Examensarbeten **2009:15**
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Foto: Jennie Sverker

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I denna rapport redovisas ett examensarbete utfört vid Institutionen för skogens ekologi och skötsel, Skogsvetenskapliga fakulteten, SLU. Arbetet har handletts och granskats av handledaren, och godkänts av examinator. För rapportens slutliga innehåll är dock författaren ensam ansvarig.

This report presents an MSc/BSc thesis at the Department of Forest Ecology and Management, Faculty of Forest Sciences, SLU. The work has been supervised and reviewed by the supervisor, and been approved by the examinator. However, the author is the sole responsible for the content.

ABSTRACT

Nitrogen (N) is considered to be the most limiting nutrient for productivity in boreal forests, and the ability of plants to complex protein during decomposition is considered to be an important mechanism by which some plants regulate the N cycle. In this study I investigated whether six boreal plant species differed in their ability to complex proteins. I hypothesized 1) that species that dominate in late successional stands would exhibit higher complexation capacities than species that dominate in young stands, 2) that individual species would demonstrate an increase in their protein complexation capacity in response to nutrient limitation, and 3) that differences in protein complexation capacity among litter types would correspond to lower rates of N mineralization from an external protein source. I collected litters from ten forest stands located in the area of Arvidsjaur, Sweden (65˚35´-66˚07´N, 17˚15´-19˚26´E) with an age range between 35 to 355 years since last major fire, and in which fertility declined with stand age. Litters from three early successional dominant species (*Betula pendula, Pinus sylvestris, Vaccinium myrtillus*), two late successional dominant species (*Picea abies, Empetrum hermaphroditum*), and one intermediate species (*Vaccinium vitis-idaea*) were collected from each stand. Their litters were extracted and protein complexation capacities measured. The data demonstrated high complexation capacities for the two early successional species (*V. myrtillus* and *B. pendula*), which was inconsistent with the first hypothesis. No species demonstrated a significant correlation between their complexation capacity and stand age (i.e. fertility) across the 10 stands, which did not support my second hypothesis. Finally, litter extracts were added to a soil with and without a protein source, in order to evaluate whether litter extracts with high protein complexation capacities would demonstrate low N mineralizaiton rates. This experiment revealed that extracts from three species (*B. pendula, P. abies,* and *V. vitis-idaea*) resulted in lower N mineralization rates relative to the control, but in all cases this was due to microbial immobilization of N rather than protein complexation. These data are therefore inconsistent with several other studies that have demonstrated that between and within species variation increases in response to nutrient limitation, or that complexation effectively reduces N mineralization. As such, the data suggest that the mechanism of protein complexation for reducing N mineralization may not be as ubiquitous in boreal forests as previously thought.

SAMMANFATTNING

Kväve anses vara det näringsämne som begränsar produktiviteten mest i boreala skogar. Växter som dominerar på magra marker tros anpassa sig till kvävefattiga förhållanden genom att utveckla en förmåga att under nedbrytningen av förnan bilda proteinkomplex. Detta har visat sig vara en viktig mekanism för att reglera kvävecykeln och säkerställa den egna tillgången på kväve. I den här studien har jag undersök sex boreala växtarters förmåga att bilda proteinkomplex. Jag har utgått från följande hypoteser i mitt arbete. 1) Arter som dominerar i sena successioner borde ha en högre förmåga att bilda proteinkomplex än arter som dominerar i unga successioner. 2) Då näringstillgången minskar borde en enskild art uppvisa en ökande förmåga att bilda proteinkomplex. 3) Om en extern proteinkälla tillförs marken borde arter med hög förmåga att bilda proteinkomplex binda proportionerligt mer av markens kväveförråd och på så sätt minska mängden mineraliserat kväve. Jag samlade in förna från tio brandsuccessioner i närheten av Arvidsjaurs, Norrbottens län. Successionerna har ett åldersspann på 35 till 355 år sedan senaste stora brand och det antas att en ökande ålder resulterat i en mer påtaglig kvävebegränsning. I varje bestånd samlade jag in förna från tre träd arter (*Betula pendula, Pinus sylvestris, Picea abies*) och tre bärris (*Vaccinium myrtillus, Vaccinium vitis-idaea, Empetrum hermaphroditum*). Förnan extraherades med vatten och extraktets förmåga att bilda proteinkomplex analyserades sedan. Data visade att två av arterna som dominerar i yngre successioner (*B. pendula, V. myrtillus*) hade hög komplexbildande förmåga, vilket motsade min första hypotes. Ingen art visade på ett samband mellan förmågan att bilda proteinkomplex och beståndsålder (dvs. näringstillgång), vilket inte heller gav något stöd för min andra hypotes. Slutligen tillsattes förna-extrakten till jordprov med och utan protein för att undersöka om extrakt från växtarter med en hög komplexbildande förmåga skulle uppvisa en låg kvävemineraliseringsgrad och även en låg markrespiration. Den mikrobiella aktiviteten tros vara beroende av mineraliserat kväve för sin tillväxt, därför borde en minskad kvävemineralisering resultera i lägre mikrobiell tillväxt och reducerad respiration. Detta experiment visade att tillsatsen av extrakt från *B. pendula, P. abies* och *V. vitis-idaea* resulterade i att mindre kväve mineraliserades i jämförelse med kontrollen. För dessa tre arter berodde den låga kvävemineraliseringen dock på mikrobiell immobilisering av kväve snarare än komplexbildning då respirationen ökade när protein tillsattes. Mina resultat motsäger tidigare studier som visat att förmågan hos växter att bilda proteinkomplex varierar med kväveförrådet i marken. Varken mellan de sex växtarterna eller mellan individer inom en enskild art hittades skillnader i proteinkomplexbildning orsakad av kvävetillgången i marken. Mina studier kan inte heller ge stöd för de studier som visat att komplexbildning minskar graden av kvävemineralisering i skogsmark. Sammanfattningsvis antyder min studie att boreala växters förmåga att bilda proteinkomplex för att reducera kvävemineraliseringen, och på så sätt stärka sin konkurrenskraft, inte är lika betydelsefull i boreala skogar som tidigare föreslagits.

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INTRODUCTION

Nitrogen (N) is considered to be the most limiting nutrient for productivity in boreal forests (Tamm 1991). Following fire disturbance, young early-successional stands exhibit high availability of N (DeLuca et al. 2002) relative to old stands, despite a much smaller total soil N pool (Zackrisson et al. 2004). Greater N availability in these younger stands is due to the higher concentrations of NH_4^+, which plants can take up and synthesize with relatively low energy expense. This inexpensive and abundant source of N in younger stands results in higher stand productivity relative to older stands. As succession occurs, N availability greatly diminishes (DeLuca et al. 2002), and the factors responsible for this decline are poorly understood. One factor thought to be of primary importance in explaining this declining N availability is the complexation of organic N into polyphenolic complexes which occurs as plant litters decompose (Stevenson 1994).

