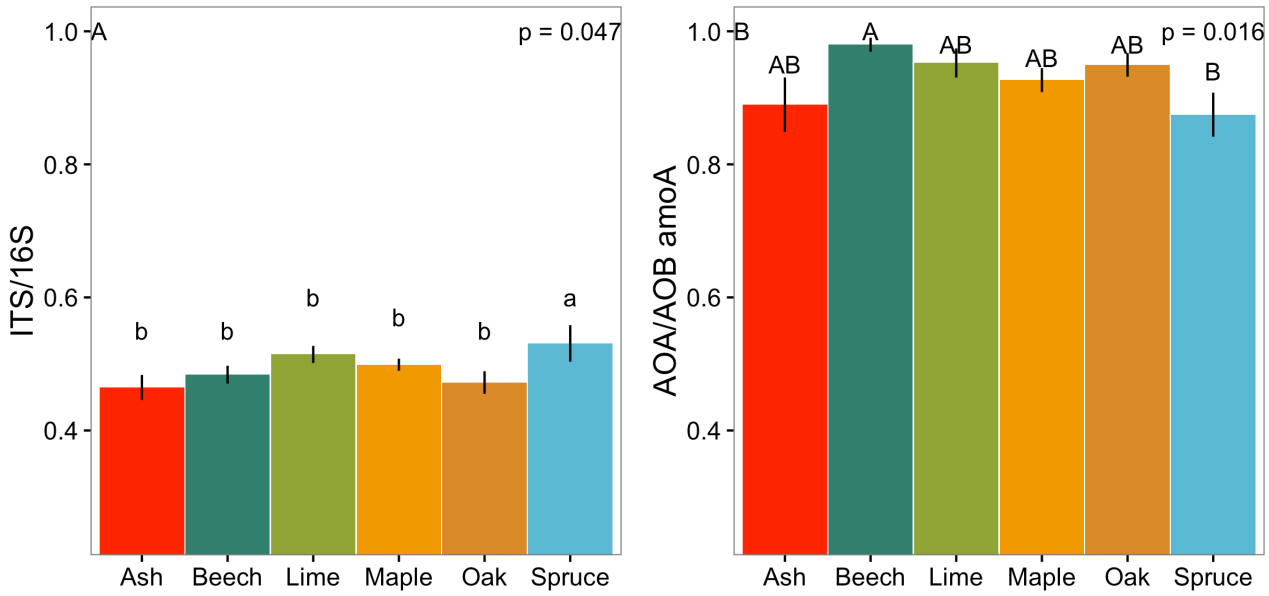


Fig. 3 Mean ± standard error of the ratios of bacterial *16S*:Fungal *ITS*, and AOA:AOB *amoA* (all data are log gene copies and averaged across sites) between the six tree species.

Principal component analysis (PCA) was used to determine the clustering of all response variables (gene abundances, nitrogen transformation rates, and soil characteristics) among the three sites and six tree species (Fig. 4). Mattrup clustered out from Vallø and Viemose, (Fig. 4). Tree species differed most along principal component 1 which explained 31% of the variation and was mainly related to pH, C:N ratio, and microbial gene abundances. The three sites differed mostly along principal component 2, which explained 20.4% of the variation and was mainly related to gross and net ammonification and nitrification transformation rates and nitrate concentrations. Together, this suggests tree species exerted a strong influence on soil pH and C:N ratio, and the soil microbial communities. Nitrogen transformations were strongly linked with apparent site differences, with faster rates of transformation at Mattrup, which was the former cropland site.

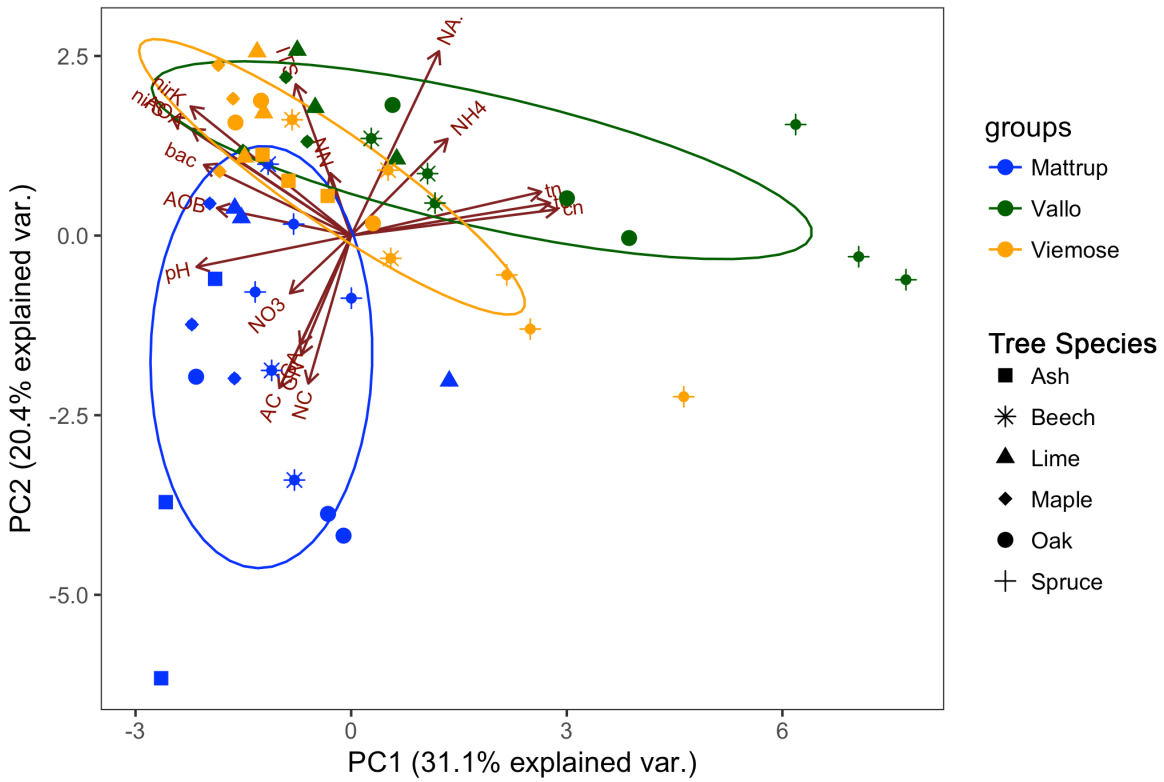


Fig. 4. PCA analysis showing the clustering of all response variables (gene abundances, nitrogen transformation rates, and soil characteristics) among the three sites and six tree species.

5. Discussion

Tree species identity influenced ecosystem function and soil microbial community composition and function, which supports our hypotheses and is consistent with previous tree species effects research (Ribbons et al. 2016; Grayston and Prescott, 2005; Hogberg et al. 2001; Hogberg et al. 2013). While net rates for nitrification and ammonification were not different between tree species, species differences emerged when gross rates of N transformation were assessed. In general, we found greater effects on NH_4^+ transformation rates, compared to NO_3^-. These observed differences clustered into groups in contrast with hypotheses based on decomposition rates (Vesterdal et al. 2008). Ash had the highest gross ammonification and NH_4^+ consumption rates while oak, maple, lime, and beech all clustered together with intermediate rates of gross ammonification and NH_4^+ consumption. Partially consistent with our hypotheses, spruce clustered out separately from other tree species for extractable NH_4^+. Spruce grouped together with oak in terms of NO^{3-} with the highest rates compared to the other four tree species. Ash,

beech, and maple were intermediate in gross nitrification and NO3- consumption rates, with lime having the lowest.

For both soil microbial community composition, and functional genes tied to nitrogen cycling, we found evidence of tree species effects. Bacterial *16S* gene copy abundance followed similar trends to ammonification rates, with the greatest abundance in ash, and the least in spruce. Fungal *ITS* gene copy abundance was greatest in lime, maple, and beech, and lowest in spruce and oak. Fungal:bacterial ratios were greatest in spruce, and the lowest in ash, corresponding to the observed trends in bacterial *16S* gene copy abundances. Spruce continued to be distinct from other tree species, occasionally clustering together with oak, but typically having the lowest gene copy abundances for both denitrifying bacteria (*nirK* and *nirS*), as well as ammonia-oxidizing bacteria and archaea (*amoA* AOB and AOA).

Ammonia-oxidizing bacteria trends were closely linked with tree species effects on soil pH, soil and leaf litter C:N ratios, and decomposition rates. This is consistent with our hypotheses about the connections between N cycling functional genes like AOB. AOA was largely consistent with these predictions as well, but a large variability in ash soils was unexpected. Szukics et al. (2012) experimentally found evidence that the contribution to ammonia oxidation by either AOA or AOB was selectively influenced by soil properties such as soil moisture, or amendment with either NH_4^+ or NO^{3-}. In their study, Szukics et al. compared two forests with contrasting N amendments and soil moisture regimes over a short-term incubation. In one forest, AOA abundance was tied to differences in water content, whereas AOB responded to the N amendment treatments. In contrast, the second forest in their experiment, the AOA abundance was tied to N addition treatments, which suggests that microbial communities selectively responded to changes in environmental factors like moisture and nitrogen availability. While we did not experimentally add N to our forest soils, the selective response of the soil microbial community to changes in environmental factors is consistent with our results.

Previous studies have found soil under ash to be high in pH and base cations (Reich et al. 2005), which correlated with higher bacterial 16S abundances, and lower fungal ITS abundances (Langenbruch et al. 2012). In our study, ash soils had the highest gross ammonification and NH_4^+

consumption rates, and intermediate gross nitrification and NO_3^- consumption rates, compared to the other tree species. Ash had the greatest bacterial 16S gene copy abundances, which is consistent with previous studies. In general, we predicted ash soil would have higher N transformation rates, similar with maple and lime, based upon these three tree species having higher soil pH and higher rates of leaf litter decomposition compared to beech, oak, and spruce (Vesterdal et al. 2008). Ash, maple, and lime can be characterized as mull-forming tree species, and are associated with greater earthworm biomass, which would aid in nutrient cycling (Neriynck et al. 1999). These mull-forming tree species generally lead to soils with higher pH, base saturation, and porosity (Neriynck et al. 1999) which would be conducive to faster N cycling.

Beech forests are known to have an association with low soil pH and a low C:N ratio (Langenbruch et al. 2012), factors which are known to lead to an increase in fungal abundances (Rousk et al. 2009; Strickland and Rousk, 2010). Compared with other deciduous tree species, beech forests are characterised by slow rates of litter decomposition, similar to oak leaves in their rates of decay (Vesterdal et al. 2008), and can be characterized as mull-moder forming tree species (Neirynck et al. 1999). These moder-forming tree species can influence soils to have lower pH and base saturation, compared with the mull-forming tree species, as well as lower soil porosity and aeration (Neirynck et al. 1999). In both the oak and beech, we would expect fungal *ITS* abundance to increase and bacterial *16S* abundance to decrease, partially as a response to recalcitrant litter inputs, as well as the lower pH of the soils.

Spruce soils are characterized by slow decomposition rates, and build up a layer of slowly decomposing leaf litter material (forest floor), which is not favoured by earthworms in contrast to the deciduous species. Spruce soil pH is typically lower than all of the deciduous species, as a result of highly acidic needle inputs. We would have expected fungal *ITS* abundance to be high and bacterial *16S* abundance to be lower due to the high pH in spruce soils. Correspondingly, we would predict slower rates of nutrient cycling in spruce soils (mineral and forest floor), as well as decreased abundances of nitrifying and denitrifying bacteria compared with the deciduous tree species. Our data confirm that spruce is indeed the most distinct tree species compared with the broadleaves in terms of N transformations, soil pH, and soil C:N ratios.

Our study supports a broad body of evidence demonstrating large differences between the broadleaf and evergreen functional groups, in relation to tree species effects on soil properties, N transformation dynamics, and soil microbial communities. However, we also observed some differences between broadleaf species, mainly beech, lime, or ash, suggesting additional functional traits such as leaf N concentration and decomposability are useful for classifying tree species effects on soils. Previous research using only net N incubations provide data that give us a single snapshot of how these soils may be responding to different tree species. Through the use of the ^{15}N pool-dilution method in conjunction with detailed qPCR analyses we were able to go "behind the curtain" of this snapshot and look at real time production and consumption of nitrogen.

6. Conclusions

We have demonstrated that tree species effects extend to mineral soil chemistry, especially influencing soil pH, and C and N concentrations. These altered soil chemical properties further alter microbial community structure in general, with bacterial community differentiating among species, while fungal community was less responsive in mineral soil. This pattern is possibly due to the fact that the forest floor of spruce may be more biologically active with regards to fungi attempting to break down spruce needles and litter input. Changes in soil pH also lead to changes in ecosystem function, as evidenced by differences in genetic potential for nitrification and denitrification, and thus specifically influencing ecosystem functions lie N cycling. This also suggests tree species effects would also differ along sites with distinct land use histories, specifically any former land-use that may significantly carry a legacy in soil pH. Furthermore, tree species effects extended to differences in soil gross N transformations, and functional genes tied to N cycling. Together, this study lends support to observed tree species effects on soil chemistry and the resulting microbial community composition and function with regards to nutrient cycling in forest soils.

Chapter V. A serenade for the role of humble roots: Tree species effects on rhizosphere soil microbial communities in monoculture and mixtures

1. Abstract

While aboveground drivers of ecosystem processes are widely researched, the field of belowground ecology and the linkages between above- and belowground ecosystems has really exploded (Wardle et al. 2004), including expanding into functional traits (Bardgett et al. 2014). The root-soil interface is considered a highly active zone for root exudation, nutrient uptake, and most likely the first area where belowground tree species effects would influence soil microbial communities. In this study, we used the Rhizotron to explore monoculture and mixtures tree species effects on rhizospheric soil communities, and whether or not this feeds back to influence tree growth. We planted Douglas-fir and red alder in even mixtures and monocultures. We also planted sycamore maple and common oak in even mixtures and monocultures. We found soil microbial communities differed from initial soil conditions, but not significantly between the tree species treatments. This indicates the afforestation of the Rhizotron altered soil microbial composition, but communities had not yet fully tuned into their respective trees and leaf litter inputs. We did however observe signs of overyielding within our experiment, such that red alder and Douglas-fir both taller and attained greater biomass in mixtures compared with their monoculture counterparts. Sycamore maple and common oak did not demonstrate any non-additive effects on growth in mixtures compared with monocultures.

2. Introduction

Plant-soil interactions play an essential role in ecosystem function and regulation (Wardle et al. 2004), and have cascading effects on the development of soil. These include tree species effects on the cycling of nutrients (Prescott et al. 2002), soil physical properties (Binkley and Giardina, 1998), soil fertility (Augusto et al. 2015), and soil microbial community composition and function (Ribbons et al. 2016). One of the ways by which plants influence soils is through the physical presence of roots, leading to a zone of soil immediately surrounding plant roots strongly

controlled by root activity, henceforth called the rhizosphere (Ryan, et al. 2001). Within the rhizosphere, plant roots exude compounds which mediate rhizospheric interactions both at the plant–microbe (plant–microbe and microbe–microbe) and the soil microbial community (plant–microbiome) levels (Jones, 1998; Huang et al. 2014). Root exudates can play important roles in regulating ecosystem properties, including the flow of elements like carbon (Farrar et al., 2003; Jones, Nguyen, and Finlay, 2009), nitrogen (Jones et al. 2005), and phosphorus (Oburger et al. 2011), litter decomposition (Kuzyakov et al. 2007), and rhizodeposition regulation along with mycorrhizal associates (Jones, Hodge, and Kuzyakov, 2004).

Another mechanism for influencing soils is through priming, whereby an increase in soil organic matter decomposition occurs as a result of inputs of available carbon (C) or nitrogen (N) sources (Blagodatskaya and Kuzyakov, 2008). Bengtson et al. (2012) highlighted the importance of differentiating between real and apparent priming, where apparent priming results in increased C and N mineralization, but not an additional increase in soil organic matter. They experimentally tested the effects of real and apparent priming via root exudates from three tree species (ponderosa pine, Sitka spruce, and western hemlock), and found evidence of a tight connection between root exudation, soil organic matter decomposition, and available nitrogen, as a result of rhizosphere priming.

A major question in ecology is how well organisms can be classified in terms of their ecosystem function, known as a functional trait (Cabido and Diaz, 2008). Plants can also be classified into varying functional groups based on their growth habits, life history characteristics (pioneer vs. late-successional), and root architecture (heart-shaped vs. tap-roots; Binkley and Fisher, 2012). At a coarse-scale trees can be classified into coniferous and deciduous, with each group largely having a suite of characteristics that contrast in terms of leaf or needle structure and chemical composition (Kattge et al. 2011). These differences in leaf and needle chemistry have cascading effects on ecosystem nutrient regulation, via litter inputs, litter decomposability (Moore et al. 1999, Prescott, 2010), organic acid concentrations within leaves and needles (Prescott et al. 2000), and their resulting influences on soil C:N ratios and pH (Vesterdal et al. 2012).

Another important functional trait associated with certain tree species is symbiotic relationships with bacteria or fungal associates. Red alders form symbiotic relationships with *Frankia alni*, which are nitrogen-fixing bacteria that form root nodules, and are able to sequester nitrogen (N_2) from the air to make it biologically available for the host plant. Mycorrhizal fungi create a greater access to organically bound nutrients for plants, which can be beneficial for nutrient acquisition and transfer. Mycorrhizal associates are another level of functional traits used to characterize tree species, with two major groups used for classification in relation to this study: ectomycorrhizae (EcM) and arbuscular mycorrhizae (AM).

