BOREAL ENVIRONMENT RESEARCH 17: 411–424
ISSN 1239-6095 (print) ISSN 1797-2469 (online)

Helsinki 30 November 2012

Epiphytes influence the transformation of nitrogen in coniferous forest canopies

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Received 13 July 2011, final version received 27 Apr. 2012, accepted 27 Apr. 2012

Woods, C. L., Hunt, S. L., Morris, D. M. & Gordon, A. M. 2012: Epiphytes influence the transformation of nitrogen in coniferous forest canopies. *Boreal Env. Res.* 17: 411–424.

The transformation of atmospherically-deposited nitrogen (N) by coniferous forest canopies has primarily been attributed to epiphytic lichens, despite microbes being present on leaf surfaces. We examined the relative contribution of live foliage with its epiphytic microbes and epiphytic lichen on N transformations in black spruce canopies. The epiphytic microbes and live foliage intercepted 27% of rainwater, 72% of incident nitrite + nitrate, and 63% of incident ammonium, while enriching throughfall with organic N by 52%. Epiphytic lichens on dead lower branches intercepted 60% of rainwater and depleted absolute amounts of inorganic and organic N, even though solute concentration increased as water passed through the lichens. Relationships were found between the number of colony forming units of epiphytic fungi and the depletion and enrichment of different forms of N in throughfall. Intensive forest management practices could result in the canopy being a net sink for N as a result of higher lichen biomass.

Introduction

The forest canopy has a large influence on nutrient cycling in forest ecosystems. The canopy partitions rainfall, with its dissolved nutrients, into throughfall, which drips from foliage, and stemflow, which is channeled down the branches and trunk (Park and Cameron 2008). As a result of rainfall partitioning, the canopy alters the chemical composition of precipitation by either enriching or depleting nutrients in rainwater. Coniferous canopies, for example, often deplete rainwater of inorganic nitrogen (N_{inorg}), and enrich rainwater with organic N (N_{org}) (Lovett and Lindberg 1993, Morris *et al*. 2003, Purainen *et al*. 1998). N_{inorg} retained in the canopy has been typically attributed to physiological assimilation by trees (Liechty *et al*. 1993), but epiphytic lichens and microbes on foliar surfaces may also contribute appreciably to canopy N assimilation and transformation (Carlisle *et al*. 1966, Friedland *et al*. 1991, Morris *et al*. 2003, Papen *et al*. 2002). To what extent epiphytic microbes and lichens affect N assimilation and transformation *in situ* in the canopy remains largely unexplored.

Canopies have been estimated to retain an average of 40% of incident N with values varying between 1 and 12 kg ha^{-1} yr^{-1}, the highest of which were found in spruce and spruce–fir stands (Lindberg *et al*. 1986, Lovett and Lind-

Editor in charge of this article: Jaana Bäck

berg 1993). If assimilated by the trees, this range of N retention could satisfy a significant portion of the annual N demands of conifers, which has been estimated to be between 5 and 50 kg ha^{-1} yr^{-1} (Cole and Rapp 1981). However, if assimilated by epiphytic lichens and foliar microbes and not by the live foliage, the canopy could act as a sink for N, thereby limiting the availability of atmospheric deposits as a substantial source of N for forest productivity. Epiphytes could also act as holding tanks of deposited N if they eventually leach the assimilated N or fall off the canopy with broken branches or in throughfall. Therefore, the deposited N retained in the canopy could either increase or limit productivity depending on its fate as it passes through the forest canopy.

The influence of the forest canopy on N cycling may be affected by the use of intensive forest management activities, such as the conversion of natural forests to plantations (Gordon *et al*. 2000), as this often changes the structure of the canopy. For example, lichens are a conspicuous component of boreal spruce forests and have been shown to preferentially colonize dead branches (Liu *et al*. 2000). The high density of trees in spruce plantations (e.g., 2200 stems ha^{-1}; Chourmouzis 1995) relative to natural spruce forests (e.g., 1740 stems ha^{-1}; Morris *et al*. 2003) reduces understory light levels resulting in a larger number of dead branches in the lower canopy (C. Woods pers. obs.). The higher number of dead branches has resulted in a higher load of epiphytic lichen in some boreal spruce plantations (Hilmo *et al*. 2009, Liu *et al*. 2000). This increase in epiphytic lichen could have large impacts on hydrologic cycles as lichen growth has been strongly correlated with water availability with lichens on average containing 1–3 g of water per g dry weight (Palmqvist 2000), and biogeochemical cycles as lichens have been shown to absorb N from solution (Lang *et al*. 1976).