Polyphenols influence N cycling through two main groups of mechanisms, by affecting microbial activity and through physico-chemical effects on the pools and forms of nutrients (Hättenschwiler and Vitousek 2000). Both mechanisms will have consequences for nutrient dynamics, species interactions and successional dynamics in late successional stages (Schimel et al. 1998). The physico-chemical effects mainly arise through complexation, where polyphenols create bonds with proteins in the litter or with extracellular enzymes from microorganisms (Hättenschwiler and Vitousek 2000). Complexation of proteins from litter during decomposition can alter N availability (Stevenson 1994) and decrease or prevent N mineralization (Northrup et al. 1995). The polyphenol-protein complexes are resistant to most decomposer organisms, and this leads to reduced plant litter decomposition rates and increased N retention in decomposing litter. It has also been suggested that polyphenolic complexes are positively associated with the release of dissolved organic nitrogen (DON) (Northrup et al. 1995, Hättenschwiler and Vitousek 2000) and greatly reduced nitrification rates (Lodhi and Killingbeck 1980). It is proposed that these mechanisms result in decreased overall ecosystem N loss by reducing the microbial mineralization step of the N cycle. This adaptation of plants to infertile soils has been proposed as an attribute that controls the fate of N and to give a competitive advantage to plant species that uptake N in organic forms (Kaye and Hart 1997).

The ability of plants to complex organic N is likely to vary greatly between species due to the enormous interspecific variation in foliar traits found within most plant communities. Different plant species possess contrasting physiological attributes that can give advantages at different chronosequence stages during succession and retrogression (Cortez et al. 2007, Quested et al. 2007). These traits can have large effects on soils and soil processes, and as such, different species can differ greatly in their effects on soil properties. As ecosystems retrogress as a result of declining nutrient availability, they become dominated by less productive plant species that input poor quality litter to the soil (Crutsinger et al. 2008). This increased nutrient limitation affects both nutrient concentration and secondary metabolites in tissues as well as plant biomass production (Wardle et al. 1997, Hattenschwiler et al. 2003, Richardson et al. 2004). Further, with increasing mineral nutrient stress during secondary succession, those plant species that begin to dominate are increasingly stress-tolerant, longer-lived and slower growing (Grime 2001) and have a greater potential to preserve nitrogen within the plant tissues and the ecosystem.

In addition to the large variation demonstrated between species, individual species also can exhibit large variation in leaf characteristics in response to nutrient availability. Within-species responses to gradients of resource availability can be driven by

either phenotypic plasticity or genotypic variation within the population (Findlay et al. 1996) (Gallet et al. 1999). Intraspecific variation of polyphenol concentrations across soils of contrasting fertility has been reported for several plant species, and there is strong evidence for production of polyphenolics in response to N limitation (Northup et al. 1998), however, this phenomenon has seldom been studied in boreal systems. It is thought that the success of some plant species in occupying a broad range of conditions is due to a high degree of phenotypic plasticity in leaf and litter quality together with genotypic selection over a large timescale (Valladares et al. 2007). Both may have implications on the litter quality input and nutrient cycling at the ecosystem level (Findlay et al. 1996), and may create competitive advantages for particular species because a high polyphenol concentration could allow nutrient conserving species to lower the nutrient availability beyond the threshold of its competitors (Aerts and Chapin 2000). By doing so, a given species may increase its own frequency within the community, and this may help drive succession, as well as greatly influence the diversity and functioning of the ecosystem. This suggests that the ability of some species to increase complexation in response to N limitation could be of great importance in ecosystem development, by influencing both plant composition and soil conditions.

In this study, I conducted experimental studies to investigate the role of polyphenols and complexation in nutrient competition theory. In doing this I tested the following three hypotheses:

(1) I hypothesized that evergreen species, which dominate in old succession nutrient poor forests, will complex protein more efficiently than will deciduous species. Evergreen species are known to contain high concentrations of polyphenols, which could give them an advantage in competition for nutrients on infertile soils by complexing nitrogen and preventing N mineralization. This means a reduction in the rate of nutrient cycling between plants and the soil, and a smaller risk of loss of mineral nutrients either by leaching or by incorporation into other organisms (Monk 1966, 1971, Thomas and Grigal 1976).

(2) I hypothesized that variation in protein complexation ability within each species will be explained by stand age, with higher complexation occurring in late successional stands. Old stands are known to be more nutrient limited than are young stands. In order to maximize their fitness, species can alter their polyphenol composition and concentration in order to improve litter nitrogen recovery and minimize nutrient losses, and thus enabling them to sustain long-term ecosystem productivity on strongly acidic and infertile soils (Northup et al. 1995).

(3) I hypothesized that extracts which have a high protein complexation capacity will result in reduced microbial respiration and reduced NH_4^+ accumulation when added to a test soil. When the plants become more nutrient efficient, less nutrients will be available to the microbes in the soil, and this will in turn lead to decreased microbial activity and mineralization of nitrogen. The overall effect of phenolic compounds in litter material should reduce decomposition rates of organic materials, resulting in reduced nutrient mineralization rates (Kuiters 1990).

MATERIALS AND METHODS

Site description

Litters used in this study were collected from 10 boreal forest locations during 8 -12 September 2008 in the area of Arvidsjaur, Sweden (65˚35´-66˚07´N, 17˚15´-19˚26´E). The ten sites formed a chronosequence, with stand age ranging from 35 to 355 years since the most recent major fire (Table 1). All sites are dominated by Scots pine (*Pinus sylvestris* L.) with a secondary occurrence of Norway spruce (*Picea abies L*. (Karst.)) and scattered individuals of silver birch (*Betula pendula* (Roth.)) and downy birch (*Betula pubescens* (Ehrh.)). The proportion of *P. abies* in the stand increases with stand age. The ground vegetation in the mature stands is dominated by ericaceous dwarf shrubs and dense carpets of feather mosses, mainly *Pleurozium schreberi* (Bird. (Mitt.)). In the young sites there is a high proportion of the grass *Deschampsia flexuosa* (L.) Trin. (wavy hairgrass), and the dwarf shrubs *Vaccinium myrtillus* (bilberry) and *Vaccinium vitis-idaea* (L.) (lingonberry) in the ground layer. Meanwhile the ground layer vegetation in the older sites have a larger proportion of *Empetrum hermaphroditum* Hagerup (black crowberry) and *P. schreberi*.