A broad-scale question remains about the effect of tree-species mixtures compared with single on soil, and resulting soil microbial community and rhizosphere development. There is much evidence that the above- and below-ground growth of juvenile trees is greater when grown in species mixtures than in monocultures, a phenomenon referred to as overyielding (Forester and Pretzsch, 2015). This effect has been attributed to differences in niche between species, mediated by differences in many functional traits including differences in foliar C:N ratios, lignin and cellulose concentrations, as well as life history traits like growth habit, and pioneer vs. closed-canopy establishment preferences. However, this evidence has been strongly focused on above-ground traits. We here use a mesocosm-scale field experiment with a common initial soil substrate (a common garden experimental design), to determine tree species effects on the development of the soil. We aimed to determine the influence of tree species identity and their mixtures on microbial community composition and function at the seedling establishment phase. We also determined the carbon and nitrogen aboveground biomass stocks for these individual trees, and species-level or functional group effects. We expected that red alders in mixtures with Douglas-fir would increase in the biomass of the Douglas-fir trees (Binkley, 2003). We expected sycamore and oak to perform better in mixtures than monocultures, due to their contrasting root-growth forms and growth habits.

To further explore these demonstrated patterns in overyielding in mixed forest stands we established a pure and mixed species experiment to look at tree species biomass, and whether differences in biomass are mirrored by differences in: carbon and nitrogen stocks in leaves, branches, roots, and rhizospheric soils, and soil microbial communities (generally through

fungal:bacterial ratios and fungal *ITS* and bacterial *16S* gene markers, denitrifying bacteria *nirK* and *nirS*, and ammonia-oxidising bacteria and archaea AOB and AOA *amoA).* In this study we addressed the specific hypothesis that differences between tree species, leading to the species-mixture effects on growth, are linked to differences in their soil microbial communities and functional traits associated with variation in their capacity to exploit physical soil space and resources.

3. Materials and Methods

3.1 Species selection

Tree species were selected based on their contrasting functional traits, and to represent a range of taxonomic, physiological, and ecological types. Species mixtures were also selected to represent commonly occurring natural forest mixtures or managed forest species. We selected sycamore maple (*Acer pseudoplatanus* L.) and common oak (*Quercus robus* L.) as model species to be grown in mixtures as they commonly co-occur in temperate broadleaf forest settings. Sycamore and oak (as they will be henceforth referred to) also differ in several key functional traits, including mycorrhizal fungal associates (arbuscular vs. ecto-mycorrhizal, respectively) and root growth forms (heart-shaped vs. tap-root), as well as several leaf traits including C:N content, base cation content, and decomposability (Vesterdal et al. 2008). In this study sycamore typically form associations with AM fungi, while oak and Douglas-fir commonly form associations with EcM. Collectively, we expect that trees with differing roots forms and fungal associates would inhabit more soil space. We also selected Douglas-fir (*Pseudotsuga menziesii*) and red alder (*Alnus rubra*) as model species to compare a common commercially grown coniferous timber species and a nitrogen-fixing broadleaf species that grow naturally together in temperate forest ecosystems of NW America (Binkley et al. 1992; Binkley, 2003).

Figure 1. Photographs of Rhizotron soil bays at the initial planting of red alder and Douglas-fir seedlings (left), compared with the red alder and Douglas-fir seedlings one year into the experiment (below).

3.2 Rhizotron study design

We used the Malcom Cherrett Rhizotron, located at Treborth Botanic gardens in Gwynedd, Wales. This unique soil laboratory allowed us to access the belowground soils and roots without destructive sampling during the experiment, through the use of access tubes within Perspex windows. The Rhiztron comprises $1m^3$ compartmentalized soil bays, which provide a metal partition to keep root systems separated between treatments. We used a total of 24 soil bays in this randomized block design experiment, with a total of 6 treatments randomly assigned within each of 4 blocks. Each treatment was randomly assigned to a soil bay with a total of 4 replicates of each treatment (the 6 treatments are 1- alder, 2- Douglas-fir, 3- mixed alder and Douglas-fir, 4-oak, 5-sycamore, 6-mixed oak and sycamore). Trees were planted at a density of 16 m^{-2}. Initial heights were recorded for each tree. Within each soil bay 16 seedlings were planted in evenly spaced rows, alternating conspecifics in the species mixture soil bays (Figure 1; see Supplementary Figure 1 for planting design).

3.3 Initial data collection and conditions in soil bays

Prior to planting trees, initial soil samples were taken to determine baseline soil conditions. An initial soil sample was frozen and stored in an -80°C freezer for subsequent analysis of pre-experiment soil microbial community composition (DNA extraction and qPCR). Initial soil samples were used immediately to assess the soil microbial biomass following the chloroform-fumigation extraction protocol (Brookes et al. 1984), soil pH, electrical conductivity, gravimetric water content, and organic matter content through loss on ignition at 550°C. We sampled 10 seedlings at the initial planting stage to use as proxies for average initial above- and belowground biomass for the 4 focal tree species. Biomass for each tree species was estimated by randomly selecting 10 individual seedlings, and harvesting both the aboveground tissue and belowground roots. All materials were dried to a constant weight in a 70°C oven to determine dry weight for biomass.

3.4 Intermediate data collection

Tree heights were assessed at intermittent time periods throughout the duration of the experiment: day of planting (May 1, 2014), and approximately every 3 months (September 2014), 15 (December 2015), and 26 (July 2016) after planting. Root collar diameters were

measured 15 and 26 months after planting, and used to estimate allometric growth equations for these tree species at sapling ages. Bulk soil samples were taken in December 2015 from a depth of 25 cm within each soil bay, and used to assess differences in bulk soil microbial community and fungal:bacterial ratios using the same qPCR methods as initial and final samples.

3.5 Final harvest and data collection

Total aboveground biomass was measured at the end of the experiments by destructive harvesting. Trees were weighed immediately during harvest to determine fresh weights for each fraction (bole, branches, leaves) and then dried at 80°C for 72 h. Roots were sampled using an 8-cm diameter soil corer to a depth of 30 cm divided into 10-cm soil core sections. For each 10-cm section, soil cores were sieved through a 2 mm mesh sieve and separated into fine (<2 mm) and coarse (>2 mm) root fractions. Duplicate soil cores were taken for each soil bay, positioned 33 cm from each side of the soil bay. Coarse roots were dried at 80°C for 48 hours, and weighed (± 0.001 g). Fine roots were scanned and analysed using WinRhizo software. Where possible roots from these cores were identified to species, which was typically only in the Douglas-fir and alder soil bays. Rhizosphere soils, identified as the soil that adhered to roots after shaking, were collected from these soil cores and used for final microbial community analyses and C:N analyses. The rhizospheric soil samples were composited within each soil bay, and as such are an average representative sample for each replicate of the six treatments. Root growth was compared between trees growing in pure and mixed species settings to determine how inter- and intra- specific competition alters tree growth, using a sub-sampling approach with root cores.

3.6 Whole tree C:N ratios

A subset of three individual trees per species per bay (n=3 for each species) were harvested and separated into bole, branch, leaf, and root fractions. All plant material was dried at 80°C for at least 72 h, and intermittently weighed to ensure no remaining mass loss occurred after 72 h. Rhizospheric soils were collected from the roots of each individual and subsamples were removed and dried at 105°C in preparation for total C and N analyses. All samples were weighed to between 0.0500g and 0.1000g for branch, leaf and root material, and between 0.1000g and 0.2000g for soil samples. Exact weights were recorded for each sample which were then

analysed for C and N concentration (which we've expressed as C:N ratios) using a Truspec CN analyser (Leco Instruments). N stocks were calculated as biomass multipled by N concentrations for each fraction (branch, leaf, root, and total biomass).

3.7 Soil microbial community- DNA extraction and qPCR analyses

Differences in bulk soil (non-rhizospheric) community composition between monocultures and mixed species soil bays were assessed after targeting total fungal ITS, bacterial 16S, and fungal:bacterial ratios in soil samples taken in April 2014 (prior to the experiment) and in December 2015. Final rhizosphere soil samples (taken June 2016) were compared between treatments, to determine if tree species altered rhizospheric communities when grown in single or mixed settings.

DNA was extracted using 0.25 g of frozen soil and a MO-BIO PowerSoil DNA isolation kit (MoBio Laboratories, Inc., Carlsbad, CA), and quality and concentration were assessed using a nanodrop spectrophotometer (Thermo Fisher Scientific Inc., Wilmington, DE) and electrophoresis in agarose gels (1% w/v in TBE). All extracts were stored at -20^{o}C prior to amplification, and ten-fold dilutions of DNA were used for quantitative polymerase chain reactions (qPCRs) to quantify gene copy numbers of two focal gene markers: bacterial *16S* and fungal *ITS*. All qPCRs were run in duplicate on an xx machine with 20 µl reactions consisting of: 10.0 µl of SYBRGreen (2x) PCR Master Mix (Life Technologies Corp., Carlsbad, CA, USA), 0.25 µl each of forward and reverse primers, 1 µl of DNA template, and 8.5 µl of nuclease-free water.

PCR conditions for fungal *ITS* were 95° C for 10 minutes followed by 40 cycles of 95° C for 15 seconds, 55° C for 30 seconds, and 72° C for 20 seconds. Standard curves for fungal *ITS* were constructed using ten-fold serial dilutions of *Fusarium aveneceum* genomic DNA, which ranged from 10^3 to 10^9 gene copies. PCR conditions for bacterial *16S* were 95° C for 10 minutes followed by 40 cycles of 95° C for 15 seconds, 53° C for 30 seconds, and 72° C for 20 seconds, and we used ten-fold serial dilutions of *Pseudomonas putida* genomic DNA, which ranged from 10^2 to 10^7 gene copies. Fungal:bacterial ratios were calculated using these ratios of log gene copies. PCR conditions for both nirK and nirS were 10 min at 95 ^{o}C and 40 cycles of 95 ^{o}C for 60

seconds, 60 °C for 60 seconds and 72 °C for 60 seconds, with fluorescence quantified at extension (Levy-Booth and Winder, 2010). The standard curve for *nirS* and *nirK* used 10-fold serial dilutions of 10^1 to 10^7 gene copies from *Pseudomonas putida.* All gene copies were calculated using exact soil extraction weights, and are presented in analyses as log gene copies/µl/g of dry weight soil. All primers and references literature are available in Table 1.

Table 1. Group-specific primers for qPCR gene quantification assays

Primer Target	*Primer Name*	*Primer Sequence (5'-3')*	*Reference*
All Bacteria (16S rRNA)	Eub338 Eub518	ACT CCT ACG GGA GGC AGC AG ATT ACC GCG GCT GCT GG	Fierer et al. (2005)
All Fungi (ITS)	BITS B58S3	ACCTGCGGARGGATCA GAGATCCRTTGYTRAAAGTT	Bokulich and Mills (2013)
AOA[a] *(amoA)*	*amoA*-23F *amoA*- 616R	ATG GTC TGG CTW AGA CG GCC ATC CAT CTG TAT GTC CA	Francis et al. (2005)
AOB[b] *(amoA)*	*amoA*-1F *amoA*-2R	GGG GTT TCT ACT GGT GGT CCC CTC KGS AAA GCC TTC TTC	Rotthauwe et al. (1997)
Cd-nitrite reductase (*nirS*)	*nirS*-1F *nirS*-3R	CCT AYT GGC CGG CRC ART GCC GCC GTC RTG VAG GAA	Braker et al. (1998)
Cu-nitrite reductase (*nirK*)	*nirK-1F* *nirK-1R*	GGG CAT GAA CGG CGC GCT CAT GGT G CGG GTT GGC GAA CTT GCC GGT GGT C	Braker et al. (1998)

[a]Ammonia Oxidizing Archaea, [b]Ammonia Oxidizing Bacteria

3.8 Data Analysis

Tree biomass, soil chemical properties, root density, and soil microbial communities were compared using a non-parametric analysis of variance, Kruskal-Wallis test, to account for unequal sample size (e.g. between pure species bays n=16, and mixed species bay n=8, when looking at target tree species effects). When significant differences were found in the main Kruskal-Wallis tests, these were followed by Kruskal multiple comparisons using Dunn post-hoc tests for paired differences. We also ran a principal components analysis on all biomass, soil and microbial data. All analyses, statistics, and figures were executed in the following packages: ggplot2 (graphs; Wickham, 2009), FactoMineR (PCA and multivariate analyses; Husson et al. 2016), gridExtra (to arrange graphs; Baptiste), wesanderson (colour palette; Ram, 2016), and

pgirmess (krusklmc; Giradoux, 2016) packages within R statistical program version 3.3.1 (The R Foundation for Statistical Computing, 2017).

4. Results

4.1 Soil chemistry

Soil analyses conducted prior to the establishment of treatments revealed characteristics of a homogenous soil (Table 2). The 0-10 cm layer of soil in the bays had a pH of 6.94 ± 0.06, carbon content of 5.99 ± 0.25 %, and nitrogen content of 0.37 ± 0.01 %. By the end of the experiment, bulk soil pH dropped to an average or 5.90 ± 0.15 across all soil bays. The soil bays containing pure alder had the greatest decrease in pH to 5.67 ± 0.35, compared with Douglas-fir (5.91 ± 0.22), sycamore (6.00 ± 0.23) and oak (6.10± 0.43), although these did not differ significantly between tree species. The mixture of alder and Douglas-fir decreased soil pH to 5.83 ± 0.24, and the oak and sycamore mixture to 5.87 ± 0.28.

Table 2. Mean soil characteristics among the soil bays, comparing initial and final soil samples.

Initial	pH	C	N	C.N
Alder fir	6.71	5.84	0.36	16.38
Douglas fir	6.73	5.99	0.37	16.38
Oak Syc	6.75	5.99	0.37	16.48
Alder	6.75	6.05	0.37	16.45
Sycamore	6.78	6.15	0.37	16.58
Oak	6.72	5.96	0.36	16.48
Final				
Alder fir	5.83	6.54	0.36	18.18
Douglas fir	5.91	6.80	0.36	18.85
Oak Syc	5.87	8.55	0.40	20.75
Alder	5.57	7.66	0.41	18.58
Sycamore	6.10	7.22	0.37	19.34
Oak	5.97	8.10	0.39	20.47

Table 3. Mean biomass (SE) for leaves, branches, stems, and the total aboveground biomass, compared among tree species. Letters indicate Dunn test paired differences following Kruskall-Wallis tests.

Tree Species	*Treatment*	*Stems*	*Branches*	*Leaves*	*Total Biomass*
p*		< 0.001	< 0.001	< 0.001	< 0.001
Alder	Alder	501 (110)*B*	238 (57.5)*B*	280 (40.5)*B*	998.7 (120)*B*
Douglas fir	Douglas fir	6.67 (1.48)*E*	16.9 (4.30)*C*	6.35 (1.43)*E*	23.98 (2.82)C
Sycamore	Sycamore	30.9 (8.63)*C*	1.93 (0.62)*D*	15.5 (2.25)*C*	48.20 (6.39)*B*
Oak	Oak	10.1 (1.46)*D*	2.17 (0.38)*D*	5.17 (0.36)*E*	17.47 (1.15)*C*
Oak	Oak Sycamore	11.9 (2.97)*D*	2.06 (0.66)*D*	6.90 (2.25)*D*	20.89 (2.48)C
Sycamore	Oak Sycamore	32.2 (9.16)*C*	2.88 (1.84)*D*	14.9 (0.92)*C*	48.87 (6.73)*B*
Alder	DougFir Alder	988 (301)*A*	478 (178)*A*	585 (98.4)*A*	1641 (309)*A*
Douglas fir	DougFir Alder	7.50 (1.59)*E*	14.5 (1.92)*C*	6.35 (1.43)*E*	21.74 (1.63)*C*

4.2 Aboveground biomass and C:N ratios

Root:shoot ratios for initial 40 sacrificed seedlings (10 per species, as the beginning of the experiment) were calculated as root/shoot mass (g) after 48 h of drying in 80°C oven. Initial root:shoot ratios were greatest for sycamore with 3.36 ± 0.94, followed by oak with 1.58 ± 0.27, alder with 0.29 ± 0.11, and Douglas-fir with 0.26 ± 0.07. By the end of the experiment we were unable to physically dig out many of the root masses, so we have no final root:shoot ratios for comparison.

Alder produced the greatest aboveground height and biomass of the four tree species, followed by sycamore, oak, and Douglas-fir (Supplementary Figure 2, Table 2). Alder and Douglas-fir both grew significantly taller in mixtures vs. their monoculture soil bays. Oak heights did not differ between mixtures with sycamores and oak monocultures. Sycamores grew marginally taller in mixtures with oaks, but not significantly different from sycamore monocultures. On average alder and Douglas-fir grown in mixtures had twice the N stocks of alders grown in monoculture (Table 3), for stems, branches, leaves, and the total aboveground biomass. In contrast, oak and sycamore N stocks in stems, branches, leaves, and the total aboveground biomass remained very similar when grown in mixtures or single-species soil bays.

Table 4. Mean N stocks for leaves, branches, stems, and total aboveground biomass compared among tree species.

