Epiphytic lichen responses to nitrogen deposition

Otilia Johansson

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Abstract
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Keywords
Lichens, air pollution, nitrogen deposition, phosphorus, growth, chlorophyll a, boreal forest, field experiment, irrigation, carbon based secondary compounds

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List of Publications

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III. Johansson, O., Palmqvist, K. and Olofsson, J. (2011), Nitrogen drives lichen community changes through the different species responses, *Manuscript*

IV. Nybakken, L., Johansson, O. & Palmqvist, K. (2009). Defensive compound concentration in boreal lichens in response to simulated nitrogen deposition. *Global Change Biology*, 15(9), 2247-2260.

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1. Introduction

1.1 Nitrogen

Nitrogen is an important nutrient in all living organisms as a building block of proteins and DNA molecules. Our atmosphere consists of up to 78% nitrogen. In physical terms, this means that there is no "nitrogen scarcity". However, the nitrogen in gaseous (N_2) form is unavailable for most life forms, thus many ecosystems are adapted to conditions of low N availability. Nitrogen gas must be fixed into other forms to become accessible. There are two general ways for our ecosystems to receive accessible nitrogen. The first is via thermal fixation, which happens when the air is heated to extreme temperatures, as occurs with lightning or combustion. The second way is via biotic fixation conducted by microorganisms (e.g. cyanobacteria). Beginning in the 1850´s, humanity has increased the anthropogenic N input dramatically by combustion of fossil fuels and agricultural activities (Galloway 2003, 2008). The fuels themselves do not contain large amounts of nitrogen, thus, in thermal fixation the nitrogen gas is forced to react with the oxygen in the atmosphere to produce nitrogen oxides (NOx). Due to these reactions with oxygen, the amounts of biologically accessible nitrogen have increased in the atmosphere. The thermally fixed nitrogen is dissolved into the atmosphere and carried by the winds, until it precipitates and forms NOx depositions. The major sources for atmospheric ammonia (NHy) deposition are agricultural activities and animal feedlot operations, such as the decomposition of urea excreted from domestic animals and fertilization of soils that can cause volatilization of NH_3 (Schlesinger 1997). In Sweden, the total N deposition ranges from 1 kg N ha^{-1} in the northern part, up to 25 kg N ha^{-1} in the southwest part. Nitrous oxides (NOx) and ammonia (NHy) contribute equally (Kindbom et al. 2001). In continental Europe, such as in the Netherlands, doses of up to 50 kg/ha or even higher is common, dominated by NHy. This will naturally affect the ecosystem, and lichens are particularly vulnerable.

1.2 Lichens

Lichens are a diverse group of organisms consisting of nutritionally specialized fungi (often ascomycetes) living in symbiosis with unicellular algae or cyanobacteria and sometimes both (Honegger 1993) (Fig 1.). Despite the symbiotic nature of lichens, they are often referred to as species (affiliation determined by the fungal partner) and will be denoted "species" throughout this thesis. Some lichen species are pioneers growing on rocks while others prefer late succession stages in the forest ecosystem. Lichens contribute significantly to diversity;

there are around 17,000 species of lichens and they occupy almost all terrestrial habitats (Nash 1996). They play important roles in the ecosystem: in primary colonization, increasing structural complexity, modifying water regimes, influencing nutrient cycling, and providing habitat, food and nest material for many animals (Rhoades 1995). Even though lichens can vary in growth forms, the general patterns are the same; the major part of the body is built up by fungal hyphae (mycobiont) and a smaller part by algal cells (photobiont). Around 1,500 species of lichens are associated with cyanobacteria (mostly Nostoc) that are able to fix nitrogen (N_2) from the atmosphere (Rai 2002; Rikkinen 2002). The photobiont provides carbon that originates from photosynthesis. In return, the mycobiont provides water and nutrients. The mycobiont also secures adequate illumination and gas exchange of the photobiont cell population by keeping them or allowing them to grow in an optimal position within the thallus (Honegger 1991; Honegger 1998).