Forest site	**Time since fire (yr)**	**Total N (%)**	**Total C (%)**	**Total tree Basal area (m^2ha^{-1})**
Njållatjivelg	35	0.99	42.2	**-**
Järvliden	41	1.05	45.8	**15.3**
Granliden	78	1.25	44.4	**20**
Avaviken	101	0.74	31.6	**25.2**
Nyvall	124	1.18	47.9	**-**
Guorbåive	171	1.04	40.8	**-**
Tjadnes	244	1.27	43.9	**20**
Vaksliden	300	-	-	**-**
Kuottavare	309	1.39	44.9	**17**
Ruttjeheden	355	1.02	42.3	**21**

Table 1. Age, total nitrogen and carbon concentration of the soil organic horizon and total tree basal area at the ten study sites (Zackrisson et al. 2004).

Field sampling

Litter from the three main tree species (*B. pendula*, *P. abies* and *P. sylvestris*) and three main ericaceous shrub species (*V. myrtillus, V. vitis-idaea* and *E. hermaphroditum*) was collected. Differences in vegetation among the sites resulted in varying availability of litter of these species, notably for *B. pendula* and *P. sylvestris* which were rare at some sites.

I collected litter from the three tree species by shaking small trees or separate branches. I spread out a large plastic bag for the litter to fall onto, beneath each tree or branch. This allowed for separation of the newly senescent leaves or needles of the desired species from other litter types. Approximately ten individuals from all tree species were used for collecting samples from each site, while for the shrubs a larger number of individuals were used. In a few sites I had difficulty to find sufficient individuals of *P. sylvestris* that were small enough to shake or with branches within reach. In these cases, I picked fresh needles from the ground and estimated the number of trees this litter came from. I collected litter from *V. myrtillus* and *E. hermaphroditum* by cutting off stems with a high percentage of dead leaves. From *V. vitis-idaea* I instead collected blackened dead leaves still adhered to the plants. All the shrubs were found in sufficient numbers in all ten sites. The litter samples were all immediately stored in room temperature for one week and thereafter put to dry in the oven at 28˚ C for two days before being sorted from other organic matter. To facilitate the sorting of *E. hermaphroditum* I shook the branches before the litter was put in the oven to dry. This made the sorting easier as the brown leaves (i.e., litter) came off at this stage while the green leaves still were attached to the branches. This collection process yielded 5 grams of litter from each species at each site.

Experimental set-up

Litter extract analysis

After doing preliminary experiments I decided to use 2 g of litter from each of the 60 samples to provide sufficiently concentrated extracts. The litters were mixed with 100 ml of deionized (DI) water and thereafter the mixtures were shaken for 24 hours and extracted through 0.2 µm disposable vacuum filters connected to a vacuum pump. Each 100 ml extract was divided into two 50 ml aliquots, and stored in the freezer until further analysis.

Several chemical properties were measured on these litter extracts. The polyphenol concentrations in the extracts were measured using the Prussian blue method (Stern et al. 1996). Because of a large difference in polyphenol concentrations between the litters types the measurements had to be repeated with diluted litter extract concentrations for *V. myrtillus* and *P. abies*. The concentration of NH_4^+ - N was measured on an Autoanalyzer III (Bran and Luebbe, Chicago, IL) using the Berthelot reaction, while simple carbohydrates was measured through reaction with anthrone (Brink et al. 1960). Dissolved organic carbon (DOC) and TN of the litter extracts was measured on a DOC/TN analyzer (Lachat Instruments).

Protein precipitation capacity of litter extracts

The method I used to determine the protein complexation capacity of the litters is similar to the Radial Diffusion Method and the method described by Joanisse et al (2008), where litter extracts are combined with an external protein source in order to assess their degree of complexation. However, our use of this approach differs from that of Joanisse et al (2008) in that they quantified the non-complexed protein remaining in the solution rather than the total N content in the precipitate.

I started the analysis by creating two parallel 15 ml centrifuge tubes for each litter sample, with one tube receiving foreign protein, and the second tube serving as a no-protein control that allowed subtraction of any background protein or absorbance interference that might have existed in the litter extracts. The protein tube received 0.5 ml of the BSA protein (Bovine Serum Albimun), while the control tube was amended with 0.5 ml of DI water. My goal was to add more protein than could be complexed by the extract solution, so that the maximum protein complexation capacity could be measured. Because the litter extracts showed a vast difference in complexation capacity, the analysis had to be repeated using higher BSA concentrations for some species.

Since it was uncertain as to how much protein the extracts could complex, I begun diluting the BSA solution to an appropriate concentration. The first concentration range was achieved by adding 0.5 ml of 1000 ppm BSA solution to 4.5 ml of extract solution, creating a 100 ppm BSA. To calculate the sample protein concentrations I then used a BSA standard curve derived from six standards of 0, 20, 40, 60, 80 and 100 ppm BSA. The tubes were vortexed and left overnight in the fridge. They were then centrifuged for 10 minutes at approximately 3000 rpm. This resulted in the tannin-protein complexes each forming a pellet in the bottom of the tube, below a clear supernatant liquid. To measure the protein content of the supernatant, I reacted a diluted portion of it with Bio-Rad protein reagent, and measured its absorbance at 595 nm on a spectrophotometer. These measurements showed that two species, *V. myrtillus* and *E. hermaphroditum*, complexed all the protein added, so the analysis was therefore repeated as above, using a 10 fold higher protein concentration.

The amount of complexed protein in each extract solution was estimated by calculating the net absorbance (difference between sorption of protein and control tube) for each sample, and converting this value to ppm protein using a standard calibration curve. The calibration curve was obtained by plotting the ppm (y-axis) of known standards against the absorbance (x-axis) for each standard. A linear equation was generated for this relationship, which was used to converted net absorbance to ppm protein. This value was then subtracted from the total concentration of protein before complexation occurred, yielding an estimate of protein complexed.

Soil samples

Forest humus was collected for use as a soil substrate for a N mineralization experiment utilizing these litter extracts. The humus was collected from a forest composed of *Pinus sylvestris* and *Picea abies* with an understory dominated by *Vaccinium myrtillus*, 0.5 km east of Skogshögskolan, SLU, Umeå. I collected the humus from two adjacent plots in the forest and sieved it immediately to remove roots and litter. The initial water content of the humus was determined to be 415 % (dry weight basis), and at this level the humus was nearly saturated. In order to achieve a less saturated moisture content, we dried the soil to 200 % by spreading the soil in four trays in an aerated oven at 28° C for three days. The addition of litter extracts to this partially dried soil brought the water content of each sample to 275%. This level of moisture in the humus does not inhibit microbial activity or gas exchange (Brady and Weil 2002).

Soil incubation

After the preliminary N analysis of the soil, I prepared 140 samples of 5.0 g of soil (dry weight equivalent), and placed it in 100 ml glass jars. The experiment consisted of a factorial combination of 7 litter treatments (i.e., litter extract from *B. pendula*, *P. abies*, *P. sylvestris*, *V. vitis-idaea*, *V. myrtillus*, *E. hermaphroditum*, and a non-litter amended treatment of DI water) x two protein treatments (added or not added). There were ten replicates for each litter x protein treatment combination, which represented the ten stands from which litter was collected. The protein-amended treatment consisted of 4.5 ml extract (or DI water for the non-litter treatment) and 0.5 ml BSA added to the soil, while the non-protein amended treatment consisted of addition of 4.5 ml extract (or DI water for the non litter treatment) and 0.5 ml DI-water.