Tree Species	*Treatment*	*Stems*	*Branches*	*Leaves*	*Total Biomass*
Alder	Alder	4.601	2.186	8.400	9.171
Douglas-fir	Douglas-fir	0.035	0.090	0.070	0.128
Sycamore	Sycamore	0.260	0.016	0.309	0.405
Oak	Oak	0.101	0.022	0.154	0.175
Oak	Oak Sycamore	0.116	0.020	0.190	0.204
Sycamore	Oak Sycamore	0.296	0.026	0.307	0.448
Alder	Doug-Fir Alder	8.154	3.945	16.38	13.54
Douglas-fir	Doug-Fir Alder	0.080	0.156	0.136	0.233

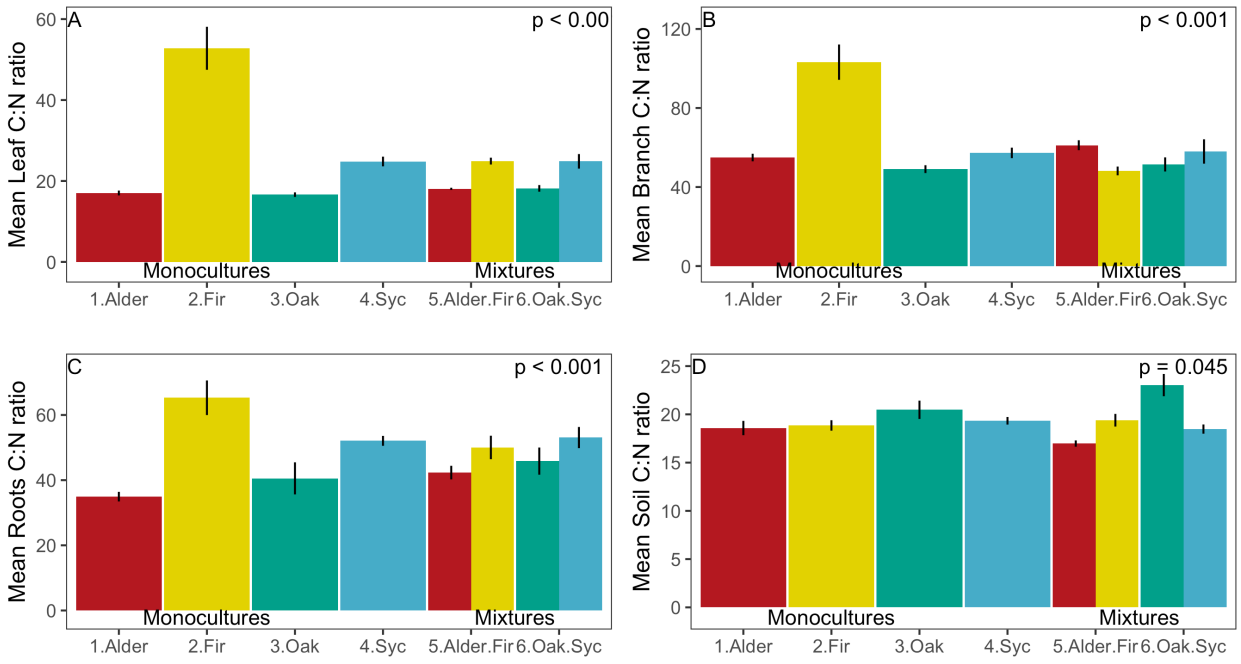


Figure 2. Carbon:Nitrogen ratios for leaves, branches, roots, and rhizospheric soils, collected at final harvest of the experiment. Maroon indicates alder, yellow indicates Douglas-fir, blue indicates oak, and green indicates sycamore.

Tree species identity and mixture of tree species influenced tree tissue and rhizospheric soil C:N ratios over the course of our experiment. We found Douglas-fir tree aboveground biomass (leaves and branches) had the highest C:N ratios compared with all other species, when grown in monoculture Douglas-fir settings (Fig. 2 a-b). Alder and oak had the lowest C:N ratios for leaves and branches when grown in monocultures vs. mixed soil bays (Fig. 2 a-b). Douglas-firs grown with alders had significantly lower C:N ratios in leaves, branches, and roots (Fig. 2 a-d), but

there was no effect of alder on rhizosphere soil for Douglas-fir. Alder had slightly elevated C:N ratios in branches, and significantly elevated C:N ratios in roots, when grown in mixtures with Douglas-fir, compared with pure alder settings (Fig. 2 a-d). Interestingly, rhizospheric soil C:N ratios in monoculture alder settings were significantly higher than alders grown in mixtures with Douglas-fir. Sycamore and oak trees did not differ in C:N ratios of leaves or branches when grown in monoculture or mixture settings. Trends towards increased C:N ratios were observed in roots and rhizospheric soils for oaks grown in mixtures with sycamores, compared with oaks in pure settings. Sycamore had slightly lower C:N ratios in rhizospheric soil, when grown in mixture with oak compared with monoculture.

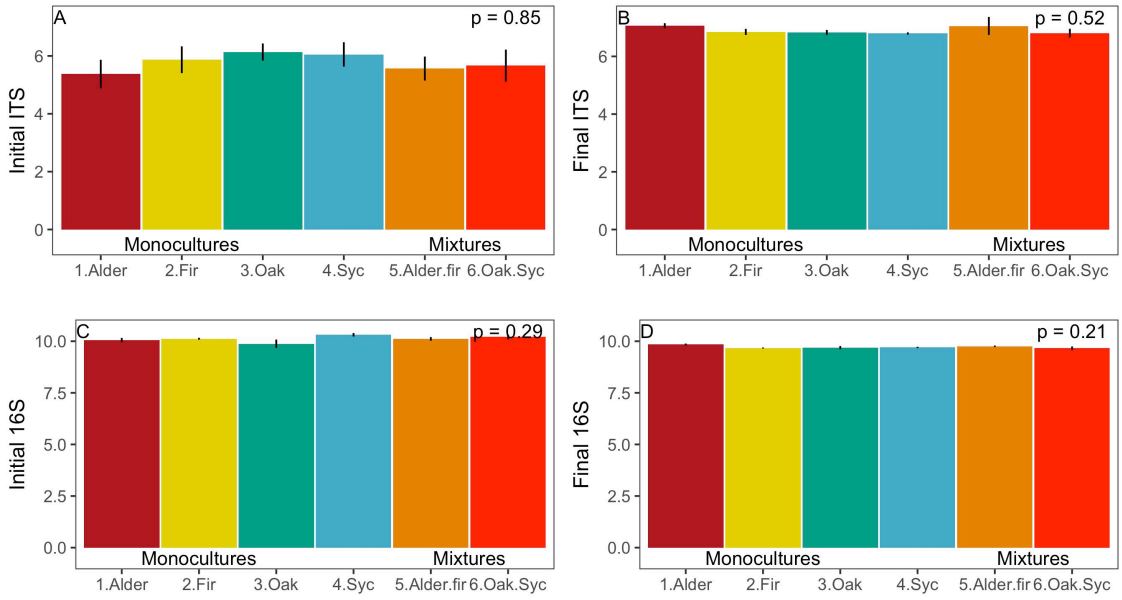


Figure 3: Treatment effects on rhizosphere soil fungal *ITS* and bacterial *16S* gene copy abundances (log gene copies/g soil), comparing initial samples and final samples collected from each soil bay.

4.3 Soil microbial community

Initial soil microbial communities did not differ among treatments for any of the 6 genes targeted, nor for the ratios of fungi:bacteria or amoA AOA:AOB. Fungal *ITS* gene copy abundances increased in all treatments, with pure alder rhizosphere soils having significantly greater abundances than other treatments (Fig.3 a-b). Final bacterial *16S* gene abundances decreased slightly in all species and their mixtures, with the greatest decline observed in oak and sycamore, and alder having greater abundances than other species and mixtures (Fig. 3 c-d).

Final *nirK* and *nirS* gene abundances increased for all tree species, with Douglas-fir having the smallest increase in *nirK*, and oak and Douglas-fir both increasing the least for *nirS* (Fig. 4 a-d). Final *amoA* AOA increased for all tree species, with no significant treatment effects (Fig. 5 a-b), whereas final *amoA* AOB was significantly different between alder and mixed oak and sycamore plots (Fig. 5 c-d). Final fungi:bacteria ratios increased for all treatments with no significant differences between treatments (Fig. 6 a-b). Final *amoA* AOA:AOB ratios increased for all treatments with some significant differences detected following kruskal-wallis tests (Fig. 6 c-d).

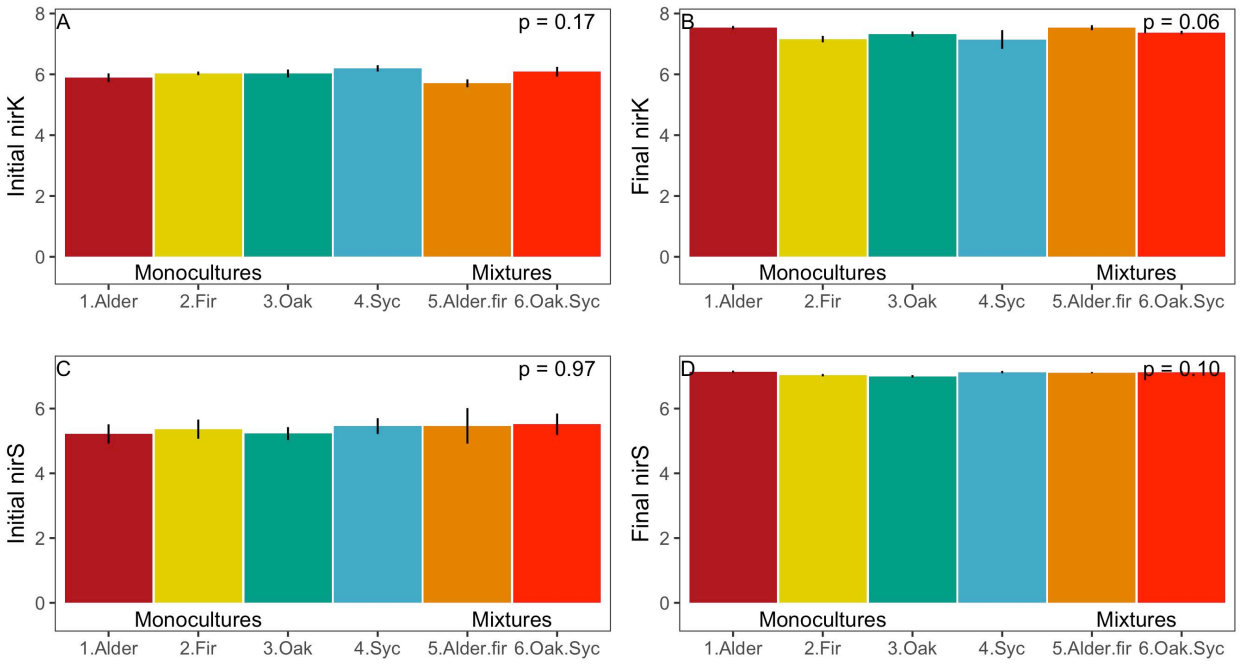


Figure 4: Treatment effects on rhizosphere soil *nirS* and *nirK* gene copy abundances (log gene copies/g soil), comparing initial samples and final samples collected from each treatment.

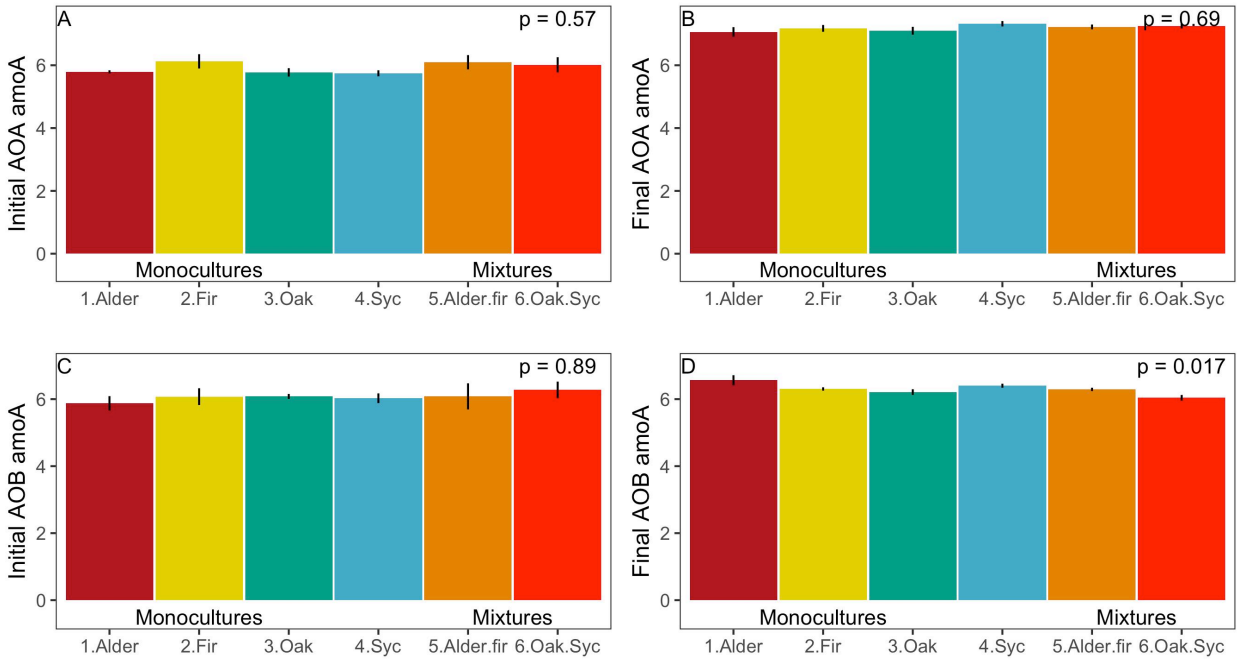


Figure 5: Treatment effects on rhizosphere soil *amoA* AOA and *amoA* AOB gene copy abundances (log gene copies/g soil), comparing initial samples and final samples collected from each treatment.

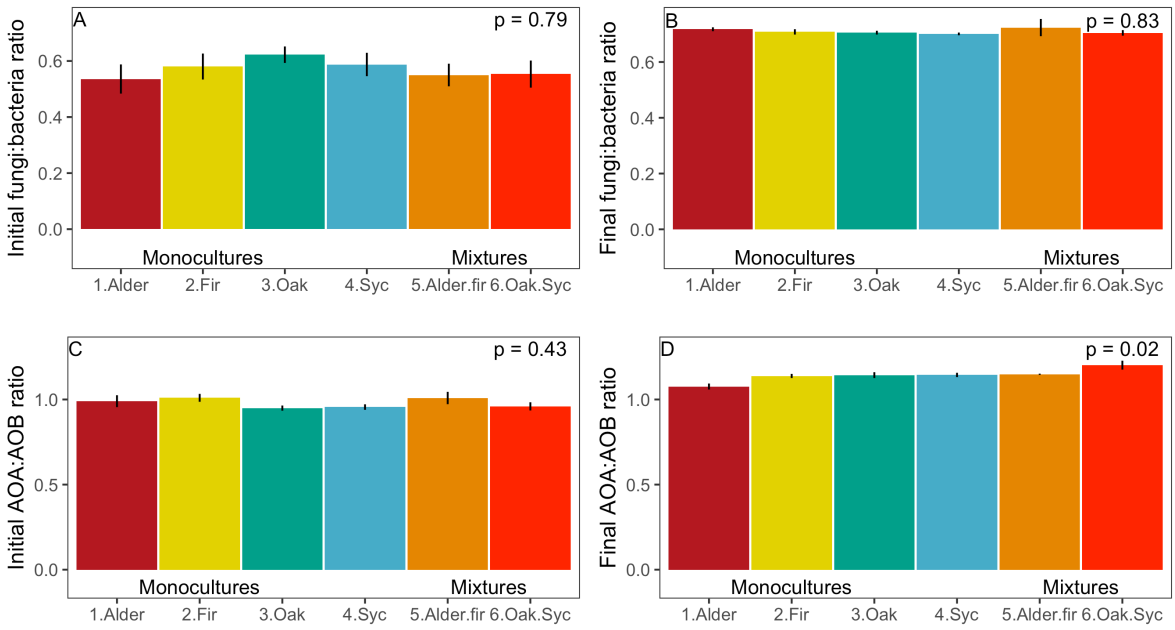


Figure 6: Treatment effects on rhizosphere soil fungal *ITS* to bacterial *16S* ratios (fungi:bacteria), and *amoA* AOA to *amoA* AOB ratios (AOA:AOB), comparing initial samples and final samples.