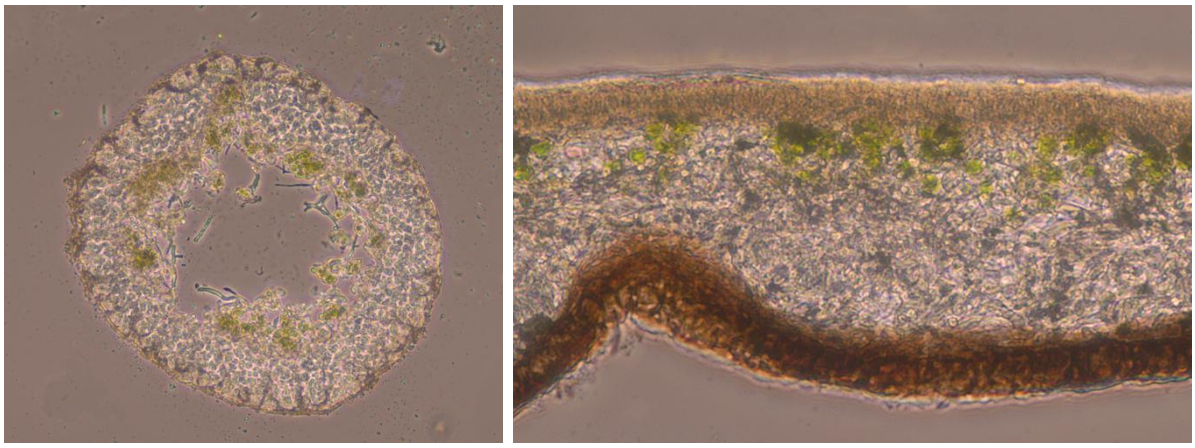

Fig 1. Tissue thin-sections of lichen thalli, a longitudinal section of P. glauca (left) and a cross-section of A. sarmentosa (right). (Photos by Otilia Johansson)

1.2.2 Growth

Lichens have the ability to survive extreme conditions, such as cold, heat or drought, so they can be found in polar regions as well as in arid deserts (Kappen et al. 1979; 1995). Their tolerance is attributable to being metabolically active only when wet (Fig 2). During the dry periods they can tolerate extreme temperature stress (Green 2007). They are poikilohydric, which means that their water content follows the fluctuations in atmospheric humidity. When the lichen is wet and metabolically active, their photosynthesis is primarily limited by light (Palmqvist and Sundberg 2000). Their respiration is controlled by water and temperature (Palmqvist 2000). Lichen growth is also dependent on the availability of the same nutrients as in plants (nitrogen, phosphorus, calcium, potassium, magnesium, etc.). However, lichens have

no roots, and are therefore dependent on the sporadic deposition of nutrients directly on the thallus, where potential sources for epiphytic lichens are autochthonous (e.g. bark decomposition, leachate of live foliage, intercepted litterfall) or allochthonous (atmospheric wet and dry deposition) (Nadkarni and Matelson 1991). Lichens effectively absorb nutrients once they are hydrated so their nutrient concentration often closely reflects the accumulated deposition (Hyvärinen and Crittenden 1998) indicating either a constant nutrient limitation or an inability to avoid excess assimilation.

Fig 2. Dry thallus (to the left) and wet and active thallus (to the right) of Lobaria pulmonaria (Photo by Kristin Palmqvist)

1.3 Lichen response to nitrogen deposition

During the 19th century, people have documented that lichens were disappearing from urban areas. This phenomenon was first attributed to coal soot. In research from the 1950's to the 1970's, a negative correlation between species diversity and sulfur dioxide (SO_2) concentrations was observed (Barkman 1958; Hawksworth and Rose 1970). During the 1980's, the effects of nitrogen deposition on lichen communities received more attention. For example, De Bakker (1989) has correlated the reduction of epiphytic lichens with the density of cattle farms in the Netherlands. Van Herk (1999) correlated the occurrence of nitrophytic (eutrophication tolerant) lichen species with maps of ammonia pollution in the Netherlands, and Van Dobben and Ter Braak (1998) related changes in the lichen flora of the Netherlands with declining sulfur dioxide (SO_2) and increasing nitrogen oxide (NOx) and ammonia-based (NHy) salts. In areas with increasing nitrogen levels, acidophytic (sensitive to eutrophication)

species have been replaced by nitrophytic ones (Van Dobben and DeBakker 1996). In addition to atmospheric N deposition, direct forest N fertilization represents a potential threat to lichens, as highlighted by the disappearance of the pendulous lichen *Alectoria sarmentosa* following 7-8 years of fertilization in a Norway spruce forest (Hesselman 1937).

1.3.1 Direct effects

N- preference

Lichens are able to take up ammonium (NH_4^+), nitrate (NO_3^-) and amino acids, although NH_4^+ generally appears to be the preferred form (Dahlman et al. 2004, Palmqvist and Dahlman 2006). Since NH_4^+ may be more physiologically harmful than NO_3^- (Britto and Kronzucker 2002), differences between species with respect to their N-preference may contribute to the differences in their N-sensitivity.

Coordinated growth

One important aspect of N compared to other pollutants is that N is an essential and limiting nutrient, thus growth is usually initially increased, even for N-sensitive species (Crittenden et al. 1994, Welch et al. 2006). The difference in response between species might also depend on the ability to coordinate growth between the photobiont and mycobiont within the lichen thallus. The N-sensitive species *Evernia prunastri* displayed decreased vitality of the mycobiont at increased N levels, caused by a reduced carbon (C) flow from the photobiont (Gaio-Oliveira *et al*. 2005). In contrast, Palmqvist & Dahlman (2006) have shown that the N-tolerant *Platismatia glauca* was able to direct a 5-fold increase in N to a 4- to 5-fold increase in chlorophyll concentration. This boosted the C-fixation capacity, allowing for a sufficiently high C flow to balance the mycobiont's increased maintenance costs of its photobiont. This may be adaptive since this can lead to increased photosynthesis and increased availability of carbon chains for growth or for binding of surplus N, thereby avoiding toxic ammonium levels. However, there might be some restrictions to growth, where other nutrients (Hogan 2010) water, or light (Jonsson Cabrajic et. al. 2010) become limiting.