Litter extracts plus BSA solutions sat for 2 hours before addition to soil to allow complexation to occur. The 100 ml jars with soil and solution were covered with perforated aluminium foil and incubated in the dark at 15˚ C. The incubation lasted for 18 days. The CO_2 concentration in the headspace of the jars was measured 5 times during this period, at day 2, 4, 7, 11 and 16, however only the first and last measurement are reported here.

To measure respiration I covered the jars with tight rubber septa to prevent CO_2 from leaking. I covered the jars with septa at one-minute intervals between each jar, allowed CO_2 to accumulate, and measured CO_2 concentration in each jar exactly 3 hours later. Samples were then injected into an Infrared Gas Analyzer (IRGA) in one-minute intervals, and the concentration of CO_2 present was compared with CO_2 standards (0, 402, 1800 ppm CO_2), which were later used to convert CO_2 concentration to μg CO_2 respired per g soil dry weight per hour.

After 18 days of incubation, I extracted the soil samples by adding 50 ml of 1M KCl to each jar, shook them for 1 hour, and vacuum filtered them through Whatman #42 filter papers. Measurements of NH_4^+ - N and NO_3^- - N were performed on these extracts as described above. Nitrate concentrations on these extracts were below detection limit, and are therefore not reported.

Statistical analysis

All data were first analyzed for assumptions of normality and homogeneity of variance required for parametric data analysis. Some data needed to be transformed (ln (X + 1)) to meet these assumptions. For the litter descriptive data, a one-way ANOVA was used to determine whether significant differences between litter extract types occurred for each chemical property. These ANOVAs were followed by the S-N-K post hoc procedure at $\alpha = 0.05$ to determine pairwise differences among treatments. For the incubation experiment, respiration and ammonium data were first compared using a two-factor ANOVA, with protein (with or without) and litter extract (*B. pendula, P. abies, P. sylvestris, V. vitis-idaea, V. myrtillus, E. hermaphroditum* and DI water) as fixed factors. This analysis was followed by post-hoc two-way ANOVA's within each species. Finally, I investigated whether any within species correlations occurred between any protein complexation capacity and incubation response variables using Spearman's rank correlation coefficients.

RESULTS

Litter descriptive data

For all chemical properties (DOC, TN, phenols, NH_4^+ or simple carbohydrates), extracts of *Pinus sylvestris*, *Picea abies* and *Vaccinium vitis-idaea* had significantly lower concentrations than did *V. myrtillus*, *B. pendula* and *E. hermaphroditum* (Figure 1). The highest concentrations for all chemical properties were found for *Vaccinium myrtillus*. *Betula pendula* extracts were similar to those of *V. myrtillus* in that concentrations of TN and NH_4^+ were significantly higher than for all other extracts. However, all carbon variables (DOC, phenols, simple carbohydrates) were significantly lower for *B. pendula* than for *V. myrtillus* (though still higher than for all the other litter extracts). *Empetrum hermaphroditum* had significantly lower values for all chemical variables than did *V. myrtillus* and *B. pendula*. However, DOC and TN concentrations for *E. hermaphroditum* were significantly higher than for all the remaining species (Figure 1).