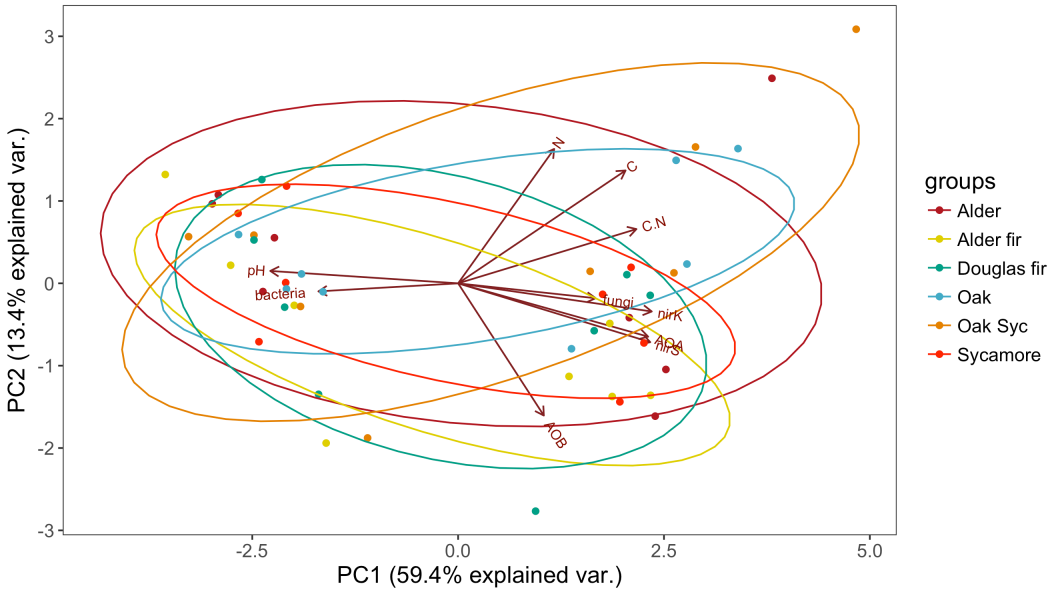


Figure 7. Principal components analysis for soil properties and gene copy abundances data compared among the six treatments.

We compared soil microbial community data and soil chemical properties among the 6 treatments at the end of the experiment. While this did not reveal any large differences among the tree species or their mixtures, we were able to differentiate trends in how these factors may be influencing soil microbial community composition. PC 1 explained 59.4% of the variation and mainly aligned with pH, bacterial *16S* and fungal *ITS* gene abundances (Figure 7). PC2 explained only 13.4% of the variation and mainly aligned with AOB *amoA* gene abundances and nitrogen concentrations (Figure 7).

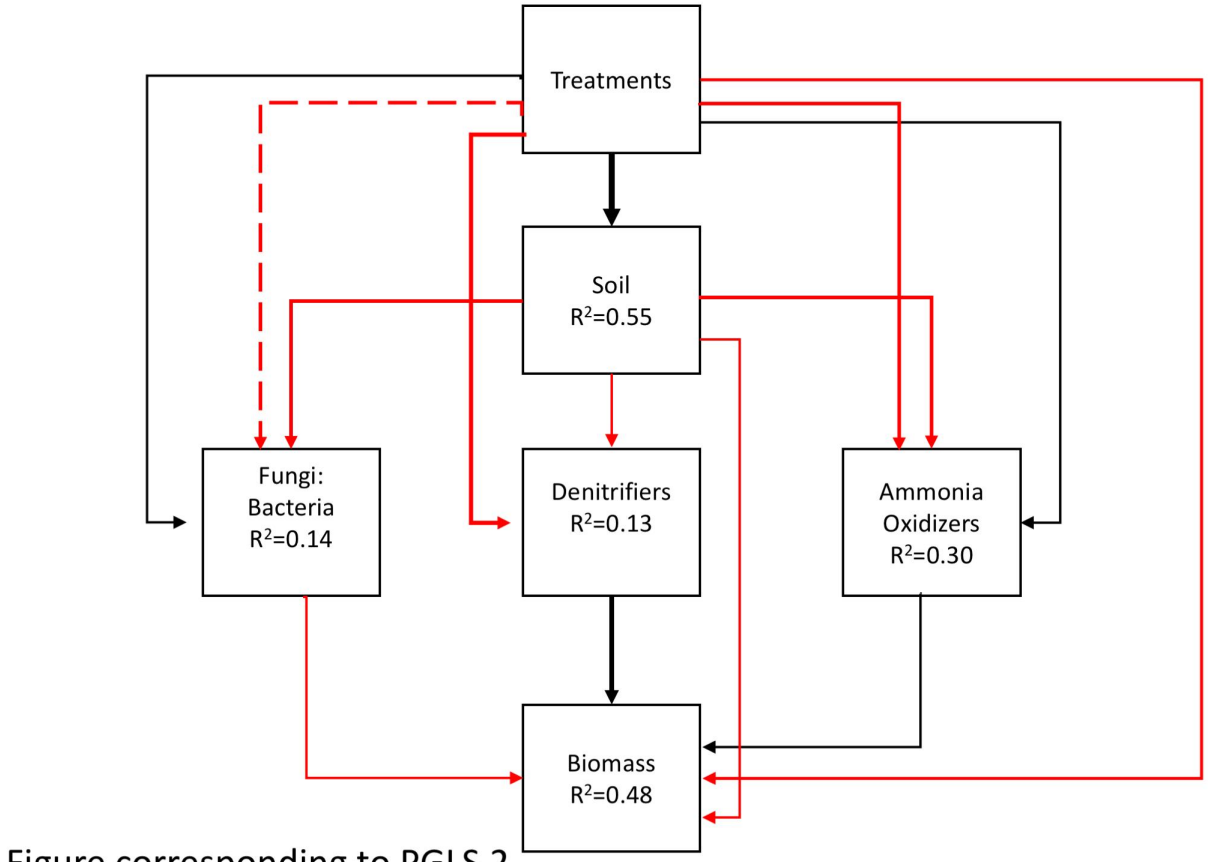


Figure 8. Partial least squares path analysis model using total aboveground biomass of all six treatments as it relates to soil pH and C:N ratios, and microbial community data [fungi:bacteria ratios, ammonia-oxidisers (*amoA* AOA and AOB), denitrifying bacteria (*nirK* and *nirS*)]. Black lines indicate positive relationships, red lines indicate negative relationships, and the width of lines is respective to the strength of those interactions.

We used partial least squares path analysis to determine causal relationships between soil microbial communities, C:N ratios, soil pH, and biomass with respect to all tree species and mixtures (Figure 8). We found strong positive relationships between the tree species and mixtures with fungal:bacterial ratios, soil C:N ratios and pH, and ammonia-oxidising archaea and bacteria. We also found strong negative relationships between tree species and mixtures with nitrifying bacteria, and total biomass.

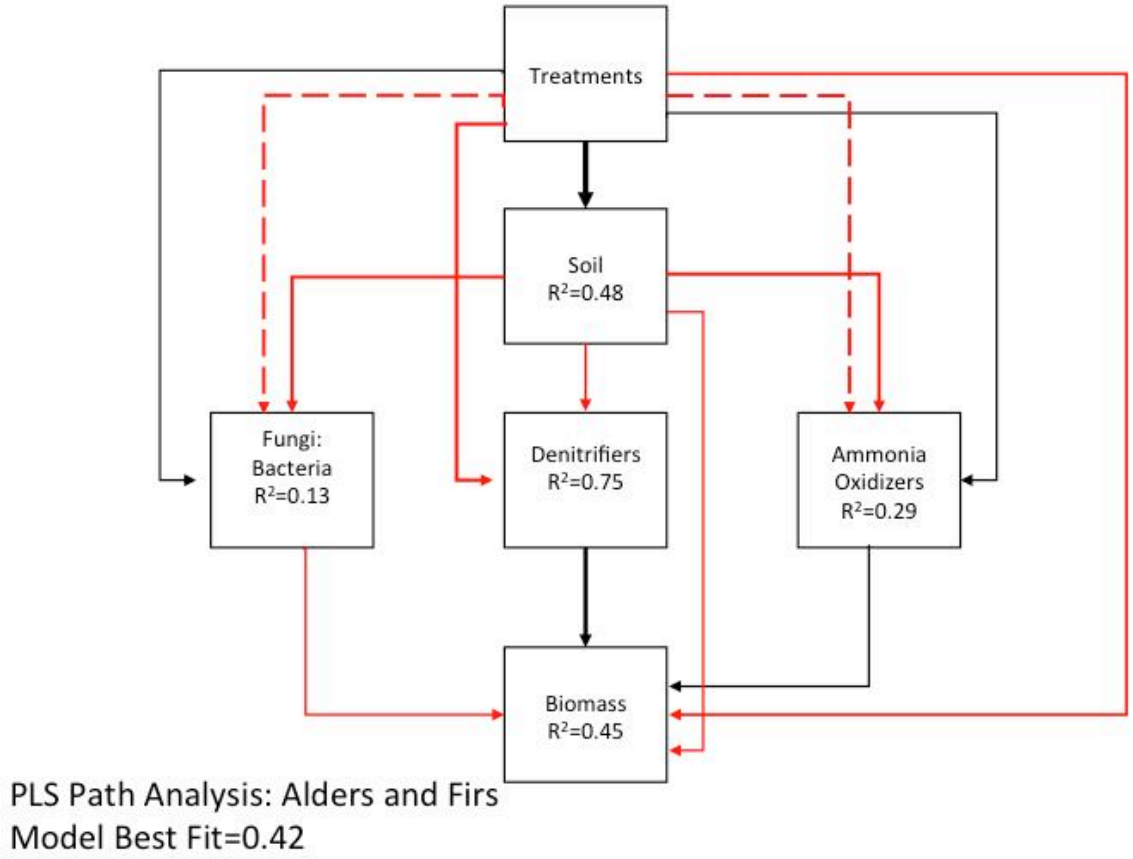


Figure 9. Partial least squares path analysis model using total aboveground biomass of alder and Douglas-fir monocultures and mixtures, as they relates to soil pH and C:N ratios, and microbial community data [fungi:bacteria ratios, ammonia-oxidisers (*amoA* AOA and AOB), denitrifying bacteria (*nirK* and *nirS*)]. Black lines indicate positive relationships, red lines indicate negative relationships, and the width of lines is respective to the strength of those interactions.

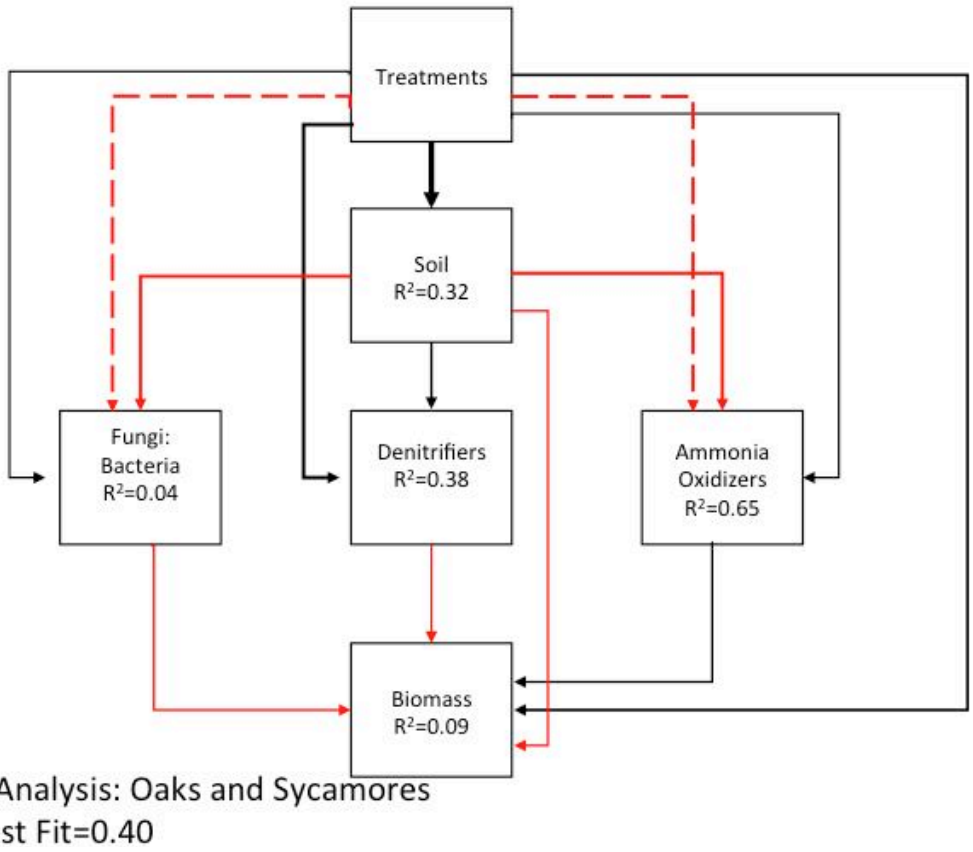


Figure 10. Partial least squares path analysis model using total aboveground biomass of oak and sycamore monocultures and mixtures as they relate to soil pH and C:N ratios, and microbial community data [fungi:bacteria ratios, ammonia-oxidisers (*amoA* AOA and AOB), denitrifying bacteria (*nirK* and *nirS*)]. Black lines indicate positive relationships, red lines indicate negative relationships, and the width of lines is respective to the strength of those interactions.

We constructed 2 additional path analysis models, a model for alder and fir monocultures and mixtures, and a model for oak and sycamore monocultures and mixtures (Figures 9-10). For the alder and fir model (Figure 9), tree species and mixtures positively influenced soil properties (pH and rhizospheric C:N; $R^2 = 0.48$), fungal:bacterial ratios ($R^2 = 0.13$), and ammonia oxidisers ($R^2 = 0.29$). There was also a strong positive correlation between denitrifying bacteria *nirK* and *nirS* on total aboveground biomass ($R^2 = 0.45$). For the oak sycamore model (Figure 10), tree species and mixtures have much weaker relationships with fungal:bacterial ratios ($R^2 = 0.04$), and biomass ($R^2 = 0.09$). We found a strong positive relationship between tree species and mixtures

on ammonia-oxidisers ($R^2 = 0.65$) and denitrifiers ($R^2 = 0.38$), and soil (pH and rhizospheric C:N ratio; $R^2 = 0.32$).

5. Discussion and Conclusion

5.1 Tree species and mixture effects on rhizosphere soil microbial community

We observed a shift in microbial community composition over time, with final soil samples showing an order of magnitude increase in fungal *ITS*, *nirK, nirS,* AOA and AOB *amoA*, and a slight decline in bacterial *16S* compared to the initial soil samples. We had the following *a priori* predictions regarding soil microbial communities:

1) Alders would have the greatest difference in N functional gene abundances as they are a nitrogen fixing species.

2) Alder and Douglas-fir would have the most distinct microbial communities, compared with sycamore and oak, due to their contrasting N characteristics.

3) Douglas-fir would have the highest fungal:bacteria ratio, and alder the lowest, with oak and sycamore intermediate.

Interestingly, we found no support for any of these predictions and no significant differences among the tree species when coming their final microbial communities. We suggest that early development of rhizospheric soil microbial communities may be indicative of larger changes as trees mature and forest soils develop. We suggest that the development of species-specific rhizospheric soil microbial communities may take longer than three years for trees, which have a longer life-expectancy compared to grasses, which may have a rhizospheric soil influence in a shorter time period.

While we did not observe any significant differences among tree species in relation to rhizospheric soil microbial communities, the large differences between initial and final soil samples are compelling and show the early influences of afforestation on soil microbial community abundance. This is perhaps not surprising since tree species effects on soils may take decades to be established, and may not be consistent over longer periods of time. For example, simulation studies based on mature forests of red alder and Douglas-fir, planted within established Douglas-fir forests, suggested that the prolonged presence of red alder could lead to

increased nitrification and NO3- leaching which would ultimately decrease forest stand biomass (Verburg et al., 2001). However, initially high N availability in red alder soils would favor increased biomass for Douglas-firs. This study underlined not only the possible temporal shifts in tree species effects, but also highlighted the context-dependency of those tree species effects when they determined site history including a land-use legacy on soil would also influence forest nutrient cycling dynamics. We did observe an increase in total biomass and tree heights, for both red alders and Douglas-firs when grown in mixtures, which is also supported by Verburg et al. (2001).

Common oak and sycamore maple are both widely distributed throughout the United Kingdom, but vary dramatically in their life history traits and have contrasting implications for forest and woodland conservation. Common oak is a slow-growing tree with great longevity, while sycamore maple is an introduced species that grows rapidly and reproduces quickly (Moorecroft and Roberts, 1999). We observed coppice sprouting on our sycamore trees even after one growing season (data not shown), which lends support to the notion that sycamores could outcompete oaks if given an equal starting opportunity in afforesting an area. We found oaks did grow less when in mixtures with sycamore, and that sycamores actually grew taller when in mixtures, compared with either grown in pure settings. This aligns with previously issued concerns for woodland conservationists (Peterken, 1996), in that sycamores clearly are outcompeting oaks in this early seedling developmental stage, although short-term studies such as this may not translate into natural seedling dynamics. While a few sycamores were able to obtain heights up over a meter in this experiment, not a single oak was able to obtain that height.

5.2 Functional traits as a diagnostic planning tool

Functional traits were explicitly used in the development and design of this experiment, in an attempt to elicit measurable responses over a relatively short time in the life of a tree. While these four tree species encompassed a large range of functional traits, the current study design does not allow us to partition out which traits might be the most dominant controlling factors in observed responses. For example, we cannot definitively state that it is sycamore's heart-shaped root growth form that was responsible for increased aboveground biomass for sycamores, compared with oak's tap-root growth form leading to decreased aboveground biomass.