Toxic effects

Increased N uptake without the possibility to use it for growth could lead to toxic effects such as membrane damage (Munzi 2009) and chlorophyll degradation (Gaio-Oliveira et al. 2004, Ridell et al.2008).

Free-living algae

Nitrogen addition may also stimulate the growth of free-living algae or liberated photobiont cells on the thallus surface (Scott 1960; Kauppi 1980; Brown and Tomlinson 1993), which could also cause breakdown of the symbiosis (Scott 1960).

1.3.2 Indirect effects

Competition

Competitive interactions are generally considered to be one of the major mechanisms explaining changes in plant community structure following increased N deposition (Grime 1979, Tilman 1988). Most often, species richness have been found to decrease in fertilized plots (Suding et al. 2005), either because the overall intensity of competition increases (Grime 1979), or as a result of a switch to competition for light when soil resources are abundant but shading is intense (Tilman 1988). In either case, large and fast-growing species outcompete small and slow-growing species following fertilization (Grime 1979, Tilman 1988). Competitive interactions seem to be important for lichen community responses to N deposition as well. One study that directly addressed this question found that nutrient application increased competition for energy and space, due to increased growth rates of some species (Welch et al. 2006).

Food-web interactions

Altered food-web interactions might also be of importance for lichen responses to increased N availability. This is a well known phenomenon reported from boreal ecosystems. For example, the effect of N addition on boreal forest floor vegetation is mediated by two types of natural enemies on the dominant shrub species, *Vaccinium myrtillus*, i.e. the pathogenic fungi *Valdensia heterodoxa* and herbivorous larvae of the genus *Opemophtera* (Strengbom et al. 2002, Nordin et al. 2009). The vegetation shifts in boreal mires following N addition is also mediated by increased disease induced by parasitic fungi (Wiederman et al 2007). Lichens, however, have historically been regarded as resistant to parasites due to their good chemical defense (Lawrey 1995). Consequently, the importance of parasites in lichen communities has been largely overlooked, and may be far more important than previously expected. Increased N availability may also influence lichen chemical defense capacity. By using fixed carbon delivered by the photobiont, the mycobiont often produces high concentrations of carbon

based secondary compounds (CBSCs) with multiple functions. These compounds may protect the thallus from herbivory (e.g. Slansky 1979; Lawrey 1983; Gauslaa 2005; Nimis & Skert 2006), detrimental radiation (e.g. Solhaug *et al*. 2003; Nybakken *et al*. 2004), competition from plants (Pyatt, 1967; Brown & Mikola 1974; Fisher 1979), and parasites (Lawrey 2000). Increased N deposition may affect cycling of C and N in the lichen symbiosis, and thus change the concentration of CBSCs.

1.4 Objectives of the thesis

The overall objective of this thesis was to understand how increased N deposition affects epiphytic lichens co-existing in a nutrient-poor boreal forest. The aim was to investigate:

1. Whether or not the lichens displayed different N assimilation patterns and subsequent increases in N concentration following a simulated N deposition, and if there were different affinities for the two N-sources, NH_4^+ and NO_3^-, which could support different critical loads for these two N forms.

2. If the response of lichens to increased N differed depending on P availability and whether nutrient limitation differed between the photo- and the mycobiont in the same lichen thallus and/or across species.

3. How N deposition affects the lichen community structure.

4. The effect of increased N deposition on the concentrations of lichen carbon based secondary compounds (CBSCs)

2. Material and Methods

2.1 Study site and experimental setup

All field studies were performed at Kulbäcksliden (64°12'N, 19°33'E), in a relatively open (basal area 24 m2 ha-1) old-growth forest stand described in detail by Tirén (1937), dominated by Norway spruce, *Picea abies* (10-15 m high and 15-30 cm diameter at breast height). The site is part of The Unit for Field-based Forest Research, Swedish University of Agricultural Sciences (Vindeln, Sweden) and is equipped with electricity (220 V) and a 700 m water pipe to the nearest road where water could be pumped. Background wet deposition of

nitrogen is c. 2 kg ha^{-1} $year^{-1}$ (Forsum 2006) and of phosphorus c. 0.03 kg ha^{-1} $year^{-1}$ (Laudon, H Pers. Comm.).

Transplants

Two of the studies were short-term studies (one growing season) with lichens transplanted on net or plastic sticks (paper II and IV). The different lichen species were transplanted in a position that mimicked their natural habitat positions. The transplant treatment units were placed in a gap in the forest so the lichens were not directly influenced by trees (i.e. leachate of live foliage) enabling greater control of influencing factors (Fig 3.).