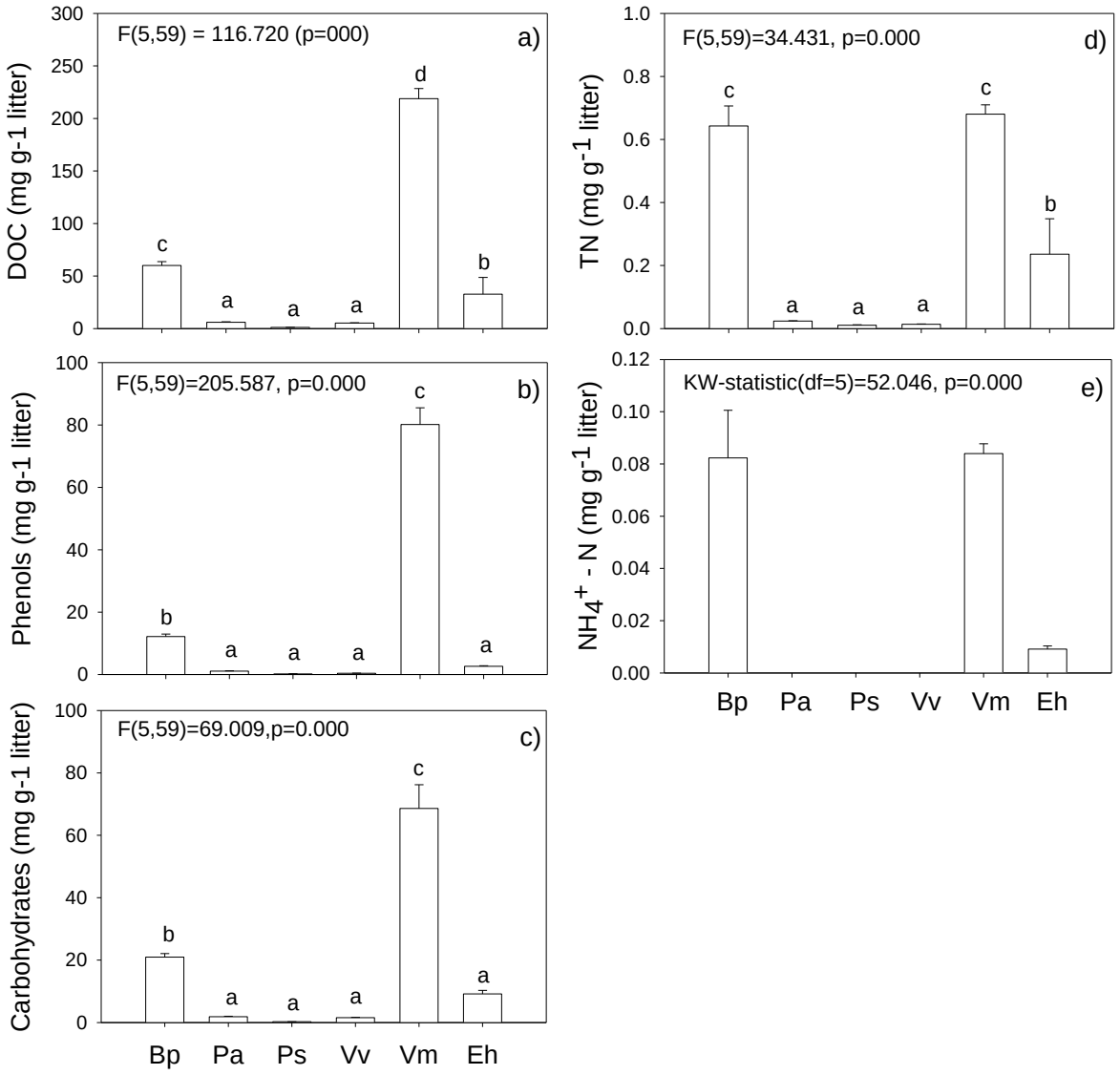

Figure 1. Mean (+SE) DOC (dissolved organic carbon), total nitrogen, phenols, NH_4^+ -N and simple carbohydrates measured from litter extracts of *B. pendula* (Bp), *P. abies* (Pa), *P. sylvestris* (Ps), *V. vitis-idaea* (Vv), *V. myrtillus* (Vm), *and E. hermaphroditum* (Eh). Letters above bars (a,b,c,d) reflect post-hoc comparisons between species.

Complexation assay results

Vaccinium myrtillus showed a significantly higher protein complexation capacity than did the five other litter extracts. Further, *E. hermaphroditum* demonstrated a significantly higher complexation capacity than did *B. pendula, P. abies, P. sylvestris* and *V. vitis-idaea*. Among these litter types *B. pendula* had a significantly higher complexation capacity than did *P. sylvestris*, and a similar capacity to *P. abies* and *V. vitis-idaea* (Figure 2a).

The complexation:phenol ratio demonstrated a different pattern than did protein precipitation capacity. *Vaccinium vitis-idaea* demonstrated a significantly higher ratio than all other species. Both *V. myrtillus* and *B. pendula* demonstrated significantly lower ratios than did all other species, whereas *P. sylvestris, E. hermaphroditum*, and *P. abies* demonstrated intermediate ratios (Figure 2b).

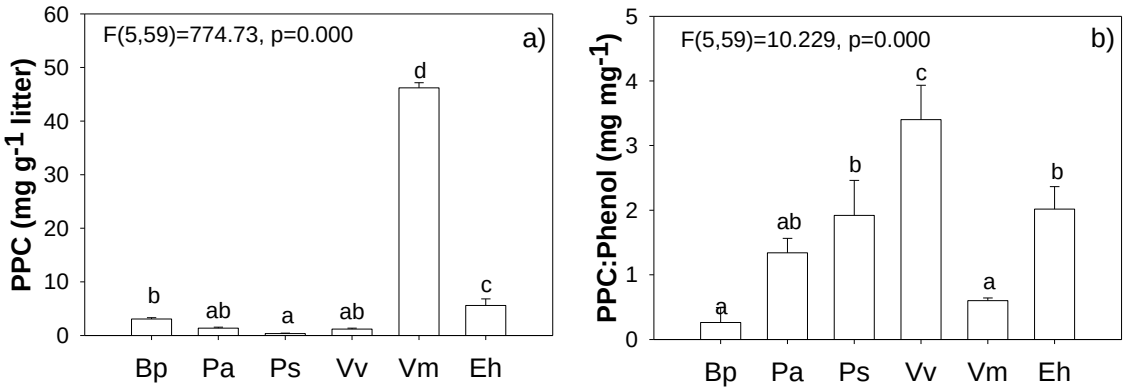

Figure 2. The mean (+SE) protein complexation capacity (PPC) (a), and mean (+SE) ratio of protein complexation capacity to phenol concentration of litter extracts from six boreal species (b), *B. pendula* (Bp), *P. abies* (Pa), *P. sylvestris* (Ps), *V. vitis-idaea* (Vv), *V. myrtillus* (Vm), *and E. hermaphroditum* (Eh). Letters above bars (a,b,c,d) reflect post-hoc comparisons between species.

Incubation data

NH_4^+ concentration in the soil extracts

The addition of protein resulted in a strong positive effect on the NH_4^+ concentration in the soil. A significant litter effect on final NH_4^+ concentration was also detected, with soils treated with *V. vitis-idaea*, *P. sylvestris* and *P. abies* demonstrating a significantly higher concentration of NH_4^+ relative to *B. pendula*, *V. myrtillus* and *E. hermaphroditum* (Figure 3). Relative to the control, the litter effect on final NH_4^+ concentration was positive in four litter types (*B. pendula, P. abie*s, *P. sylvestris, V. vitis-idaea*), and did not differ from the control for two litter types (*E. hermaphroditum* and *V. myrtillus*). *Vaccinium vitis-idaea* showed the strongest litter effect, closely followed by *P. abies*. *Pinus sylvestris* had a strong positive litter effect compared to *B. pendula* and *V. myrtillus*, both of which had similarly weak effects. Significant protein by litter extract interactions were detected for several species (Table 2). Three species, *B. pendula*, *P. abies*, and *V. vitis-idaea*, demonstrated negative extract by protein interactions, whereas *E. hermaphroditum* demonstrated a positive interaction. No significant interactive effect between protein and extracts was detected for *P. sylvestris* and *V. myrtillus* (Table 2, Figure 3.).

	Litter	**Protein**	**Litter x Protein**
Initial 2-Way ANOVA	**13.2 (<0.001)**	**10.4 (0.058)**	**7.3 (<0.001)**
Post hoc 2-Way ANOVA			
Betula pendula	3.8 (0.058)	1.5 (0.234)	**14.6 (0.001)**
Picea abies	**55.5 (<0.001)**	**13.0 (0.001)**	**35.3 (<0.001)**
Pinus sylvestris	**14.4 (0.001)**	1.2 (0.272)	0.0 (0.941)
Vaccinium vitis-idaea	**27.4 (<0.001)**	**13.0 (0.001)**	**24.4 (<0.001)**
Vaccinium myrtillus	1.2 (0.287)	0.2 (0.668)	3.8 (0.060)
Empetrum hermaphroditum	0.8 (0.376)	0.8 (0.376)	**8.7 (0.006)**

Table 2. ANOVA tables (F and P values) evaluating the effect of protein and litter, and their interaction, on extracable NH_4^+ - N at the end of a soil incubation experiment. An initial 2-way ANOVA was done to evaluate the effect of Protein (present or absent) and Litter (six groups: *Betula pendula, Picea abies, Pinus sylvestris, Vaccinium vitis-idaea, Vaccinium myrtillus, and Empetrum hermaphroditum*), and their interaction (alpha = 0.05). Post hoc 2-way ANOVA's were performed for each litter type in order to evaluate individual interactive effects of each litter type with protein. The alpha level for post hoc ANOVA's were Bonferoni adjusted (0.008), with the significant values put in bold.