The fate of N in precipitation as it moves through the forest canopy has been difficult to determine. Because throughfall collectors are typically placed on the forest floor (Lovett and Lindberg 1993, Mahendrappa and Ogden 1973, Morris *et al*. 2003), measures of N transformations in the canopy include the combined effect of all canopy components (i.e., live foliage, epiphytic microbes, and epiphytic lichen) and, as a result, the relative importance of interactions with different canopy components remains unclear. To date, no study has measured the effect of different forest canopy components on N flux to the forest floor.

In this study, we examined N transformations and assimilation potential of epiphytic microbes on live foliage, as well as epiphytic lichens on dead branches in black spruce forest canopies in order to determine if these epiphytes are acting as a sink for N or an eventual source of N upon death or falling from the canopy. Our study was conducted in boreal black spruce plantations, where the amount of dead branches with epiphytic lichens is high, in order to effectively compare the effects of epiphytic microbes and epiphytic lichens on canopy N cycling. We used a unique stratified canopy throughfall design to differentiate the effects of different canopy components on *in situ* N cycling.

Our general hypotheses were as follows: (1) if the canopy is not homogenous with regard to rainfall interception and throughfall N transformations, then different canopy levels (particularly the live canopy with its associated microbes *vs*. the lichen-covered dead branches) will differ in their rates of rainwater interception and depletion or enrichment of various forms of N; and (2) if epiphytic microbes are important to N-cycling within the canopy, then the depletion or enrichment of different forms of N in throughfall will correlate with the abundance of fungi grouped by form of N used.

Material and methods

Site description

The study was conducted in a 50 year old black spruce plantation in northern Ontario, Canada (49.5°N, 88°W). This site is a part of a larger study evaluating nutrient fluxes of managed plantations and was chosen based on the results of a throughfall study conducted in 2003–2005 that showed depletion of N_{inorg} and enrichment of N_{org} by the canopy (S. Hunt unpubl. data). The plantation also acts as a simplified model forest

for nutrient cycling studies, as variation in factors such as tree species, canopy height, needle biomass, and epiphytic lichen biomass between replicate trees is minimized. The plantation is found on an upland, naturally well-drained, silty-clay site. Rain accounts for 72% of the mean annual precipitation of 760.3 mm with 46% of the rain falling between the warmer months of May to August and the remaining precipitation falling mostly as snow in the winter months (Environment Canada 2008). This confined our throughfall study to the short summer between May and August. The mean annual temperature for the Nipigon region is 2.4 °C with the highest temperatures found between May and August (Environment Canada 2008).

The average height of the trees was 12.5 ± 0.73 m (mean ± SE), with the top two thirds of the canopy consisting of live foliage and the lower third consisting of dead branches covered in epiphytic lichen. The upper canopy of black spruce consists of a high density of needles that decreases with decreasing canopy height. The live portion of the canopy had no epiphytic lichens making this plantation an ideal system in which to separate the effects of the live foliage and its associated epiphytic microbes from the dead branches covered in epiphytic lichen on N transformations and assimilation potential. There are both fruticose and foliose lichens in this plantation but no cyanolichens (lichens that fix N_2, Berg 2008) suggesting that the only source of N for these epiphytic lichens is either dry or wet deposition. The epiphytic lichen community is dominated by *Usnea* spp., *Parmelia sulcata*, *Tuckermannopsis americana*, *Evernia mesomorpha*, *Hypogymnia physodes*, *Bryoria furcellata*, *Bryoria lanestris*, *Bryoria trichodes*, and *Bryoria* spp. (Berg 2008). To date, no information is available on epiphytic microorganisms in this plantation, but other studies examining fungi associated with black spruce foliage have found the fungi to be primarily saprophytic or pathogenic (Johnson and Whitney 1992, Sokolski *et al*. 2007).

Throughfall collection

We established three 50-m^2 plots in each of which three randomly chosen trees were set up for throughfall collection. In each tree, throughfall collectors, consisting of plastic funnels (254.5 cm^2) attached to 4-l polypropylene jugs with polyvinyl chloride (PVC) tubing and held aloft on wooden brackets strapped to the tree trunk, were set up at three different heights: upper canopy (average height of 8.4 ± 0.61 m), mid canopy (average height 5.5 ± 0.48 m), and lower canopy (average height 1.0 ± 0.2 m). The upper throughfall collector was placed under the high density of foliage in the upper canopy, and the mid-canopy throughfall collector was placed at the base of the live foliage. Because there were no lichens in the upper- and mid-canopy levels, any change in N concentration from below the mid canopy to below the lower canopy was attributed to epiphytic lichen. Bulk precipitation was collected in an open field adjacent to the plantation. Glass wool was used in all collection funnels to prevent debris from entering the collecting jugs.