Fig 3. The transplantation experiment year 2006. (Photo by Otilia Johansson)

In study II, irrigation started in the morning when lichens are naturally active to minimize the effect of the irrigation itself. In study IV, active time was manipulated by irrigating at different lengths and frequencies, and at different times of the day (night, morning, mid-day) thereby varying the magnitude of net C gain. In combination with a varying N concentration during irrigation, the lichens were exposed to widely varying combinations of C and N. One advantage of using transplants was the possibility to follow their growth, since their weights were measured before the treatment started. Another advantage of transplants was the ability to divide thalli, as for study IV where all thalli were divided into two parts: one piece for chemical measurements at start (before transplantation) and one for transplantation. In this way, all thalli had their own control.

Native lichens

The other two studies (Paper I and III) are from an experiment designed for long-term responses, aiming to examine epiphytic lichen responses to N-deposition under natural conditions over a longer time period. To study the effects of N-deposition on lichens in their native environment, 15 old-growth *Picea abies* trees that were densely populated with both sensitive and more resistant lichens were selected as treatment units at the field site. Wooden towers were built around each tree and a sprinkler system surrounding each tree was placed on top of the tower (at 6 m height) (Fig 4.). Each tower was equipped with two platforms at 4 m height above ground level to allow for sampling of native lichens (Paper I) and a non destructive inventory of the lichen community (Paper III).

Fig 3. One of the irrigation towers for the long-term experiment. (Photo taken in autumn 2005 by Kristin Palmqvist).

Irrigation

To simulate "natural" N deposition, an irrigation system was constructed. In all studies, a circular irrigation tube equipped with smaller sprinklers, connected to its respective treatment solution tank, was used. The sprinklers were positioned to create an evenly dispersed spray of the solution over the lichens. Nitrogen was added in the form of ammonium nitrate (NH_4NO_3) and dissolved in water. The treatment mimicked a mixture of dry and wet deposition via condensed fog, so the lichens were less wet following treatment than after rain. For practical reasons, the irrigation could not commence for the season before the road conditions had improved (by the first weeks of June) to allow heavy transports of water. Tanks, tubing and

pumps were emptied before winter conditions set in at the end of the season. Our 4-month treatment period each year overlapped the active growth period for the forest vegetation. The growing season in this part of Sweden starts by the end of May to early June and continues until late September to early October. All other months can have freezing temperatures. Sub-zero temperatures are dominant from November to April. The N applications were controlled by an automated system consisting of a PLC205 D2-240 AutomationDirect DirectLOGIC Modular Programmable Logic Controller, with Directsoft software programming version 2.0c from Koyo Electronics Industries Company, LTD, Tokyo, Japan.

2.2 Study species

This thesis focused on epiphytic species of lichens that co-exist in a nutrient-poor boreal forest and differ in their nutrient acquisition mechanisms and/or tolerances.

The two species *Alectoria sarmentosa* and *Platismatia glauca* were used in all four studies (Paper I-IV). *Alectoria sarmentosa* (Ach.) Ach., is a pendulous, fruticose green algal lichen associated with open, old-growth spruce forests (Esseen et al. 1996). It is intolerant of heavy shade, and presumably has low N requirements as its N uptake is rather low (Dahlman et al. 2004) and has decreased after N fertilization (Hesselman 1937). The foliose green algal *P. glauca* (L.) W.L. Culb. & C.F. Culb., has a wide ecological amplitude, and is common on birch (*Betula pubescens*) and Norway spruce in oligotrophic boreal forests. We know from earlier studies that it can assimilate high amounts of N without negative effects (Dahlman et al. 2004; Palmqvist & Dahlman 2006). Thus, in all four studies *A. sarmentosa* was used as a model species for a N-sensitive lichens and *P. glauca* as a relatively more N-tolerant lichen. Both species have *Trebouxia sp.* as the photobiont.

In paper II we also used *L. pulmonaria* which is a broad-lobed and foliose tri-partite lichen (Fig. 2), with the green algae *Dictyochloropsis reticulata* as the primary photobiont and the N_2-fixing cyanobacteria *Nostoc sp.* in internal cephalodia.

All epiphytic macrolichens present on the branches of treated trees were inventoried in paper III: *Alectoria sarmentosa* (Ach.) Ach, *Bryoria capillaris* (Ach.) Brodo & D.Hawksw, *Bryoria fremontii* (Tuck.) Brodo & D. Hawksw., *Bryoria fuscescens* (Gyeln.) Brodo & D. Hawksw., *Bryoria simplicor* (Vain. Brodo & D. Hawksw), *Tuckermanopsis chlorophylla* (Willd. in Humb.) Vain., *Hypogymnia physodes* (L.) Nyl., *Hypogymnia tubulosa* (Schaer.) Hav., *Mycoblastus sanginarius* (L.) Norman, *Parmelia sulcata* Taylor, *Parmeliopsis ambigua*

(Wulfen) Nyl., *Parmeliopsis hyperopta* (Ach.) Arnold, *Platismatia glauca* (L.) W.L. Culb & C.F. Culb, *Usnea fillipendula* Stirt., *Usnea subfloridana* Stirt., and *Vulpicida pinastri* (Scop.) (J.-E.Mattsson & M.J.Lai,).