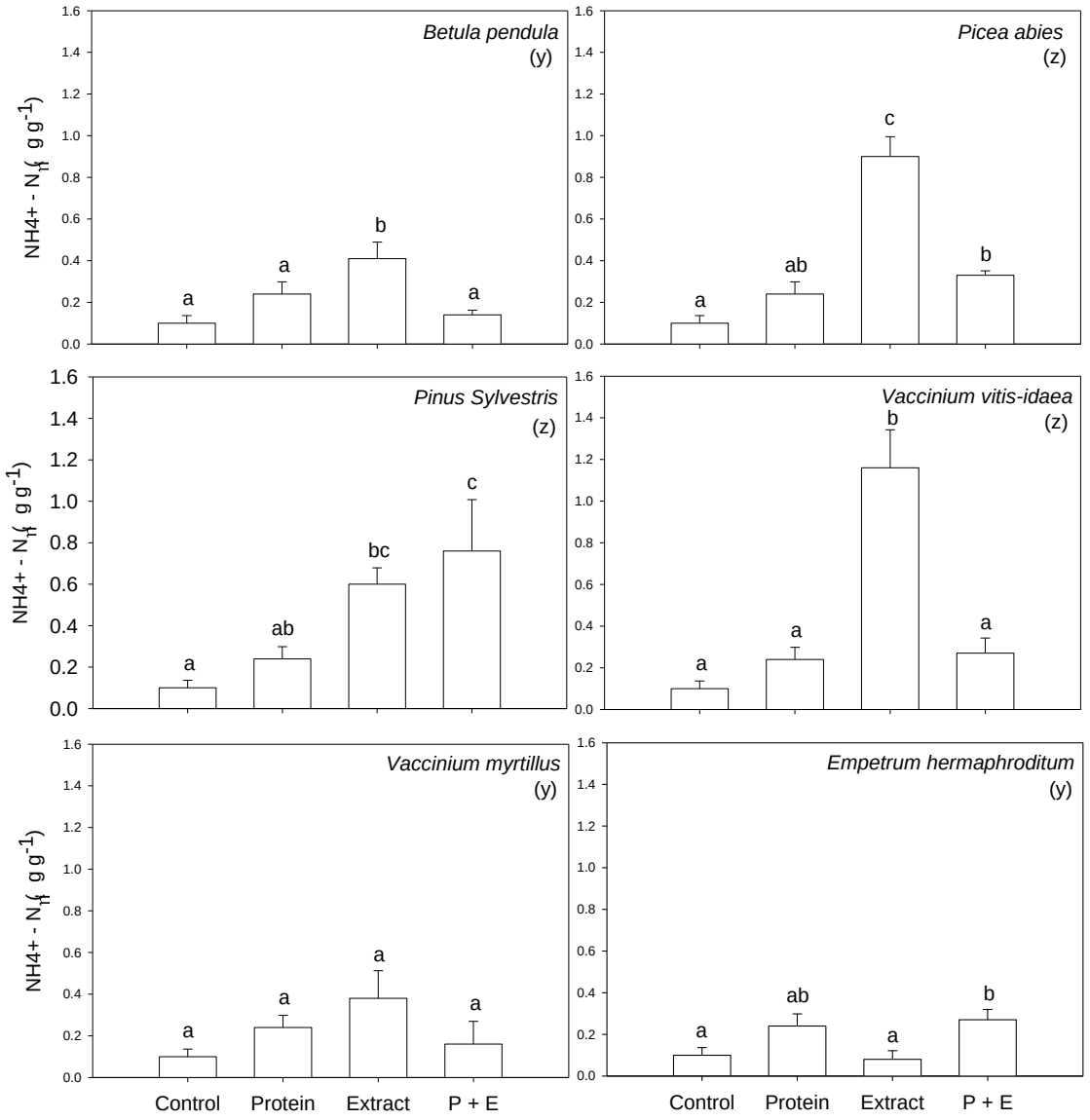

Figure 3. Mean (+SE) ammonium concentration at the end of a soil incubation experiment, where soils were treated with a factorial combination of six litter extract types (*B. pendula*, *P. abies*, *P. sylvestris*, *V. vitis-idaea*, *V. myrtillus*, and *E. hermaphroditum*) and two protein treatments (with and without). Letters above bars indicate post-hoc differences within each species. Letters beneath each species name indicate post-hoc differences in mean species effect (x,y,z).

Respiration results

The addition of protein resulted in a strong positive effect on respiration at day 1 (Table 3, Figure 4). A significant difference between litter types was detected at day 1, with *V. myrtillus* and *B. pendula* causing significantly higher respiration than *P. sylvestris* and *V. vitis-idaea*. Relative to the control, *P. abies*, *P. sylvestris* and *V. vitis-idaea* showed significant negative effects, and *V. myrtillus*, *E. hermaphroditum*, and *B. pendula* demonstrated significant positive effect on respiration at day 1. All litter types also demonstrated a significant positive interactive effect with protein at day 1.

The positive effect of protein on respiration remained strong at day 18 (Table 3, Figure 4.), whereas the effect of litter weakened. A significant difference between species was still detectable, with *P. sylvestris* and *V. vitis-idaea* showing higher respiration than *V. myrtillus* and *E. hermaphroditum*. Relative to the control, *P. sylvestris*, *V. vitis-idaea* and *E. hermaphroditum* demonstrated a weak positive effect, whereas *B. pendula*, *P. abies* and *V. myrtillus* did not demonstrate any significant effect on respiration. At the end of the incubation, a significant interaction between litter extract and protein was only present for *E. hermaphroditum* and *V. vitis-idaea*, which demonstrated weak, positive interactive effects at day 18.

	Time	**Litter**	**Protein**	**Litter x Protein**
Initial 2-Way ANOVA	Day 1	**117.0 (<0.001)**	**91.9 (<0.001)**	**4.2 (0.001)**
	Day 18	**4,1 (0.001)**	**59.2 (<0.001)**	0.8 (0.587)
Post hoc 2-Way ANOVA				
Betula pendula	Day 1	**86.8 (<0.001)**	**36.1 (<0.001)**	**36.1 (<0.001)**
	Day 18	0.2 (0.675)	**70.3 (<0.001)**	3.2 (0.081)
Picea abies	Day 1	2.6 (0.118)	**11.0 (0.002)**	**7**.3 (0.010)
	Day 18	0.3 (0.619)	**13.4 (0.001)**	1.2 (0.290)
Pinus sylvestris	Day 1	**16.7 (<0.001)**	**29.9 (<0.001)**	**20.3 (<0.001)**
	Day 18	1,6 (0.215)	**72.4 (<0.001)**	5.5 (0.024)
Vaccinium vitis-idaea	Day 1	**9.9 (0.003)**	**39.2 (<0.001)**	**29.2 (<0.001)**
	Day 18	3.0 (0.093)	**71.3 (<0.001)**	7.4 (0.010)
Vaccinium myrtillus	Day 1	**222.1 (<0.001)**	**15.9 (<0.001)**	**13.5 (0.001)**
	Day 18	4.9 (0.033)	**16.7 (<0.001)**	0.0 (0.976)
Empetrum hermaphroditum	Day 1	**44.1 (<0.001)**	**13.3 (0.001)**	**9.8 (0.003)**
	Day 18	**21.8 (<0.001)**	**102.0 (<0.001)**	0.029 (0.866)