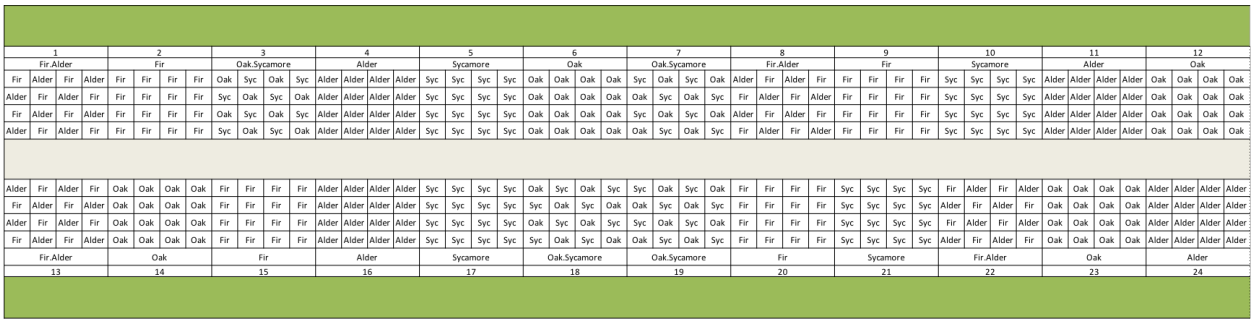
Functional traits can be a useful approach for categorizing tree species, and a whole field of biology is devoted to meticulously documenting plant functional traits (Kattge et al. 2011). Certainly functional categories such as nitrogen-fixing species, were useful in the design of our study, and additional data on plant functional traits can continue to be a useful tool for designing experiments in the future.

5.3 Future directions

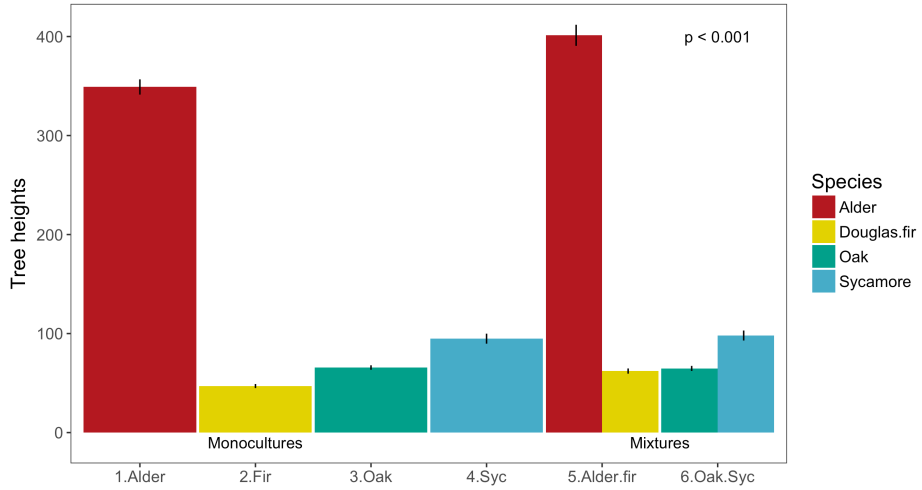
Early stage tree species effects were noticeable in our experiment, and future research should explore the sapling to young mature forest development of species in mixtures and monocultures growing in common garden soils. Large-scale biodiversity tree species addition studies such as BioDivNet (Kattge et al. 2011) and BIOTREE (Scherer-Lorenzen, et al. 2011) and soil nutrient studies such as NutNet (Borer et al. 2014). There are many other components to the soil community aside from fungi and bacteria, including notably earthworms, ants, mites, and collembolans. While we observed there were on average equal abundances of these organisms distributed throughout our compartments both at the initial and final stages of our experiment, it is worthwhile to pursue similarly taxon-focused research programs in larger pure and mixed species forests.

Future studies would benefit from focusing their designs on mechanisms and processes to specifically test hypotheses relating to rhizospheric effects on microbial communities. One possibility might be to perhaps use a chronosequence approach to capture the development of microbial communities as it corresponds to the development of the forest from early-stages to intermediate development after a decade or two. For studies that are confined to shorter time periods, controlled manipulative laboratory experiments may be a useful approach for isolating causal mechanisms among plant and soil communities. Isotope-tracing studies would be a useful approach for determining whether belowground inputs from a conspecific tree actually lead to uptake and enhanced growth of a neighboring tree.

Supplementary Figures



Supplementary Figure 1. Map of planting design for each individual tree seedling, as well as their physical location in relation to neighboring plots.



Supplementary Figure 2: Total aboveground height for each of the four species: red alder, Douglas-fir, oak, and sycamore, grown in pure or mixed species soil bays.

Chapter VI. Soil microbial communities are slow to respond one decade after afforestation in Bangor Diverse experiment

1. Abstract

The benefits of increased forest biodiversity extend from increased resilience to disturbances, and enhanced ecosystem function resilience as well. We explored forest biodiversity and mixtures effects on soil microbial communities and nitrogen cycling in the mineral soil underneath a young factorial forest experiment, called Bangor diverse. Within this experiment we sampled monocultures of red alder, birch, European beech, and the pairs and full mixture of all three. We sampled the mineral soil and assess soil microbial community abundances using qPCR for bacterial *16S*, fungal *ITS*, and functional genes associated with nitrogen cycling. Soil samples were also used to determine net N transformation process rates, and explore links between N process rates and soil microbial communities. We found few differences among the tree species, with no observations of non-additive or synergistic effects in the mixtures, compared with monocultures.

2. Introduction

Strategies to promote forest biodiversity and replace monocultures are gaining popularity worldwide (Verheyen *et al*. 2016). Enhanced biodiversity can provide a plethora of benefits from enhanced ecosystem stability and resilience to disturbances (Holling 1973, Loreau and Manzancourt 2013), e.g. in connection with species-specific pests and pathogens (le Mellec *et al.* 2009, 2011; Haase *et al.* 2015). Tree species affect belowground processes via organic matter inputs from above- (eg. Hobbie 1992, Jewell *et al.* 2016) and below-ground (Godbold *et al.* 2006), altering soil microbial community structure and function (Hackl *et al*. 2005, Aponte *et al*. 2013, Purahong 2016), and cascading effects on nutrient cycling (Spohn *et al*., 2013; Ribbons *et al*. 2016). The question remains as to how species mixtures, or increasing tree species diversity, will influence belowground processes. Determining the effects of tree species diversity of mixed forests through the use of controlled factorial experiments, is an important platform for the development of forest management strategies (Verheyen *et al*. 2016).

Previous studies have documented that single tree species influence nitrogen cycling rates and the soil microbial community associated with that ecosystem function (Levy-Booth et al. 2014, Ribbons et al. 2016). In this paper we aim to determine if two- and three-species mixtures alter soil microbial communities and ecosystem processes related to N turnover in soil compared to single species stands of the same tree species. We aimed to address the following research questions:

1. Do tree species influence soil microbial community composition?
2. Does increased tree species diversity alter N processes?
3. Do tree species with contrasting functional leaf traits (C:N ratios, decomposition rates) alter nitrogen cycling functional genes, as assessed by denitrifying bacteria and ammonia-oxidizing bacteria and archaea?

We hypothesized that the functional genes of soil microbes involved in nitrogen cycling processes would increase with increasing tree species diversity. Given the contrasting functional traits of the three tree species in this experiment, we predicted that alder would have the highest N cycling rates, followed by birch, and beech. We predicted there would be a corresponding shift in soil microbial functional gene abundances, and fungal ITS and bacterial 16S rDNA gene abundance. We hypothesized: 1) beech decreases soil pH and abundance of the bacterial 16S rDNA gene marker and increases soil C:N ratios, 2) alder would increase soil N and decrease C:N ratios while increasing rates of nitrification; and 3) birch would increase the abundance of soil bacterial communities (*16S* genes) compared with beech.

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3. Materials and methods

3.1 Study design

Soil samples were taken from the BangorDIVERSE forest diversity experiment located in Abergwyngregyn, North Wales (53°14'15"N, 4°1'4"W). The site was established in March 2004 and consists of replicated (n = 4) plots of one, two and three tree species mixtures at a density of 10,000 stems ha^{-1} (Godbold *et al*. 2014; Ahmed *et al.* 2016). Within BangorDIVERSE we sampled seven treatments in 2016: monocultures of alder (*Alnus glutinosa*), birch (*Betula pendula*), and beech (*Fagus sylvatica*), and two and three species mixtures of alder + birch,

beech + birch, alder + beech, alder + birch + beech. These species were chosen based on their contrasting litter properties, mycorrhizal status and N-fixing ability (Table S1). For DNA isolation, composite samples of soil were taken from each replicate plot, sieved to pass 2 mm and immediately frozen at -80°C. For N process rate measurements, composite samples were taken from each replicate plot, sieved to pass 5 mm and stored at 4°C prior to use.

3.2 DNA isolation and qPCR

DNA isolation, and targeted gene abundances were quantified following the protocol described in Ribbons et al. (in review, Chapter 3). Briefly, microbial DNA was extracted from 0.10 g of soil, using a Power Clean® soil extraction kit (Mo-Bio Laboratories Inc., Carlsbad, CA). DNA extracts were quantified using a nanodrop spectrophotometer, and 1:10 (v/v) dilutions of DNA extracts were used for downstream analyses. qPCR conditions for bacterial 16S, fungal ITS, denitrifying bacteria *nirK* and *nirS,* and ammonia-oxidizing bacteria and archaea (*amoA* AOB and AOA, respectively) are shown in Table 1.

Table 1. qPCR conditions for bacterial *16S*, fungal *ITS*, denitrifying bacteria (*nirK* and *nirS*), and ammonia-oxidizing bacteria (*amoA* AOB) and archaea (*amoA* AOA).

Primer target	*Primers*	*Primer Sequence (5'-3')*	*Reference*
All Bacteria (16S rRNA)	Eub338 Eub518	ACT CCT ACG GGA GGC AGC AG ATT ACC GCG GCT GCT GG	Fierer et al. (2005)
All Fungi (ITS)	BITS B58S3	ACCTGCGGARGGATCA GAGATCCRTTGYTRAAAGTT	Bokulich and Mills (2013)
AOA[a] *(amoA)*	*amoA*-23F *amoA*- 616R	ATG GTC TGG CTW AGA CG GCC ATC CAT CTG TAT GTC CA	Francis et al. (2005)
AOB[b] *(amoA)*	*amoA*-1F *amoA*-2R	GGG GTT TCT ACT GGT GGT CCC CTC KGS AAA GCC TTC TTC	Rotthauwe et al. (1997)
Cd-nitrite reductase (*nirS*)	*nirS*-1F *nirS*-3R	CCT AYT GGC CGG CRC ART GCC GCC GTC RTG VAG GAA	Braker et al. (1998)
Cu-nitrite reductase (*nirK*)	*nirK-1F* *nirK-1R*	GGG CAT GAA CGG CGC GCT CAT GGT G CGG GTT GGC GAA CTT GCC GGT GGT C	Braker et al. (1998)

3.3 Nitrogen process rates

Soil net nitrification rate was determined according Beck (1976, 1979). Briefly, 1 ml of a 50 mM NH_4SO_4 solution was added to 10 g of field-moist soil and the samples incubated for 7 d at 25°C. Subsequently, the soils were extracted with 100 ml of 0.05 M K_2SO_4 (200 rev min^{-1}, 1 h), filtered, and NH_4^+ and NO_3^- concentrations in the extracts determined colorimetrically according to the methods of Mulvaney (1996) and Miranda et al. (2001). Soil net ammonification rate was determined using the anaerobic incubation method of Waring and Bremner (1964) and Keeney (1982). Briefly, field-moist soil (5 g) was incubated with 12.5 ml of distilled water in the absence of O_2 with continuous shaking (100 rev min^{-1}) at 40°C for 7 d. The soils were then extracted and the amount of NH_4^+ accumulated determined as described above.

3.4 Statistical analysis

Analysis of variance (ANOVA) was used to determine differences between the seven treatments for the soil gene abundances and the N process rates (alpha = 0.05). Aside from the log of gene copies data, no transformations were performed, and as no significant main effects were observed no post-hoc tests were completed. All analyses and figures were conducted in R version 3.3.2 (R Statistical Group, 2016) using the vegan (Oksanen et al. 2016) and ggplot2 (Bates et al. 2016) packages. Principal components analyses (PCA) were used to explore forest type effects on soil microbial communities, in addition to nitrogen process rates, and soil physical properties.

4. Results

Microbial community abundances in relation to fungi and bacteria did not differ significantly between the seven treatments (Fig. 1a fungal *ITS*, Fig 1b bacterial *16S*). Functional genes tied to nitrogen cycling including markers for denitrifying bacteria (*nirK* and *nirS*), and ammonia-oxidizing archaea and bacteria also did not differ between the 7 forest types (Fig. 1c-f). Alder had significantly greater rates of net nitrification than mixture of alder + birch, and alder + beech. There were no differences in net nitrification rates between beech, birch, birch + beech, or alder + birch + beech. Birch had significantly higher rates of ammonification from beech, and birch + beech. There were no other differences in net ammonification rates between the tree species mixtures. Alder had higher gross potential nitrification than alder + birch, but there were no other differences between tree species mixtures.

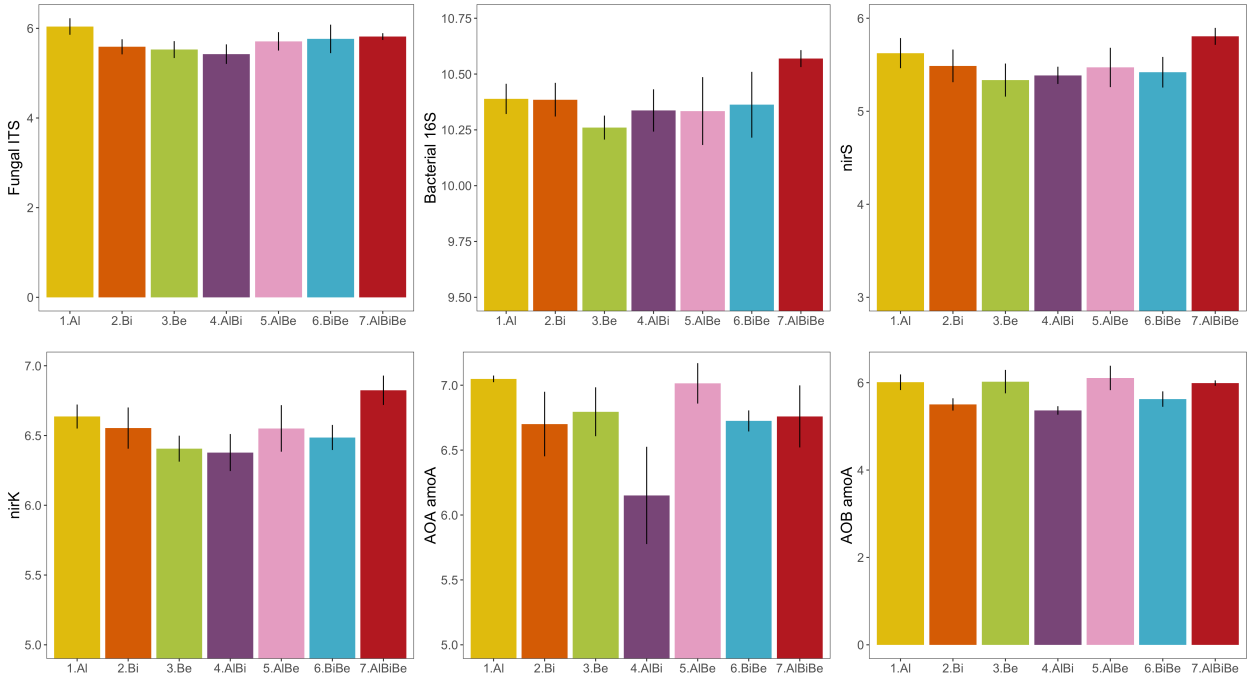


Figure 1. Total abundance of bacterial 16*S*, fungal *ITS, nirK, nirS,* ammonia-oxidizing archaea and bacteria *amoA* AOA and AOB, presented as log gene copies compared across the seven treatments. (means ± SEM, $n = 4$).

Three variations of principal components analyses were performed, the first contained all genes, soil chemistry, and N process rates data, the second contained genes and soil chemistry data, and the third contained only genes data (Fig. 3a-c). For the first model- Principal component 1 explained 33.1% of the variation and was mainly associated with fungal *ITS* and ammonia-oxidizing archaea (AOA *amoA*) (Fig. 3a). Principal component 2 explained 17.2% of the variation and was mainly associated with NH4+ concentrations and nitrification rates (Fig. 3a). For the second model- Principal component 1 explained 40.2% of the variation and was mainly associated with ammonia-oxidizing archaea (AOA *amoA*) (Fig. 3b). Principal component 2 explained 21.4% of the variation and was mainly associated with NH4+ concentrations and pH (Fig. 3b). For the third model- Principal component 1 explained 57.2% of the variation and was mainly associated with ammonia-oxidizing archaea and bacteria (AOB and AOA *amoA*) (Fig. 3c). Principal component 1 explained 17.3% of the variation and was mainly associated with niK (Fig. 3c).