In the last study (paper IV) we also included one N-fixing species *Lobaria scrobiculata* (Scop.) and one N-tolerant species *Xanthoria aureola* (besides the two model species: *A. sarmentosa* and *P. glauca). Lobaria scrobiculata* is a foliose, epiphytic, cyanobacterial lichen. It depends on old forest habitats in areas with low N-depositions (Gauslaa 1995; Kuusinen 1996). *Xanthoria aureola* (Ach.) Erichsen is a foliose, green algal lichen that grows in full sun exposure on seaside rocks. Lichens from the genus Xanthoria are nitrophytic. *Xanthoria aureola* was recently separated from *X. parietina* (Lindblom et al. 2005), therefore earlier studies may have mixed the two species. As the two species are assumed to respond similarly in relation to N, the results reported from *X. parietina* and *X. aureola* will be discussed as though from the same species. *X. parietina* is tolerant to N pollution (Gaio-Oliveira et al. 2004). It can deal with both high and low N concentrations in nature (Gaio-Oliveira et al. 2001; Van Herk 2001) and has the capacity to meet the C demands associated with high N assimilation (Gaio-Oliveira et al. 2004).

2.3 Growth measurements

For dry weight measurements (Paper II and IV), the samples were dried in darkness in a climate-controlled room until no further loss in weight was detected, then measured to the nearest 0.1 mg. The same procedure was done both before transplantation and after harvest.

2.4 Chemical analysis and lab measurements

The total N concentrations and the isotope ratio of ^{15}N to ^{14}N (Paper I, II, IV) were analyzed with an elemental analyzer-isotopic ratio mass spectrometer (EA-IRMS) by Colorado Plateau Stable Isotope Laboratory (CPSIL, Northern Arizona University, USA) for Paper I and by laboratories at the Faculty of Forest Sciences (Swedish University of Agricultural Sciences, Umeå, Sweden) for Papers II and IV. The phosphorous (P) concentrations were analyzed by CPSIL using a Lachat Instruments QuikChem 8000 Series FIA+ equipped with a XYZ autosampler for Paper I, and by "Department of Geology and Geochemistry, Stockholm University" using ICP-OES (inductively coupled plasma-optical emission spectroscopy) for Paper II.

Chlorophyll *a* (Chl *a*) was used as an indirect marker for the amount of photobiont in the lichens, as in Palmqvist et al. (2002; 2008) (Paper I, II, IV). In paper II, biont proportions were obtained by measuring the relative proportion occupied by each biont in thin cross sections (Paper II, Fig 1.). In that paper, the potential photosynthetic activity of thalli (Fig. 2) was measured using an IMAGING-PAM M-series Chlorophyll Fluorometer with a MINI-Head IMAG-MIN/B measuring head (Heinz Walz GmbH, Effeltrich, Germany).

In Paper IV, carbon-based secondary compounds (CBSCs) were analyzed in the lab at the Norwegian University of Life Sciences on a 1100 Series HPLC (Agilent Technologies, Waldbronn, Germany) as in Nybakken & Julkunen-Tiitto (2006).

2.5 Species abundance inventory

In paper III, lichen development on each branch was recorded each autumn with a digital camera, beginning one year before the treatments started. The images were analyzed using ArcGIS 9.2 ESRI (Environmental Systems Resource Institute, Redlands, California, USA) and the scale was set using the coordinates on a background scale grid in each picture. A grid interception method was used to estimate species abundance.

2.6 Statistical analysis

In paper I, Analysis of variance (ANOVA) was used to analyze differences in N and P concentrations and N to P ratios in response to the treatments and an Analysis of covariance ANCOVA was used to analyze differences in N-uptake between the two N-forms. In paper II, a Students t-test was used to analyze differences between untreated control and Control for the estimated parameters. Differences between the four different fertilizer treatments were then analyzed with a full-factorial ANCOVA (Two-way ANOVA). The relationship between the proportion of algal cells and concentrations of Chl *a* was examined by linear regression. In paper III, differences at start were analyzed using ANOVA. Effects of time, height and branch quality on species abundance were analyzed separately for each species using a hierarchical repeated measure ANOVA. All statistical analyses for paper I, II and III were performed using the statistical package R (R Development Core Team 2009). In paper IV, stepwise multiple linear regressions were used to analyze which factors influenced the concentrations of CBSCs, using the statistical package Statistix 7 (Analytical Software, Tallahassee, FL, USA).