Table 3. ANOVA tables (F and P values) for the effect of protein and litter, and their interaction on soil respiration ($\mu g\ g^{-1}\ hr^{-1}$) at the beginning and end of a soil incubation. An initial 2-way ANOVA was done to evaluate the effect of protein (present or absent) and litter (six groups: *Betula pendula*, *Picea abies*, *Pinus sylvestris*, *Vaccinium vitis-idaea*, *Vaccinium myrtillus, and Empetrum hermaphroditum*), and their interaction (alpha = 0.05). Post hoc 2-way ANOVAs were performed for each litter type in order to evaluate individual interactive effects of each litter type with protein. The alpha level for post hoc ANOVAs were Bonferoni adjusted (alpha = 0.008), with the significant values in bold.

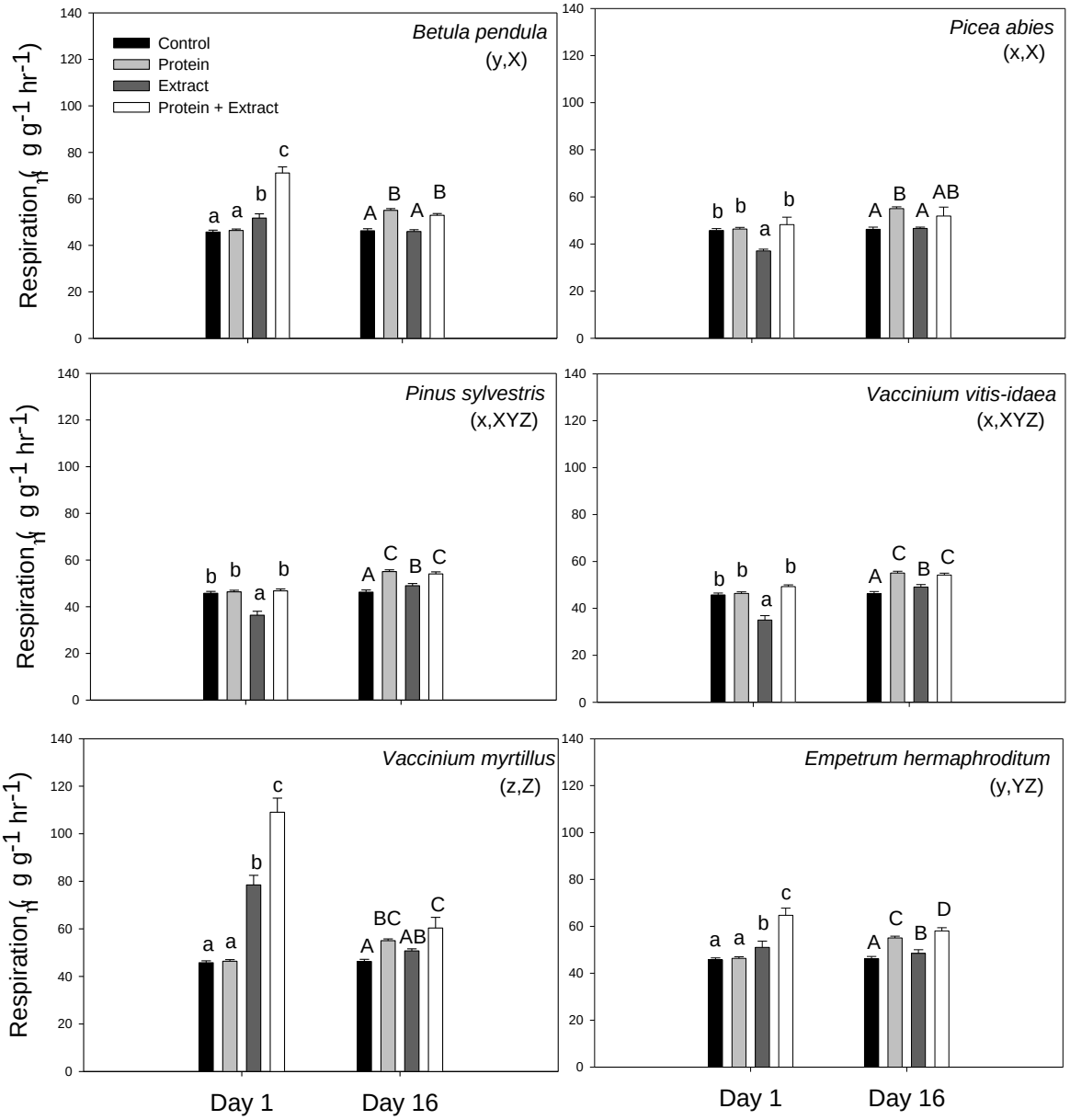

Figure 4. Mean (+SE) soil respiration at the beginning and end of a soil incubation experiment, where soils were treated with a factorial combination of six litter extract types (*B. pendula, P. abies, P. sylvestris, V. vitis-idaea, V. myrtillus, and E. hermaphroditum*) and two protein treatments (with and without). Letters above bars (a,b,c,d) reflect post-hoc comparisons within species, and letters beneath each species name reflect post-hoc comparison of the species effect (x,y,z). Lower case letters are used for day one, and upper case letters for day 18.

DISCUSSION

There is currently substantial interest in understanding how variation of key plant traits drives ecological processes (Kuiters 1991, Hobbie 1992, Northup et al. 1995, Hättenschwiler and Vitousek 2000). My study directly addressed how variation in one important chemical trait, namely protein complexation, influences a key process in boreal forests, namely N mineralization. Specifically, I evaluated whether six boreal plant species (*Pinus sylvestris, Picea abies, Betula pendula, Vaccinium myrtillus, Vaccinium vitis-idaea,* and *Empetrum hermaphroditum*) differed in protein complexation capacity, and the implications of these differences for N mineralization. Additionally, I investigated whether individual species increase their protein complexation capacity in response to decreasing nutrient availability across a well described fire chronosequence.