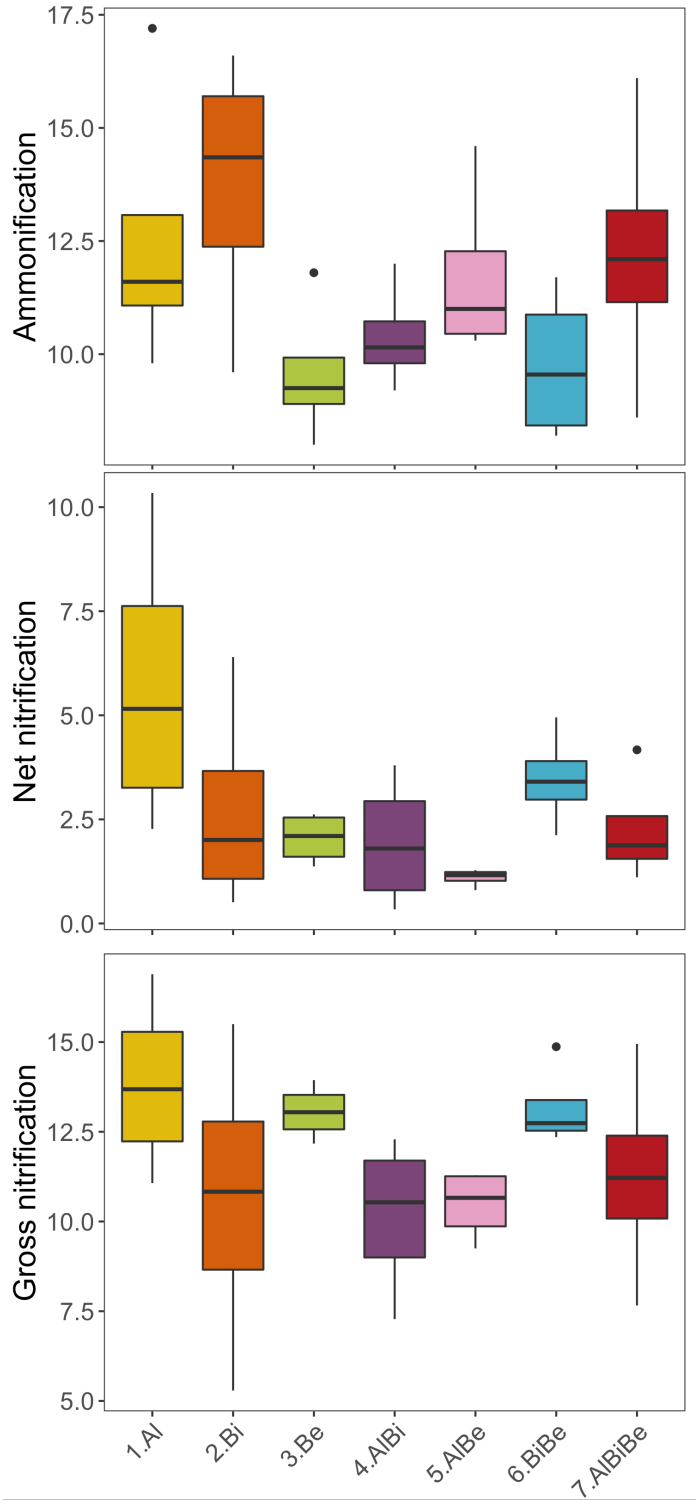


Figure 2. Nitrogen cycling process rates among the seven treatments. Nitrification rates are from an aerobic incubation with NH_4SO_4, whereas ammonification rates are from an anaerobic incubation (means ± SEM, $n = 4$).

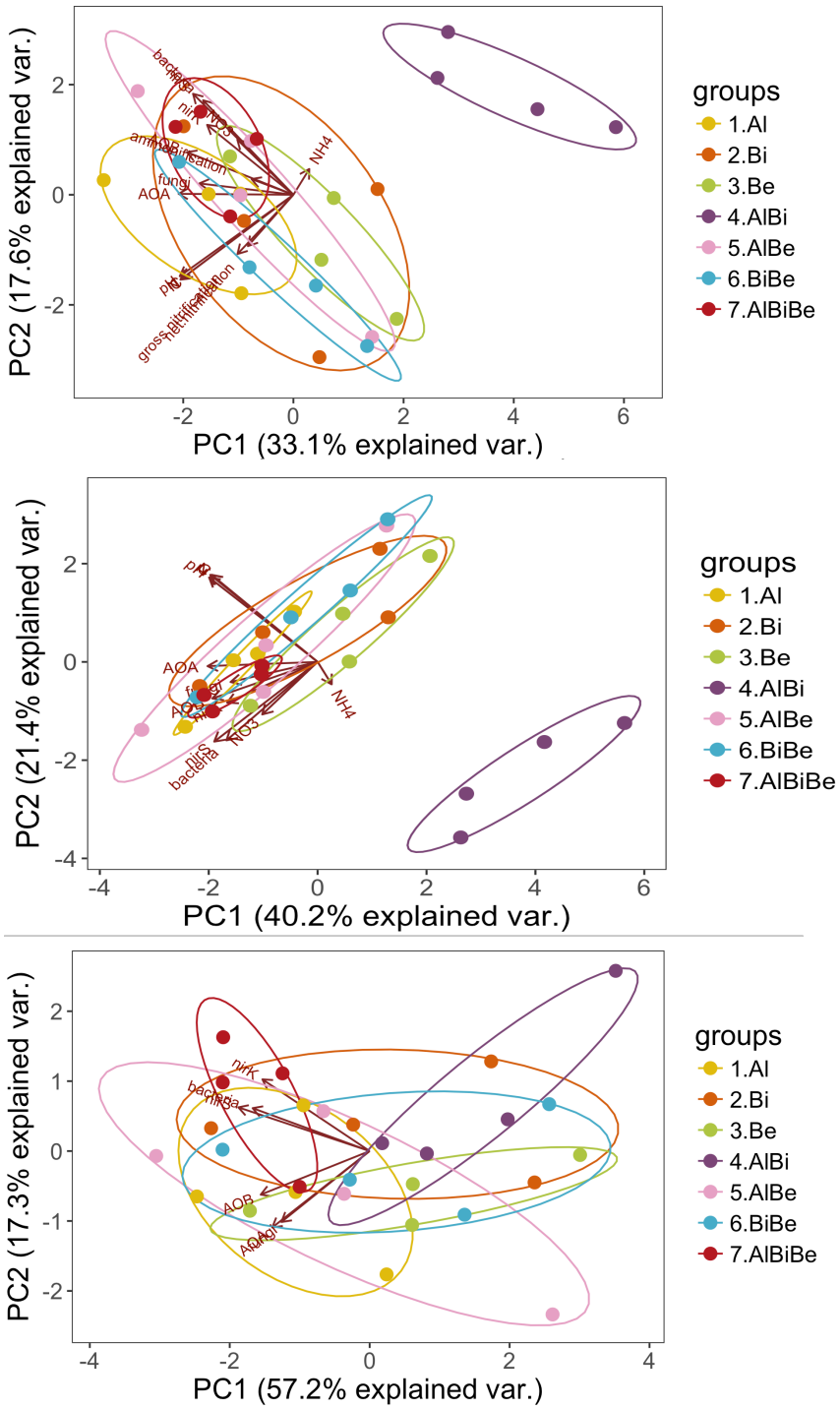


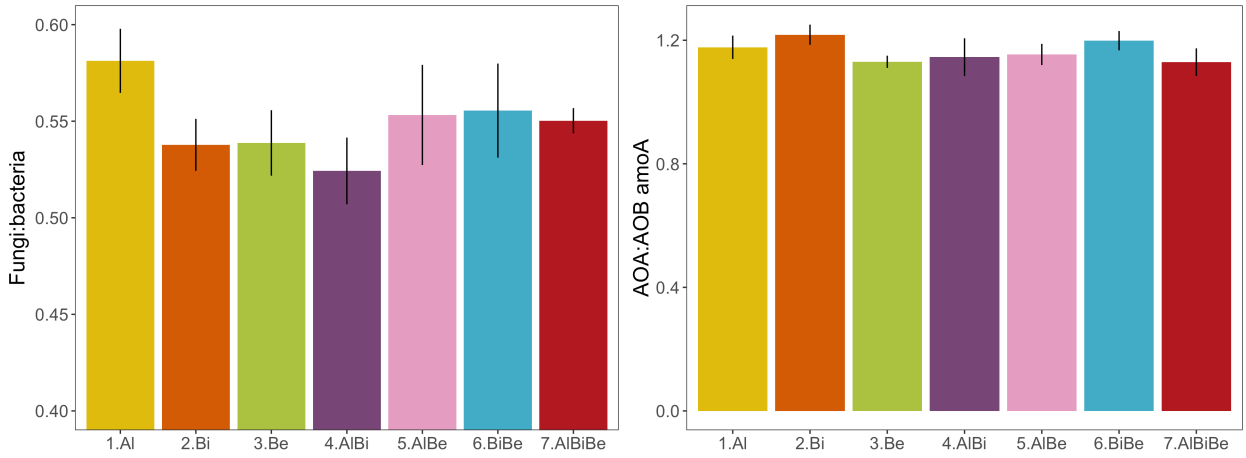
Figure. 3. Principal Components Analysis of all gene copies, N process rates, and soil chemistry data as dependent variables, grouped among the 7 forest treatments as the explanatory variables (a), with gene copies and soil chemistry data only (b), and with only gene copies grouped by the 7 forest treatments (c).

5. Discussion and Conclusion

Tree diversity in our young forest stand, and associated differences in litter quality had no impact on microbial community composition, functional gene abundances, or ecosystem process rates. We found no support for our first hypotheses: there was no documented tree species or mixtures effects on soil microbial community abundances (H1) as assessed by fungal *ITS* and bacterial *16S*. We did observe a tree species but not a mixture effect on N process rates (H2), specifically with the nitrogen fixing alder having higher rates of net nitrification. We found support for out third hypothesis that functional traits alter N process rates, but this was not evidenced in the functional genes (denitrifying bacteria *nirK* and *nirS* and ammonia-oxidising bacteria and archaea AOB and AOA *amoA*) tied to nitrogen cycling (H3).

This is slightly in contrast with recent research on soil microbial communities within this same experiment (Gunina *et al.* 2017), which found a few weak differences in microbial group composition in the upper 10 cm of soil. Gunina et al. (2017) found the addition of beech into any species mixtures resulted in different PLFA biomarkers compared to other tree species, generally with a decrease in bacterial biomarkers. They also found the nitrogen-fixing alder did not have dramatically different fungal or bacterial PLFA biomarkers. Our results may be partially explained as a lag in response time, where soil microbial communities are responding to different litter and nutrient inputs, and take time to stabilize. For example, soil C and N stocks initially increase as forests establish (Noavara et al. 2014), reach peak accumulation rates which decline as forests reach maturity (DeLuca and Boisvenue 2012). Similarly, C and N rates stabilize 30-50 years after afforestation (Gunina et al. 2017), which suggests forest ecosystems may take several decades to reach a dynamic equilibrium and is consistent with the lack of tree species effects observed in this study.

Supplementary Tables and Figures



Supplementary Figure 1. Ratios of fungi:bacteria and ammonia-oxidizing archaea to bacteria (AOA/AOB *amoA*) gene copies compared across the 7 Forest treatments.

Supplementary Table 1. Soil properties C, N, pH, NH4 concentration, and NO3 concentration among the 7 forest treatments (means ± SDEV, n =4).

	C	N	pH	NH4	NO3
Alder	29.97 (1.77)	3.11 (0.17)	5.29 (0.10)	2.49 (1.14)	19.06 (3.31)
Birch	33.75 (4.10)	3.21 (0.52)	5.36 (0.06)	4.01 (1.20)	16.91 (2.27)
Beech	24.78 (2.67)	2.53 (0.36)	5.23 (0.11)	5.73 (6.73)	14.83 (2.49)
Alder Birch	NA	NA	NA	4.10 (1.21)	15.94 (9.34)
Alder Beech	31.54 (3.97)	3.06 (0.31)	5.35 (0.25)	3.05 (0.98)	21.37 (4.42)
Birch Beech	34.65 (3.54)	3.23 (0.41)	5.38 (0.14)	2.91 (0.80)	13.87 (3.73)
Alder Birch Beech	30.02 (3.57)	3.07 (0.29)	5.40 (0.17)	3.39 (0.68)	15.39 (2.56)

Chapter VII. Discussion

"Education, if it means anything, should not take people away from the land, but instill in them even more respect for it, because educated people are in a position to understand what is being lost. The future of the planet concerns all of us, and all of us should do what we can to protect it. As I told the foresters, and the women, you don't need a diploma to plant a tree."

— Wangari Maathai**,** Nobel Peace Prize Laureate

1. Identifying the links between soil microbial communities and ecosystem processes: a synthesis across studies

Refined scientific methods and technologies provide researchers with a variety of tools to explore the mechanisms and processes tied to historical observations of patterns in naturally occurring or experimentally manipulated tree species habitats. From in-situ measurements of root exudate production (Wilkinson et al. 2014) to isotope-tracing studies coupled with the use of quantitative microbial community assessments (Chapter 3, Ribbons et al. 2016); these complementary methods can be used to dig deep into the soil processes occurring under different forest canopies. While decades of foresters, naturalists, and biologists can attest to tree species preferentially found in certain habitats, performing better in some soils, it is the recent explosion in molecular tools that has really added to our general understanding of forest ecosystem functioning and ability to explore specific ecosystem processes and microbial communities associated with those processes (e.g. N cycling (Levy-Booth et al. 2014)). These naturally occurring patterns of tree species site presences extends to processes that link the aboveground with belowground, such as decomposition, has been confirmed through numerous transplant experiments and molecular techniques (Strickland et al. 2009), and linked with modelling studies (Keiser et al. 2014). For leaf litter decomposition this is formally referred to as the home-field advantage hypothesis (Ayres et al. 2009), whereby microbial communities are adapted to leaf

litter inputs form their home vegetation type. Within the Rhizotron experiment, the potential indirect effects of home-field advantage in relation to the fostering of soil microbial communities uniquely adapted to the aboveground plant communities, was only implicitly tested.

The study of nitrogen and its cycling within terrestrial ecosystems is crucial for understanding how plants take up essential nutrients. In a very immediate sense, the entire food and nutrition industry for humans and other organisms is entirely dependent on the ability to access and acquire nutrients. Some of the key processes in the nitrogen cycle include the decomposition of organic nitrogen into ammonia/ammonium (ammonification), and nitrification, which is the oxidation of ammonia/ammonium into nitrite then nitrate. Several key methods were developed to explore net rates or nitrification and ammonification (Binkley and Hart 1989), and advance to understanding gross rates of nitrification and ammonification (Davidson et al. 1991, Hart et al. 1994) Nitrification and denitrification was recently evaluated along a vegetation gradient in Alaska (Petersen et al. 2012).

Gross production and consumption of NH_4^+ and NO_3^- (using the 15N pool dilution method of Hart et al. (1994)) rates were quantified within forest floors of four widespread and commercially important coniferous species in the EP571 common garden experiment (Chapter 3). These same techniques were further refined to explore similar research questions with a new suite of widespread and abundant temperate forest tree species in Denmark (Chapter 4), looking at mineral soil microbial consumption and production of NH_4^+ and NO_3^- after modifying the ^{15}N isotope pool dilution method. Both of these experiments were designed the soil sampling and laboratory experiment techniques and analytical approaches to be comparable so that general trends in N transformations might be observed across these distant forests.

Microbial communities responsible for controlling ecosystem processes like N transformations are increasingly explored to understand their functional roles. Zhao et al. (2014) used simulation models to understand the effects of global changes, and explore the mechanism of microbe-mediated soil feedbacks. They found soil transplant experiments, with specifically quantified microbial communities' functional potentials, to be a useful proxy for exploring possible mechanistic links between soils and their functional processes. Additional studies on interactions

between tree species fungal associated and soil microbial communities such as those by Herman et al. (2012) on arbuscular mycorrhizae are also warranted for increasing clarity in understanding ecosystem functions. Nuccio et al. (2013) used a nanometre-scale secondary ion mass spectrometry (NanoSIMS) to fing arbuscular mycorrhizae altered soil bacterial communities and nitrogen cycling during decomposition. This suggests the arbuscular mycorrhizal fungi modify soil microbial communities and ecosystem processes.

Rhizospheric soils are a hotspot of biological diversity (Griffiths 1994, Kuzyakov and Blagodatskaya 2015). Kuzyakov reviewed the heterogeneity of biodiversity hotspots within the rhizosphere, as well as other localized regions within ecosystems, such as decomposition hotspots. Jones et al. (2004) explored how dissolved organic nitrogen (DON), and found microbial consumption of nitrate was largely to satisfy microbial C requirements rather than their N needs. Additional studies in the rhizosphere are needed to evaluate mechanisms between tree species and soil microbial communities. These include additional simulation experiments have explored the specific relationships between soil microbial community compositions and the direct effects of root exudates (Strickland et al. 2015). Furthermore, research on soil organic matter priming effects (Kuzyakov 2010, Zhu et al. 2014) within the rhizosphere are a good approach for linking tree species with soil microbial communities and ecosystem processes.

1.1 Exploring microbial communities

This dissertation relied heavily on the use of quantitative PCR techniques to determine fungal:bacterial ratios in soil samples, which was intended to serve as a snapshot of the microbial community broadly categorized into these two groups. Fungal:bacterial ratios are often considered a useful metric for examining nutrient cycling potential, in that high fungal:bacterial ratios indicate slower N cycling (since it is generally considered that fungi are better able to slowly extract nutrients over time, whereas bacterial populations turnover quickly and are better suited to quickly take up nutrients (Chapin et al. 2000)). Fungal:bacterial ratios were intended to provide a snapshot of the composition of the soil microbial community, prior to more detailed microbial sampling and analyses.