3. Major results and discussion

Paper I

The specific aim of Paper I was to follow the initial responses over three years, of the less N-sensitive foliose lichen *P. glauca*, and the more N-sensitive pendulous lichen, *A. sarmentosa*, when exposed to an N-deposition gradient. We examined whether the lichens displayed different N assimilation patterns and subsequent increases in N concentration following the simulated N deposition. We also wanted to determine if changes could be related to different affinities for the two N-sources, NH_4^+ and NO_3^-, that could support different critical loads for these two N forms. The thallus N concentrations increased significantly in both lichen species with increasing N-deposition level, and the differences in thallus N concentrations between the treatment levels increased over time. The increase in thallus N concentrations was attributed to the fertilizer application, and there were strong linear relationships between the N concentrations and uptake of both NH_4^+ and NO_3^- that were independent of year. The increase in thallus N concentration also resulted in an increase of the chlorophyll *a* concentration, which is indicative of an elevated photobiont concentration in the thallus, and is consistent with results of previous N-fertilization experiments with lichens. When related to the accumulated N load over the three years, there was a curvilinear relationship between N exposure and chlorophyll *a* concentrations in both species. In *P. glauca*, the chlorophyll *a* concentration increased to 2 mg g^{-1} DW at the highest accumulated N load, i.e. 100 - 150 kg N ha^{-1}. In *A. sarmentosa*, the thallus was saturated with photobiont cells at a chlorophyll *a* concentration of 1.5 mg g^{-1} DW, with no further increase beyond an accumulated N load of 50 kg N ha^{-1}. This saturation for photobiont density in the thallus may be caused by continued thallus expansion or arrestment of photobiont division. The photobiont concentration can only increase linearly with increasing N until limited by morphological constraints, water, light or other nutrients. A likely candidate for a nutrient to become limiting is phosphorous (P), which decreased in *A. sarmentosa* during the third year of treatment.

Significant responses in lichen chemistry were detected at inputs of 12.5 kg N ha^{-1} $year^{-1}$ or higher, suggesting that resources other than N limit lichens at higher N levels. However, the data also suggests that N saturation may be cumulative over time, even at low N levels.

Paper II

Responses to simulated N deposition with or without added P were investigated for three contrasting lichen species: the N-sensitive *A. sarmentosa*, the more N-tolerant *P. glauca* and the N_2-fixing *Lobaria pulmonaria*. For *A. sarmentosa*, total thallus growth was increased by P, but not by N fertilization. *Platismatia glauca* did not display any positive growth response to P, and it exhibited a negative response to N with a 10% weight loss in the N and a 40% loss in the NP treatments. No significant growth effects on thallus by treatment were found for *L. pulmonaria*. Nitrogen stimulated algal growth in all three species. The mycobiont was P-limited in one species, *A. sarmentosa*, but the growth response of the mycobionts was complex, since fungal growth is also dependent on a reliable carbon export from the photobiont, which may explain the decrease of the mycobiont with N addition in *P. glauca*. Our results showed that both N and P can be limiting nutrients for epiphytic lichen growth in boreal forests and that the balance between N and P supply is important for the symbiotic interaction. Our findings also showed that P availability is an important factor when studying the effects of N deposition. Effects of P are both positive and negative: with P availability, lichens have a higher ability to use the additional N for growth since they are not limited by P (positive effect), while the symbiotic lifestyle may turn negative. We conclude that, in general, algal cells are N-limited, while the fungal growth is more complex, since it can either increase or decrease in response to nutrient additions. There were also species-specific differences: *A. sarmentosa* and *L. pulmonaria* exhibited both algal and fungal growth increases with N addition, while *P. glauca* exhibited only increased algal growth, suggesting a disruption in the symbiosis. Surprisingly, it was the green algal lichen *A. sarmentosa* that was P-limited and not the N_2-fixing species *L. pulmonaria*. This shows that the response to nutrient enrichment is not only species-specific, but also dependent on the thallus concentrations and relative proportions of N, P and other nutrients, as well as other environmental factors, such as light availability and water supply. Hence, under conditions of nutrient enrichment, species-specific responses could be reflected in changes in species composition of lichen communities in boreal forests.