The data demonstrated that all species were able to complex proteins to at least some extent, but large differences in N complexation occurred between species. One plant species, *V. myrtillus*, exhibited an order of magnitude greater protein complexation capacity than did all other species, with intermediate complexation demonstrated by *E. hermaphroditum* and *B. pendula,* and lower complexation capacities demonstrated by *V. vitis-idaea, P. sylvestris,* and *P. abies* (Figure 1). A main goal of the study was to evaluate whether differences in protein complexation capacity among the species corresponded with their pattern of dominance along a well described successional gradient (DeLuca et al. 2002). Boreal forests are N-limited, and N mineralization and availability both greatly diminish over time (DeLuca et al. 2002). Young stands are thus relatively less N limited, and are dominated by species that grow relatively fast, and produce litters that decompose relatively quickly (e.g., *B. pendula, P. sylvestris,* and *V. myrtillus*). As stand age increases, species with slow growth rates and poor quality litter dominate (e.g., *P. abies* and *E. hermaphroditum*). An additional species, *V. vitis-idaea,* demonstrates an intermediate growth strategy, and its relative abundance changes relatively little during succession. Because polyphenol concentration in the litter is thought to be strongly negatively correlated with release rates of both mineral and organic forms of nitrogen from the litter, complexation has been proposed as an adaptive mechanism for low N availability (Northrup et al. 1995). I predicted that species dominating in late successional stands would exhibit a higher protein complexation capacity than would species in young stands, but my data did not provide any support for this hypothesis. *Vaccinium myrtillus* and *B. pendula,* both of which are dominant in young productive stands, demonstrated the first and third highest protein complexation capacities respectively among all the species. This pattern is inconsistent with several other studies that have identified late-successional species to have higher protein complexation capacities than earlier successional ones (Northrup et al. 1995, Northup et al. 1995).

The data did not support my first hypothesis, which predicted that late successional dominant species would complex more protein per unit leaf mass than would early successional dominant species (Figure 2a). The high complexation capacities demonstrated by *V. myrtillus* and *B. pendula* appears to be driven by significantly higher concentrations of phenolic compounds in these species (Figure 1). Despite these high concentrations, the data indicates that the phenols of the evergreen species are more efficient at complexing proteins than are the phenols of *V. myrtillus* and *B. pendula*. One possible explanation is that late successional species, which have lower carbon assimilation rates, allocate a relatively high portion of their carbon budget to the production of protein-complexing phenols. In contrast, early successional species that are less N efficient allocate a much smaller portion of their carbon budget to the production of these compounds. One factor that likely contributes to the higher concentrations of most foliar chemical properties of the two deciduous species is their large surface area and lower structural integrity relative to the

evergreen species. This difference could make a comparison between litter extracts somewhat misleading, as evergreens may release their polyphenolic compounds during the more advanced stages of decomposition than would the deciduous species (Kuiters and Sarink 1986, Gallet and Lebreton 1995, Wardle et al. 2003).

An additional aim of my study was to determine whether the protein complexation capacity of each species increased in response to nutrient limitation during succession. The data demonstrated that none of the chemical variables that I measured for any species demonstrated a significant correlation with stand age (data not shown), failing to support my second hypothesis. This finding stands in contrast to several other studies that have found numerous leaf chemical properties and protein complexation capacity to increase in response to nutrient limitation. For instance, Northup et al. 1995 showed that declining soil fertility increased polyphenol production in *Pinus muricata*, which resulted in higher release rates of organic N and lower rates of release of mineral N. Several other studies also found leaf secondary metabolites to increase and nutrient content to decline with increasing time since disturbance (Vitousek et al. 1995, Wardle et al. 1997, Crutsinger et al. 2008). A potential reason as to why I may not have detected a change in leaf chemical properties in response to nutrient decline is because I performed the extractions on whole leaves. It has been shown for many species that leaf area relative to leaf mass (Leaf Specific Area, LSA) declines in response to nutrient stress (Garnier et al. 2004, Cortez et al. 2007). This lower surface area per leaf mass could result in the reduced release of soluble chemicals from the leaf surface, and therefore possibly offset any increased concentration of soluble compounds within them that have resulted in response to nutrient stress. An alternative approach could be to grind leaves prior to leaf extraction, which would make it possible to simultaneously hold the extacted surface area and leaf mass constant. This approach has been used in several other studies, and may in part explain the strong correlation between site fertility and leaf chemical properties detected in those studies (Northrup et al. 1995, Northup et al. 1995, Vitousek et al. 1995, Crutsinger et al. 2008). However, it should be noted that whole leaves as opposed to ground leaves better represent reality in nature since litter falls to the ground in a fairly intact form and are only fragmented slowly over time (Kuiters and Sarink 1986).

In order to investigate whether these differences in protein complexation capacity led to differences in N mineralization rates from an added protein N source, I conducted a soil incubation experiment in which litter extracts and protein were added individually and in combination. For any litter extract treatment, the presence of a negative interaction with protein on both the final NH_4^+ concentration and soil respiration would collectively indicate that protein complexation was the mechanism responsible for reduced N mineralization. The data revealed that three species (*B. pendula, P. abies,* and *V. vitis-idaea*) exhibited negative litter extract by protein interactions on final NH_4^+ concentration, two species demonstrated no significant interaction (*V. myrtillus* and *P. sylvestris*), and one species demonstrated a significant positive interaction (*E. hermaphroditum*) (Table 1, Figure 3). Of the three species that exhibited negative interactions with protein on final NH_4^+ concentration, all demonstrated positive interactions with protein on soil respiration. This pattern strongly suggests that protein complexation did not reduce N mineralization in the manner predicted by my third hypothesis. Alternatively, these data suggest that carbon substrates in these litter extracts stimulated microbial immobilization of N. These results are consistent with numerous other studies that have reported temporary net immobilization of N following the addition of fresh litter with a high C/N ratio (Keeney 1980, Kuiters 1991, Scott and Binkley 1997).

Conclusions

My data are inconsistent with several studies that have investigated the role of protein complexation in regulating decomposition and N mineralization, and the suggested importance of these processes for plant competition in N limited environments. It has been proposed that plant species compete for N by synthesizing polyphenols that complex proteins (Hättenschwiler and Vitousek 2000), which are thought to then reduce access by the plant's competitors to inorganic forms of N. It has also been suggested that evergreen species, which dominate in nutrient poor sites, possess a greater ability to complex N than do deciduous species, providing them with a competitive advantage when N is limiting (Monk 1966, 1971, Thomas and Grigal 1976). Likewise, it has been demonstrated that some species increase their protein complexation in response to nutrient stress, through either phenotypic plasticity or genotypic variation (Valladares et al. 2007). In contrast to those studies, my data did not provide any evidence that between species differences or within species variation in complexation increases in response to nutrient limitation. Instead, my data showed that two early successional species had high complexation capacities, and that complexation did not vary with stand age for any species. Additionally the data provided little evidence that protein complexation was a viable mechanism for reducing N mineralization rates, but instead indicated that microbial immobilization was a potential mechanism by which litter extract could reduce N mineralization rates. As such, these data suggest that protein complexation may be a less important competitive strategy in N limited boreal environments than has often been suggested.

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