Fungal:bacterial ratio analyses were followed by targeted functional genetic markers as a means to explore a soil's genetic potential for denitrification and ammonia-oxidisation (Chapter 3-7, Ribbons et al. 2016). Four functional genes, whose expression is important for enzymes used in N transformation processes, were use in these studies. These functional genes included two denitrifying nitrate-reductase genes, *nirK* and *nirS*, and two ammonia-oxidising genes, ammonia-monooxygenase *amoA* AOA (archaea) and *amoA* AOB (bacteria). Denitrification is a pathway in the N cycle where N returns to the atmosphere from soil or water via nitrate reduction to nitrous oxide (Henry et al. 2004). Ammonia-oxidising bacteria (AOB) and archaea (AOA) perform the first and generally rate-limiting step on the nitrification process, with recent interest in the role of *amoA* archaea in soil potentially surpassing that of *amoA* bacteria (Petersen et al. 2012).

Tree species effects were observed in relation to fungal *ITS*, bacterial *16S*, *nirK, nirS, amoA* AOA and AOB in several studies, but not all genes differed by forest type within each experiment. In general, tree species effects on microbial gene abundances were context dependent. For example, the bacterial *16S* gene abundances were greater in San Juan forest floors compared with Fairy Lake (Chapter 3, Figure 3, Table 3) at EP571. This was attributed to differences in N status at these two sites, with San Juan generally falling into a high N and Fairy Lake a low N site (Chapter 3, Figure 1). Within the Danish tree species trials we also found tree species effects on microbial communities for bacterial *16S, nirK, nirS,* and *amoA* AOA, with the largest differences observed between spruce and the broadleaved trees (Chapter 4, figure 2). Somewhat surprisingly, no differences between the tree species in the Danish experiment in relation to fungal *ITS* were observed. This is perhaps attributable to a large portion of spruce forest floor containing a thick matted layer of fungal hyphae which was not comparable with the broadleaves that lacked any forest floor development, although for Chapter 4 only mineral soil data and results were presented.

In contrast to these two more established forests (EP571 and the Danish tree species trials), the seedlings of the Rhizotron (Chapter 5), and the 10-yr old Bangor diverse experiment (Chapter 6) showed few tree species effects on soil microbial communities. The nitrogen-fixing alder dominated the Rhizotron experiment in terms of productivity, but this was not mirrored by a larger shift in microbial communities abundances of fungal *ITS*, bacterial *16S*, or any of the

nitrogen functional genes (Chapter 5, Figures 2-5). While shifts in microbial communities from pre- and post-treatment within the Rhizotron were observed, these did not extend to significant differences among tree species or mixtures. Within the Bangor Diverse experiment there were also a series of tree species mixtures, which also included an alder species, but again no significant differences were observed between the forest cover types (Chapter 5). These mixtures appeared to result in only additive effects on the microbial communities meaning the effects of two species was equal to the average of those two species, as opposed to non-additive or synergistic effects where the sum of the two is greater than the average combination from each tree species. In general, it can be concluded that stand age of length of time since dominant vegetation cover last changed, may play an important role in relation to the development of forest soil microbial communities. Furthermore, while these tree species may at some point develop specific microbial communities they are not currently captured by qPCR techniques.

1.2 Current understanding of microbial biodiversity

One of the benefits of using microbial community analyses, coupled with ecosystem process rates studies (e.g. gross rates of ammonification and nitrification) is the ability to connect the field of community ecology and microbiology. Nemergut et al. (2013) carefully bridged the divide between community ecologists and microbiologists in a review paper that identified key hypotheses for microbial community assembly. They called for a unified conceptual framework of microbial community assembly (interestingly, see Fierer et al. (2009) for their quest for a unifying principle in soil ecology), in an effort to shape the direction of future microbiological research. They first highlighted the unique aspects of microbial communities, namely passive dispersal via wind and air, dormant states (which are much less common in macro-organisms, and much more widespread across phylogenies for microorganisms), and possible phenotypic plasticity. While dispersal mechanisms were not explicitly tested in this dissertation, the spatial variation in microbial communities was explicitly examined in several chapters (Chapters 3-6). Nemergut et al. (2013) noted that microorganisms are typically not studied for their spatial and temporal patterns as closely as their macroorganismal counterparts, which the authors suggest should be addressed in future studies (Nemergut et al. 2013). This dissertation specifically included additional field sampling strategies to capture spatial variation in microbial communities.

Expanding upon studies focused on the distribution of microbes, Martiny et al. (2006) reviewed the global biogeography of microorganisms. Given the large literature base they supported the idea of free-living microbes exhibiting biogeographic patterns such as the Baas-Becking hypothesis (which states the environment selects which microbes would persist in a given set of environmental characteristics). Martiny et al. (2006) presented four alternative hypotheses useful for the exploration of microbial biogeographical patterns: H1. A null hypothesis that microbes are randomly distributed over space, H2. Microbes may be globally distributed, ubiquitous, and implies microbes have enormous dispersal potential which can override evolutionary selection pressures, entitled the Baas-Becking hypothesis (also known as the concept that "everything is everywhere"), H3. All spatial variation in microbial communities is due to lingering effects of historical events such as past environmental conditions or dispersal limitations, and H4. The distributions of microbial taxa reflect the influences of both past events and current environmental conditions.

Martiny et al. (2006) concluded that future studies were really needed to tackle the mechanisms that generate patterns in microbial diversity and distribution. This dissertation specifically aimed to combine explorations of soil microbial communities and in conjunction with studies with ecosystem processes like N cycling (Chapters 3, 4, and 6), or forest growth as an indirect effect of potential differences in soil microbial communities (Chapter 5). Causal relationships between tree species and soil microbial communities, ecosystem process, and the soil microbial community associated with changes in ecosystem functions were explored, to various degrees among the studies in this dissertation. A chief objective in this dissertation was to determine mechanistic links between coniferous tree species and gross nitrification and ammonification rates. In British Columbia (Chapter 3) for example, it is worth noting the significance of forest floor C:N ratios on functional soil microbial community gene abundances. Links between gross nitrification and ammonification rates and mineral soil microbial communities, but with a stronger connection to soil pH across three sites, were also found in Denmark (Chapter 4).

While this dissertation does span across large geographic ranges (Canada-UK-Denmark), the Baas-Becking hypotheses was not explicitly tested. However, it is important foundational

knowledge to allow for the further understanding of microbial diversity and global distribution as context for this dissertation. Finlay (2002) delved into the Baas-Becking hypothesis, and found that the abundance of individual microbial taxa is so great that geographic barriers are not likely to restrict their dispersal. Finlay concluded that if dispersal is ubiquitous microbial species richness might be low at a global scale. This was supported by a group of well-studied organisms, free-living ciliates, which have about 3,000 species (Finlay et al. 1996). This number stands in stark contrast with global terrestrial arthropod diversity for example, which is estimated at 6.8 million (Stork, 2015), and is likely due to diversification as a result of geographically limited ranges (Finlay 2002).

Bacterial diversity and biogeography within soils attracted a great deal of scientific interest, with Fierer and Jackson (2006) presenting a continental-scale description of soil bacterial communities across North and South America using 98 soil samples collected from across those regions. They found bacterial diversity was not related to site temperature, latitude, or many of the typically strong plant diversity predictor variables. Ecosystem type played an important role in shaping soil community bacterial richness and diversity (Fierer and Jackson 2006). They found bacterial diversity decreased from neutral to acidic soils. They suggested that microbial biographical patterns were strongly constrained by edaphic variables, and not as closely tied to latitude or geographic distance the way plant and animal diversity is.

Fungal biodiversity was recently reviewed by Tedersoo et al. (2014) who explored the vast global diversity and biogeography of fungi using DNA metabarcoding analysis of hundreds of globally-distributed soils samples, containing 80,485 operational taxonomic units (OTUs). They found the greatest diversity in tropical ecosystems except for ectomycorrhizal fungi, which were most abundant in temperate and boreal systems. They also found plant diversity and fungal diversity patterns were divergent, but climactic factors were the best predictor of fungal diversity. This raises additional concerns for important symbiotic relationships, such as those between ectomycorrhizal fungi, and many temperate and boreal forest tree species, in world where the climate that strongly controls fungi is changing rapidly.

1.3 Links across studies in relation to N transformations

We were able to discern both the intuitive relationships (such as a strong correlation between gross ammonification and bacterial *16S* gene copies), and observe surprising patterns (such as a lack of any significant relationship between ammonium consumption and bacterial *16S* gene copies). We found forest floor C:N ratios and microbial C:N ratios were also informative predictors of N transformation rates (Chapter 3), which was a consistent trend across multiple studies. Similar approaches with multiple linear regressions were used to determine the best predictors of N transformation rates within the Danish tree species trials (Chapter 4). Bacterial *16S* gene copies were great predictors of gross ammonification rates (Chapter 4) in the Danish tree species trials. In contrast to EP571, there were more relationships between fungal *ITS* gene copies and N transformation rates (including gross and net ammonification, and gross ammonium and nitrates consumption), than the singular instance of fungal *ITS* as an informative predictor for gross ammonification (Chapter 3). These patterns could suggest that fungal *ITS* plays a more discerning role in N transformations in the Danish forests compared to the Canadian forests in these experiments. This may be due to increased N in Danish forests, as a result of widespread industrialization, compared with the more pristine British Columbian forests that lack an urban industrial legacy.

The addition of partial least squares path analysis for the Rhizotron work (Chapter 5) was used to shed light on causal relationships between the four tree species and their rhizospheric soil microbial communities. Specifically the path analyses were used for comparing and contrasting the effects of single and mixed species on biomass, rhizospheric and bulk soil properties like pH and C:N ratios, and soil microbial communities. Positive correlations between monocultures and mixtures on soil microbial communities using path analyses were consistently observed, where Kruskal-Wallis tests did not discern differences. The alder and Douglas-fir path analysis showed stronger correlations between tree species and soil functional genes tied to N cycling, as well as rhizospheric C:N ratios, compared with the common oak and sycamore maple path analyses. These findings suggest that increases in alder and Douglas-fir growth can be directly linked to functional gene abundances relating to N cycling, which is logical as alders have symbiotic relationships with N-fixing bacteria.

2. Tree species effects on soil properties

Centuries of foresters have documented the legacy of tree species associations with specific soils, habitats, and environmental features (Binkley and Giardina, 1998). This thesis has been especially focused on tree species effects on nitrogen dynamics and soil microbial communities associated with the transformation of nitrogen in forest floors and mineral soils. Nitrogen is one of the essential nutrients, a critical component of amino acids and proteins, and is often a limiting resource in terrestrial environments (Vitousek, 1999). While forests are not often considered a large reservoir of nitrogen, their importance for global C dynamics is well respected, and they do harbour great potential to transform landscapes after afforestation or through targeted shifts of tree species compositions. For example, afforestation of former agricultural landscapes could lead to increased long-term sustainable production through soil stabilization and decreased soil erosion common to arable agricultural lands.

Augusto et al. (2002) reviewed the scientific literature relating to tree species effects on soil in European temperate forests. They noted that conifer plantations have been used extensively for afforestation throughout Europe as a result of policy drivers since the 19th century. These efforts led to an increased need for research on overstory tree species effects on forest ecosystems in general. More recently, Augusto et al. (2014) re-examined overstory tree species effects on soil fertility and forest functioning. They considered physical, chemical, biological, climatic, and anthropogenic characteristics of sites as context, including atmospheric nutrient deposition and nutrient losses (including K, Na, Ca, Mg, N, and S), C:N ratios, soil pH, modifications to understory vegetation communities, soil macro- and microfauna, and physical features like soil structure. Many of these same soil properties were altered by tree species, as demonstrated by this dissertation.

Tree species effects typically take decades to develop in the forest floor and the upper 10 cm of mineral soil or near roots. The Rhizotron and Bangor Diverse experiments clearly lacked many of the expected results in relation to soil microbial communities, which is likely due to the length of time those soils were exposed to their respective forest overstories. Augusto et al. (2002) summarized their results across tree species by ranking species with respect to their potential effects on soil fertility, and highlighted that testing and refining these rankings is a necessary step

in furthering our collective understanding of tree species effects. Coniferous tree species like spruce, pine, fir, and Douglas-fir were found to have the most pronounced soil acidifying effect, with oak, beech, and birch intermediate, and the maple, ash, lime, and hornbeam species having the least influence on soil pH. Augusto et al. (2002, 2014) findings support my own findings that coniferous tree species decreased soil pH more compared with deciduous tree species in Denmark (Chapter 4), compared with the beech, oak, maple, ash, and lime. Similarly, the Canadian coniferous tree species trials (Chapter 3) did demonstrate a strong influence on soil pH, although there were no deciduous trees in those same sites to explicitly test the divide between deciduous and coniferous tree species.

While tree growth is often limited as a result of N, P, Ca, Mg, or K deficiencies, central Europe does have an atmospheric deposition and industrial legacy, which alleviates the naturally occurring soil N deficiencies (Gundersen et al. 1998, Nadelhoffer et al. 1999). Potassium deficiencies are common on calcareous soils and associated with drought (Landmann et al. 1995). This is similar to observations for Ca and Mg except both Ca and Mg deficiencies are exacerbated in acidic soils, where K typically is not exacerbated in acidic soils. This suggests tree species-mediated soil acidification could in fact lead to an increase in nutrient-limited growth as Ca and Mg become more limited in acidified soils, which may be less prominent than K deficiencies or likely P deficiencies. Augusto et al. (2002) were unable to establish a general principal about overstory tree species effects on the composition of understories and soil physical features, and instead reiterated the need to maintain close links with forest management goals and objectives. Over a decade later, Augusto et al. (2014) concluded that forests dominated by evergreen gymnosperms functioned notably differently from forests dominated by deciduous angiosperms, specifically noting that N cycling was typically higher in deciduous angiosperm forests. Augusto et al. (2002, 2015) both summarized their findings by underscoring the significant abilities of tree species to modify their environments through organic inputs and interactions with soil microbial communities. They further highlighted the need to include tree species as one of many factors in forest management planning, and as a potential mitigation tool. The main objective of this dissertation was to explore tree species N dynamics, which Augusto et al. (2015) further reinforced as being poorly understood.

3. Tree species effects in pure stands and mixtures

The legacy of mixture effects on plant species on soil properties is not a recent scientific discovery, as noted by Darwin and Wallace (1858), "We know that it has been experimentally shown that a plot of land will field a greater weight if sown with several species and genera of grasses, than if sown with only two or three species." This was studied more explicitly in a stand of pure Douglas-fir was compared with an alder and Douglas-fir mixed plantation, both 30-years old, on the Wind River Experimental Forest (Tarrant and Miller, 1962). The soils beneath the mixture had significantly greater amounts of nitrogen in the forest floor and mineral soil compared with the pure Douglas-fir plantation. Tarrant and Miller (1962) found 425 kilograms more nitrogen per acre underneath the Douglas-fir and alder plantation, compared to the pure Douglas-fir plantation. This resulted in 16 additional kg of N accumulation per acre per year in the mixed plantation, indicative of a non-additive or synergistic effect in this experiment. Similarly, the Rhizotron mixtures of alder with Douglas-fir resulted in increased rhizospheric soil N concentrations, compared with Douglas-fir monocultures (Chapter 5).

The benefits of tree species mixtures on forests extend beyond soil development and nutrient accumulation, and extend to aboveground biomass accumulation and merchantable timber (Hooper et al. 2005; see also a meta-analysis by Zhang et al. 2012). Forests also provide a large range of broader ecosystem services including water purification, regulation of pests and diseases, which can be generally summarized as the diversity-productivity relationship (Millennium Ecosystem Assessment, 2005).

Mixtures of common European tree species including spruce and beech or oak and beech forests yield more biomass than pure stands, which is typically ascribed to reductions in competition because of complementary niches occupied by conspecifics in mixtures (Kelty 1992). This could also be referred to as the niche complementarity hypothesis, which postulates that a positive response to increased diversity is attributable to increased resource use and nutrient retention via niche differentiation or partitioning and intraspecific facilitation (Tilman 1999; Loreau et al. 2001; Hooper et al. 2005). One prominent pattern leading to increased productivity in mixtures vs. monocultures is called overyielding (Garnier et al. 1997). There is mixed support for increased diversity always leading to increased productivity with some examples perhaps

highlighting single tree species effects (e.g. the Amoroso and Turnblom (2011) example of Douglas-fir mixed with western hemlock), and others indicating species interactions along abiotic gradients may play a more dominant role in the outcome of those interactions (Chamberlain, Bronstein, and Rudgers 2014).