Paper III

The aim of paper III was to study how N deposition would affect the epiphytic lichen community composition in a naturally N-poor boreal forest. This was done by daily fertilization (during the growing season) of spruce trees with a rich lichen flora at five N levels during four consecutive growing seasons. The simulated N deposition caused significant changes for *A. sarmentosa*, *Bryoria spp.* and *H. physodes*. *Alectoria sarmentosa* increased over time in the 0.6 kg N ha^{-1} and 6 kg N ha^{-1} treatments, while it was more or less stable in the 12.5 kg N ha^{-1} treatment and decreased substantially in the 25 kg N ha^{-1} and 50 kg N ha^{-1} treatments. *Bryoria spp* showed a positive response to 6 kg N ha^{-1}, but a negative response to 12.5 kg N ha^{-1} and higher loads. *Hypogymnia spp.* decreased over time for all treatments except in 12.5 kg N ha^{-1} where it only decreased during the first year and then increased after 2007. The cover of *Platismatia glauca* increased over time, independent of treatment. Our results show that four years of simulated N deposition caused an alteration of the epiphytic lichen community. Responses to the treatments differed among species, reflecting their different N optima. Many of the responses we recorded in this experiment are in agreement with what have previously been recorded in forest fertilization studies or descriptive studies along nitrogen deposition gradients (Söchting 1995, Van Herk 1999, Van Dobben and Ter Braak 1998, Wolseley et al. 2006, Davies et al. 2007, Rogers et a.l 2009, Jovan 2008, McCune and Geiser 2009). A substantial decrease in frequency of *A. sarmentosa* was found in the two highest treatments (25 and 50 kg ha^{-1} $year^{-1}$). The decrease in *A. sarmentosa* is related to an increased N concentration in their thalli. The decline of *A. sarmentosa* coincides with thallus N concentration values above 6 mg N g^{-1} DW (Paper 1) i.e. *A. sarmentosa* declined between 2006 and 2007 in the two highest N deposition treatments where the lichen thalli had N concentration of 7 mg g^{-1} in 2006, while no decrease could be detected in treatments with thalli concentrations below 6 mg g^{-1}. The reason for the decline is still not clear. A direct toxic effect of N is not plausible, since paper II showed that N concentrations around 10 mg g^{-1} are not directly toxic, as positive growth was seen in *A. sarmentosa* with concentrations as high as 17 mg g^{-1}. Therefore, we suggest that the two most reasonable explanations for the observed decline of *A. sarmentosa* are a reduced stability of the lichen thalli or an increased susceptibility to diseases.

Since differences in N optima among epiphytic lichens recorded in this long-term field experiment largely coincide with the results from descriptive studies along pollution gradients, we conclude that N is an important pollutant for many lichen species.

Paper IV

The aim of paper IV was to study the effect of increased N deposition on the concentrations of carbon based secondary compounds (CBSCs) in lichens. None of the studied species reduced their concentrations of secondary compounds during the experimental period, but in *P. glauca* the concentrations of all compounds were significantly lower in nitrogen-treated thalli compared with those that received rainwater only. Our results indicate conservative and robust chemical defense systems in *A. sarmentosa*, *L. scrobiculata* and *X. aureola* that were not influenced by environmental factors. These species may have a high degree of genetically determined defense. They have rather specific habitat requirements, presumably adapted to predictable environmental stress levels (sun radiation, herbivore pressure etc). However, we don't know if these lichens are able to sustain a high level of protection over several years after new tissue is formed.

4. Summary discussion

The results in paper I showed that at ecologically relevant N concentrations and doses, there was no significant difference in uptake of the two N forms by either lichen species, regardless of thallus N concentration and accumulated N load. The filamentous *A. sarmentosa* lichen reached chlorophyll *a* saturation more rapidly than the foliose *P. glauca*. This indicates that something is limiting a higher photobiont concentration in *A. sarmentosa* and thus provides a mechanistic reason for the sensitivity of this species detected in earlier N fertilization studies. One possibility is that other nutrients (e.g. phosphorus, magnesium or iron) may have become limiting for either continued photobiont investments, or thallus growth, or both in *A. sarmentosa*. A likely candidate for a limiting nutrient is phosphorous (P), which decreased in *A. sarmentosa* during the third year of treatment (Paper I). The results in Paper II indicate that *A. sarmentosa* is P-limited and not N-limited as previously expected.

Paper II highlights P as an important factor when studying the effects of N deposition, since P can both mitigate and intensify the negative effects of N on epiphytic lichens. The lichenized algal cells were generally N-limited, since N stimulated algal growth in all three species. However, the growth response of the mycobionts is complex and inconclusive, since fungal growth is also dependent on a reliable carbon export from the photobiont. The decrease of the

mycobiont in *P. glauca* is an example of nutrient addition disturbing the symbiotic balance by favouring one of the partners over the other. This is consistent with theoretical modelling showing that symbiotic interactions should occur in particularly in harsh conditions (Travis 2005, 2006). When the conditions are advantageous, *i.e.,* with a good supply of water and nutrients, the symbiotic lifestyle may turn negative (Paper II). Secondary compounds produced by fungi, such as atranorin, may also be involved in symbiotic regulation by suppressing photobiont growth in older parts of the lichen thallus (Stephenson & Rundel 1979; Honegger 1993; Fahselt 1994). Atranorin concentrations decreased in *P. glauca* after N fertilization and with increasing chlorophyll concentrations (Paper IV). This could explain why the photobiont flourished at the expense of the mycobiont in paper II. However, the loads in paper II were unrealistically high, and when N was simulated in more realistic loads in their native environment, no negative effect was found on the abundance of *P. glauca* (Paper I, III). This highlights the importance of combining short-term studies and long-term studies with more realistic simulations.

Alectoria sarmentosa did not show any decreased vitality in the short-term treatment (Paper II) and no effect of N addition on CBSCs was found (Paper IV). In contrast, in the long-term study, a substantial decrease in frequency of *A. sarmentosa* was found in the two highest treatments of 25 and 50 kg ha^{-1} $year^{-1}$ (Paper III). The reason for the decline is still not clear, thus, we suggest that the two most reasonable explanations for the observed decline of *A. sarmentosa* are an increased risk of fragmentation and an increased vulnerability to diseases. The increased N concentration likely reduces the stability of the lichen since it increases the photobiont:mycobiont ratio (Paper II), where it is the mycobiont that is responsible for the structure and stability of the thalli. Besides a reduced mycobiont ratio, it could also be a change in the quality of the fungal cellwall that reduces the stability of the thalli. The cellwall of fungi consists of both cellulose and chitin, where the latter is more brittle than the former, particularly when the molecule is hydrated (Duchesne & Larson 1989). There are indications that increased chitin concentrations in the fungal hyphae of *A. sarmentosa* results from increased N supply (unpublished data), thereby causing the thalli to fragment more easily. Increased infections of parasitic fungi may also explain the rapid decline in *A. sarmentosa*, since at least one species of parasitic fungus (Ström 2011) has increased with high N treatments. Moreover, the response of lichens to N treatments changed over time. *Alectoria sarmentosa* increased faster in the two highest N treatments during the first year, but declined

rapidly thereafter in both treatments. Long-term experiments are necessary to reveal such non-linear responses of species to environmental manipulations.

5. Conclusion

The following conclusions can be drawn from this thesis:

- At ecologically relevant N concentrations and doses, there was no significant difference in uptake of ammonium and nitrate by *A. sarmentosa* or *P. glauca.*

- P availability is an important factor when studying the effects of N deposition, since P can both mitigate and intensify the negative effects of N on epiphytic lichens.

- Since differences in N optima among epiphytic lichens recorded in this long-term field experiment largely coincide with the results from descriptive studies along pollution gradients, we conclude that N is an important pollutant for many lichen species. Moreover, the response of lichens to N treatments changed over time. *Alectoria sarmentosa* increased in the two highest N treatments during the first year, but declined rapidly thereafter in both treatments. Long-term experiments are necessary to reveal such non-linear responses of species to environmental manipulations.

- All investigated lichens seem to have rather robust chemical defence systems despite considerable manipulation of the environmental conditions. However, we don't know if these lichens are able to sustain high levels of protection over several years as new tissue is formed.

6. Outlook

Even though we have found direct evidence for negative effects of N deposition on lichens (i.e *A. sarmentosa*) we have not determined the exact mechanism behind the decline. Further studies could focus more on mycobiont responses during increased N availability. A robust method for measuring mycobiont vitality needs to be created, which could include labeling the CO_2 with ^{13}C - to follow the carbon flow in thalli. It would be interesting to measure chitin

concentrations and tensile strength of *A. sarmentosa* in the long-term experiment, both on thalli in the canopy and on fragmented lichens on the ground, to test the hypothesis that N increases the risk of fragmentation. It would also be interesting to do further studies on the lichenicolous parasites to get an increased understanding of their influence on lichens both at the physiological and community levels (Fig. 5).

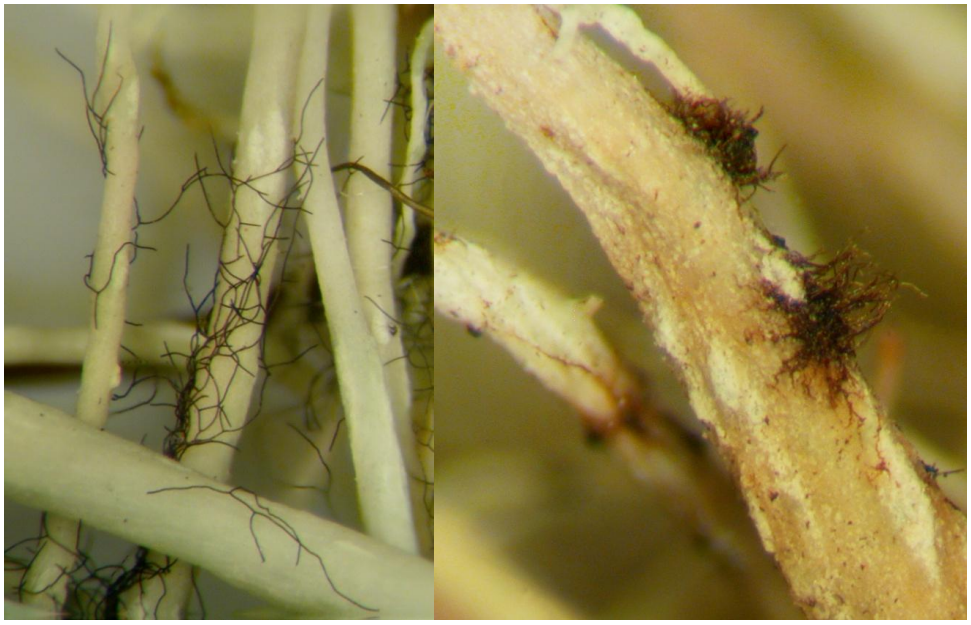

Fig 5. Two unidentified hyphomycetes growing on *Alectoria sarmentosa* (Photos by Otilia Johansson).

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