Lu et al. (2016) found overyielding of mixed forests in the Netherlands was partially dependent on the inclusion of evergreens or only deciduous tree species into the mixtures. They researched a suite of tree species including two of those used in the Rhizotron experiment (Chapter 5), Douglas-fir and common oak, as well as Scots pine, European beech, and silver birch. They predicted they would find support for the niche complementarity hypothesis, with stronger overyielding observed in forests comprised of species with a greater contrast in leaf phenology and shade tolerance. Lu et al. (2016) observed transgressive overyielding in Douglas-fir and European beech mixtures, and Scots pine-common oak mixtures (each compared to their respective monoculture stands). This study supports the notion that leaf traits and phenology (e.g. deciduous vs. coniferous) are important factors leading to consistent overyielding, even when thinning history, site quality, and stand development were factored in. They concluded by suggesting the inclusion of evergreens into deciduous mixtures is a valid forest management strategy. While Lu et al. (2016) primary focus was overyielding as it relates to productivity, it is a useful concept for applying to additive and non-additive effects of tree species mixtures on other aspects of forest ecosystems including N cycling and soil microbial community gene abundances (e.g. Chapter 5).

Mixture tree species effects along site productivity gradients were evaluated in a recent study using the French National Forest Inventory (Toigo et al. 2015). They examined overall productivity of 5 2-species mixtures and monocultures with five common European tree species: sessile oak, Scots pine, European beech, silver fir, and Norway spruce. They observed overyielding in the beech and Norway spruce, beech and silver fir, and silver fir and Norway spruce mixtures. The greatest positive effects of mixtures were observed on sites with low productivity, such as the highlands in this study. Toigo et al. (2015) found that mixed forest overyielding patterns diminished as site productivity increases, which suggests stand age may be a more important consideration for long-term forest management. This negative link between

mixtures and site productivity thus also supports the stress-gradient hypothesis (Bertness and Callaway 1994), which suggests that communities shift from competitive to facilitative interactions when transitioning from favourable to harsh environments. This does not mean nutrient competition only occurs in nutrient limited environments, but can also result from species-specific traits (see Binkley and Giardina 1998 for further discussion on frayed connections between trees and soils).

The stress-gradient hypothesis is, in essence, a further refinement of Grime's (1977) proposed competitor, stress-tolerant, and ruderal triangle of plant strategies, commonly referred to as the C-S-R theory for plant biology. While the observations of these diversity-productivity relationships are interesting, the ability for this pattern to occur in naturally assembled communities is debatable (Adler et al. 2011). Perhaps the most compelling research is refining the mechanistic links between net biodiversity effects and ecological and ecosystem processes (Cardinale 2011). This dissertation aimed to elucidate some of those mechanistic links between tree species and soil microbial communities and ecosystem functions.

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4. Species influences on nitrogen cycling

One major assumption in many terrestrial ecosystem models is that soil microbial communities placed into a common environment would function identically regardless of community composition. This is partially in contrast to the home-field advantage hypothesis (Ayres et al. 2009), but is more explicitly meant to highlight that high microbial diversity in soils might enable a quick alteration of their functions given a new set of environmental conditions. Strickland et al. (2009) used litter decomposition experiments to explicitly test whether a home-field advantage existed in relation to an important ecosystem process, C mineralization. They found that the origin of soil microbial communities played a significant role in a given soils C mineralization capacity (supporting the home-field advantage), and highlighted the need to include more accurate representations of soil microbial community composition into larger biogeochemical models (Strickland et al. 2009), especially for C and N cycles and in light of changing climatic conditions. While C mineralization was not explicitly evaluated in this dissertation, the study by Strickland et al. (2009) highlights the potential for soil microbial

communities to quickly adapt to changes in environmental characteristics, like afforestation or new tree species.

This dissertation aimed to explore soil biodiversity as it relates to tree species identity across a range of species with contrasting functional traits and geographic distributions (Chapter 3-6). The effects of forest mixtures compared with those of monocultures (Chapters 5,6) on soil biodiversity were also explored in this dissertation. The functional microbial community was specifically targeted to link soil microbial communities to N cycling processes. Smith et al. (2015) clearly articulated the importance of soil biodiversity in relation to biogeochemical cycling and ecosystem services. They used the Millennium Ecosystem Assessment (2005) framework to review the current understanding of biogeochemical cycles and soil biodiversity divided into provisioning, regulating, supporting, and cultural ecosystem services. They summarized that in relation to nitrogen, soil fertility was a critical provisioning ecosystem service facilitated by nutrient recycling from organic to plant available forms, physical-chemical properties which control their absorption, and strongly influenced organic matter (Smith et al. 2015). Importantly, the largest flux in N is from the internal cycling from plants to soils, with N availability typically being the limiting resource in terrestrial environments. They suggest an integrated approach to nutrient management that would focus on preserving existing organic matter stocks in soil and reducing nutrient losses.

Tree species, and the soil legacy of the land in which those trees grow, had large influences on N cycling processes in this dissertation. EP571 demonstrated the importance of tree species identity on gross ammonification rates, which coupled tightly with changes in microbial community gene abundances. This is supported by research on net and gross mineralization and nitrification rates in other forest studies (Verchot et al. 2001;Zeller et al 2007). More recently, Nelson et al. (2016) used soil metagenomes to characterize the distribution of microbial N traits across the globe, partitioned as the frequency of eight N-cycling pathways including: ammonia assimilation, assimilatory nitrate to nitrite, assimilatory nitrate to ammonium, nitrogen fixation, nitrification, dissimilatory nitrate to nitrite, denitrification, and dissimilatory nitrate to ammonium (Nelson et al. 2016, Figure 2A-B).

Nelson et al. (2016) found broad biogeographic patterns across all N cycling traits combined, with great variation among different soil samples. Habitat type was typically an important predictor of the frequencies of the eight N pathways when examined individually. Nelson et al. (2016) also found ammonia assimilation frequency increased with soil N in temperate forests, but decreased in tropical forests. Soil carbon was not a good predictor of the frequency of the individual pathways for bacteria. Archaea were generally worse at predicting individual pathways, compared with bacteria, although this is possibly due to a lack of sampling resolution for archaeal compared with the abundance of publically available bacterial data (Nelson et al. 2016). This dissertation showed greater tree species effects on archaeal ammonia-oxidiser gene abundances than bacterial ammonia-oxidisers, which suggests additional data on archaea might change the story presented by Nelson et al. (2016).

The legacy of former agricultural soils, and in turn those legacy effects on soil pH mediated tree species effects in the Danish tree species trials, all of which influenced N cycling processes. While habitat type was a useful predictor for the individual N cycling pathways in Nelson et al. (2016), genera that were dominant in one habitat type typically extended to dominating other habitats, and didn't correlate with pH, which is contrast with other studies. Thoms et al. (2010) explored links between tree species diversity and soil microbial communities using PLFA, in addition to pH, soil nutrient status. They found pH explained most of the variation within microbial community, suggesting that indirect effects from tree species (such as changes in pH) play an important role in regulating soil microbial community composition. Nerlson et al. (2016) finding is also surprising given the other datasets in this dissertation as well, which demonstrated strong connections between pH and soil microbial community composition and abundance. Nelson et al. (2016) concluded that assessing the links between genes and process rates using metagenomic traits is an important step forward.

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5. The importance of forest context and biodiversity

Under which conditions do [tree] species influence soils the most is a research question that continues to come to the forefront of ecology research, especially in light of an increasing necessity to understand ecosystem functioning and possibly respond to climate change with

adaptive management strategies. Methodologically, mesocosm experiments like the Rhizotron (Chapter 5), which aimed to look at early development of soil rhizospheric communities, are an important step in the process of understanding the full development of rhizospheric microbial communities. The use of mircorbial DNA extraction techniques continues to advance, with careful consideration to the best uses and limitations of those techniques (Blazewicz et al. 2013). The DNA extraction methods used also likely influence not just DNA yield, but will further influence our ability to accurately and precisely quantify bacterial, fungal, and archaeal abundances (Wagner et al. 2015).

What are the implications of studies established at a single site in an even-aged setting (which may represent large increases in site-level productivity) compared those set up across a range of sites (which may present much smaller changes in productivity)? Prescott and Grayston (2013) acknowledge the context-dependency of many tree species effects, stating that many factors are only relevant under certain circumstances, and that the real challenge is knowing under which circumstances mixing effects will have significant consequences on forest functions. This is consistent with the findings of Chapter 3 (EP571) that reinforced the context-dependency of tree species effects on nitrogen cycling and soil microbial communities, albeit EP571 is a single tree species study and not a forest diversity experiment. Similar methods were deployed in Chapter 4 (Danish tree species trials), which allowed for the exploration of the mechanisms behind tree species identity effects on gross nitrogen transformation processes and nitrogen cycling functional genes, with the inclusion of multiple sites along a pH gradient.

The methods used in this thesis partially address concerns raised by Forrester and Pretzsch (2015) about the consistency of tree species effects on multiple sites, for mixed tree species or diversity effects. Forrester and Pretzsch (2015) summarized various case studies and remind us that correlation does not equal causation, a well-worn axiom in science. They suggest the use of the production ecology equation (Monteith 1977), but perhaps more strongly advocate for the mass balance approach suggested by Binkley and Menyailo (2005) when attempting to quantify mixing effects in forests. In essence the mass balance approach is a useful tool for calculating predicted effects of species mixtures on ecosystem process rates, for example. Binkley and Menyailo's (2005) efforts to look for general patterns and generate equations to explain these

patterns are an example of the on-going quest in science to collect and evaluate evidence, to test and refine hypotheses, and achieve a greater understanding of how to world works. One area of interest with this in mind is the continued development and application of methods like structural equation modelling and path analyses (Chapter 5), which can be viewed as a means to explore causation in experimental studies.

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6. Global climate change effects on forests

Global climate change is one of the greatest threats to forest ecosystems, and mixed-species forests are considered an excellent mitigation tactic (Messier et al. 2013). The evidence to support this is mixed, with some studies suggest that high plant diversity is required to maintain ecosystem services (Isbell et al. 2011), while others (Grossiord et al. 2014) indicate forest ecosystems do not benefit from increased drought-resistance, which suggests some context-dependency of forest biodiversity effects. In an experimental platform in Finland, early stages of forest biodiversity effects in a 11 year old "established forest" were examined (Ampooter 2014). They found tree species identity influenced understory vegetation composition, diversity and temporal turnover, and these effects were strongest in monocultures with mixtures often diluting tree species effects (Ampooter 2014). The Bangor diverse experiment was designed to explore the effects of species mixtures and monocultures (Chapter 6), and we similarly found stronger effects in monocultures than mixtures. Additional large-scale tree species mixtures studies (Scherer-Lorenzen 2007, Baeten et al. 2013) are an important step forward in determining additive and non-additive effects on forest function over time.

Increased tree diversity may also influence insect communities, including potential ecosystem benefits such as reduced herbivory by forest pest insects (Jactel et al. 2007). Forest pests are one of the pressing concerns for global forest health, in addition to deforestation and global change. In Canada forest pests such as mountain pine beetle have ravaged dominant tree species and dramatically altered forest composition, and have important carbon feedbacks, which will exacerbate the effects of climate change (Kurz et al. 2008). The Skimikin plots (Thomas and Prescott, 2000) were sampled as a proposed portion of this dissertation, but this interior Canadian field site had been devastated and the lodgepole pine mortality and reforestation by neighbouring

tree species would confound potential tree species effects. Subsequent sampling in the remaining tree species plots was undertaken, but has not been included in this dissertation. Tree species identity may play a more important role on certain insects such as oribatid mites (Eissfeller et al. 2013), or ants (Del Toro 2013ab), which provide multiple ecosystem benefits and actually provide supporting ecosystem functions within forests including soil perturbation, increased N mineralization (Del Toro, Ribbons and Pelini 2012; Del Toro, Ribbons, and Ellison 2015), and possible influences on other insect guilds (Marquis et al. 2014).

A recent study by Mayor et al. (2017) explored ecosystem cycling of C and N along elevational gradients along temperate forested mountain ranges. They found that declining air temperatures coupled with increasing elevation reduced average plant community nitrogen concentrations, which coupled with soil organic matter content (C:N ratios) and microbial communities (measured through PLFA). This suggests that future changes in climate could disrupt ecosystem functions across mountains. While this dissertation was not established along an elevational gradient, changes in climate will also influence nutrient cycling dynamics in ecosystems across the globe. It is important to study nutrient cycling dynamics to understand how ecosystems are currently functioning.

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7. Forest age effects on soil properties, N cycling, and microbes

Forest age or stand development stage can be important co-factors with relation to nitrogen cycling, although there are mixed data on the length of time required for trees to influence rhizospheric vs. bulk soil properties. While a chronosequence approach was not used in this thesis, which would allow one to specifically test age effects, experiments were undertaken in forests of contrasting ages, from 3 to 60 years old. The long-term common garden experiments located in Canada (Thomas and Prescott 2000; Prescott and Vesterdal 2005) showed differences in soil physical and chemical properties such as changes in forest floor C:N ratios and distinct microbial communities under western red cedars. Further exploration of these same forests through the use of PLFA (Grayston and Prescott 2005) provided insights into tree species effects on some microbial patterns. In comparison with Grayston and Prescott (2005), these differences in microbial community composition were more pronounced in the recent qPCR work on the

same sites (Ribbons et al. 2016), which may suggest the longer a forest canopy is established the greater the effects on soil properties of that species or species mixture. The addition of the isotope pool-dilution studies of nitrogen transformation rates (Ribbons et al. 2016) showed contrasting microbial communities in relation to specific ecosystem functions developed in those soils over time as well, lending further support for the hypothesis that time is an important factor in establishing tree species effects on forest soils.

The Bangor Diverse tree species diversity experiment further elucidated the effects of forest age on soil development and is a younger forest manipulation established in 2004 (Ahmed et al. 2016). Ahmed et al. (2016) found bulk soil C stocks differed under contrasting forest types established just over a decade prior to sampling. Within that same experiment, few tree species or diversity effects were observed on soil microbial community abundance or differences in functional genes tied to N cycling (Chapter 6), which suggests that soil microbial communities may require a longer exposure period to contrasting litter and root inputs than soil C stocks. Alternatively, the soil microbial genetic pool may be highly resilient to shifts in the overstory community, and thus have a lag-time in response to forest changes, and resulting effects on carbon and nutrient inputs, that is longer than a decade (Liu et al. 2015). Another consideration is whether or not the effects of forest age are consistent over time, or if they reach a dynamic equilibrium after maturity (Gunina et al. 2016).

While it was not directly tested in this dissertation, it is also true that in many environments, seasonality influences soil microbial community function and composition (Thoms and Gleixner 2013, Bell et al. 2009), so shifts in seasonal inputs of leaf litter for example, could have large influences on soil microbial communities over time. Seasonal pulses in water, nutrients, and soil moisture can exert strong controls of microbial community structure and function (Blazewicz 2014). Succession of plant communities could play an especially important role in determining the long-term trajectories of soil microbial communities (Shi et al. 2015). This was supported by phylogenetic analyses of rhizospheric taxa showing a home-field advantage soil community that is adapted to plant species inputs.

"Unless someone like you cares a whole awful lot,
nothing is going to get better,
it simply will not."
The Lorax, Dr. Seuss

8. Conclusions and future directions

Tree species examined in this dissertation fostered unique soil microbial communities, which in turn transformed nitrogen at different rates and predominately in different forms (NH_4^+ vs. $NO_3^=$). Many of the 13 focal tree species influenced soils in terms of developing physical and chemical differences in soil properties like pH, C:N ratios, and microbial biomass. This dissertation adds to the larger body of scientific knowledge on species interactions, plant-soil dynamics, and nitrogen cycling in forests. A case study in British Columbia was used, which demonstrated that underlying site factors like N status can moderate tree species effects on forest floor N cycling, and soil microbial communities. Danish forest trials across a pH gradient showed that tree species have strong influences on nitrogen cycling gene abundances, but not fungal community gene abundances. The Rhizotron experiment showed early effects of overyielding in forest mixtures compared with monocultures, with the greatest gains noted in mixtures containing a nitrogen-fixing alder. However, little difference was observed in the Rhizotron's rhizospheric soil microbial community, suggesting more time may be needed for soil communities to adapt to tree species unique characteristics. Similarly small differences were observed been mixtures and monocultures in the Bangor diverse experiment, although N process rates did show mixture effects. Collectively, these studies show the importance of individual tree species effects on microbial communities and the ecosystem functions performed by these microbes.

This dissertation built upon a solid foundation of literature on patterns observed in forests, and part of its significance lies in its contribution to scientific understanding of how nitrogen is cycled in temperate forests underneath the thirteen tree species studied herein. This dissertation

has demonstrated tree species effects on microbial community structure and function, in single and mixed species forest environments, across a range of stand development from seedlings, saplings, to maturity. Evidence was gathered on the mechanistic link between tree species effects on soils, microbes, and nitrogen cycling dynamics in a variety of forests across multiple continents. This dissertation adds to the scientific communities' forest of knowledge by demonstrating the continued need for mechanistic-based research, the importance of context-dependency in understanding species interactions.

Future research should focus on filling the knowledge gaps further highlighted by this dissertation, which mostly centre around increasing direct observations of ecosystem process and microbial activity, rather than relying on proxies. 1. Active assays of soil microbial communities through RNA sequencing (DeAngelis and Firestone 2012) or metagenomics approaches (Fierer et al. 2007). This will provide a sharper insight into the active soil microbial community, which would go beyond the genetic potential of soils, and steer the field towards direct observations of processes rather than relying on the assumptions associated with enzyme encoding genes (Rocca et al. 2015). 2. Identify under which environmental contexts are relic DNA most commonly observed, and focus active assays on these areas to refine working theories on how these microbial communities actually function (Carini et al. 2017).

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