Throughfall and bulk precipitation samples were collected on an event basis (i.e., 24 h after each rainfall event) for three events every 2–3 weeks beginning in early June, and frozen until analysis. Each rain event consisted of several rainfalls (each ranging in duration from 1–48 h) and was considered an event if rainfall stopped for at least 24 h. These rainfalls were approximately evenly distributed across the 2–3 weeks in between each rainfall event; thus, the amount of N originating from dry deposition is assumed to be equal for each rainfall event. Total rainfall over all three collections was 101.6 mm, which was distributed evenly across each event, and is similar to the 10-year average rainfall in June and July of 98 ± 10.6 mm (Environment Canada 2008). Funnels and collecting jugs were washed and replaced after each collection. Total rainfall and throughfall volume was converted to millimeters and the interception rate at each canopy level was calculated using the following equation:

$$I = [(P - T)/P] \times 100\ (\%) \qquad (1)$$

where I is the interception rate, P is the amount of water in bulk precipitation (mm), and T is the amount of water in throughfall (mm). Because

black spruce canopies partition precipitation primarily into throughfall and interception with less than 1% as stemflow (Mahendrappa and Ogden 1973, Price *et al*. 1997), and a similar result was found in the current plantation (S. Hunt unpubl. data), we did not measure stemflow in our study.

The samples were filtered through Fisherbrand P2 filters after which they were analyzed for nitrite + nitrate (NO_2^--N + NO_3^--N) and amonium (NH_4^+-N) by colourimetry at the University of Guelph, Guelph, Ontario, Canada. Total nitrogen (N_{tot}) was determined using a persulfate digestion (Qualls 1989) and analyzed by colourimetry. Dissolved organic nitrogen (DON) was calculated as $N_{tot} - (NO_3^-\text{-N} + NO_2^-\text{-N} + NH_4^+\text{-N})$.

Fungal extraction and counts

Needles from the previous year's growth (i.e., one-year-old needles) without evidence of damage were randomly sampled 24–48 h after each throughfall collection from three randomly chosen trees in each of the three plots from the upper canopy (average height [mean ± SE] of 8.4 ± 0.61 m) and the mid canopy (average height 5.5 ± 0.48 m). Black spruce in Canada's southern boreal forest tend to have 5–7 age classes of needles (Lamhamedi and Bernier 1994); we chose to sample from one needle age class to minimize variability, and because it was beyond the scope of our study to assess the influence of needle age. The first two needle samples were collected from non-throughfall trees so as not to affect throughfall, and the final needle samples were collected from the same trees used for throughfall measurements. Needle samples were collected using pole pruners sterilized with 95% ethanol, transferred into sterile bags, and kept at 4 °C until microbial analysis (within 24 h). The three needle samples from each plot at each canopy height were homogenized into one sterile sample bag (resulting in 18 needle samples in total: upper and mid canopy for three plots for each of three sampling dates). Only epiphytic fungi were examined in this study as few epiphytic bacteria and yeast, and no fungal endophytes were found in needle extracts from a preliminary study conducted in this black spruce plantation in 2006 (C. Woods unpubl. data).

The purpose of this study was not to distinguish between direct foliar uptake of N and uptake by epiphytic fungi on the live foliage. Rather we sought to determine to what extent the fungi that colonize the needle surface metabolize different forms of N in order to gain insight into their role in the N transformations commonly observed as rainfall passes through black spruce canopies (Lovett and Lindberg 1993, Morris *et al*. 2003). The role of lichen in N transformations was assessed based on throughfall data, as our vertically stratified throughfall collection design allowed for the isolation of the dead lower branches with their epiphytic lichens.

In order to determine the extent of colonization of the needle surface by epiphytic fungi and the use of different N forms by these fungi, 1 g of needles was shaken in 9% w/v sterile NaCl solution, and 100 ml of needle extract was plated into 96-well plates containing either ammonium, nitrite, or N_{org} sole N sources (Korner 1999, Woods 2008). The first row of the 96-well plates contained only media as a control for media contamination and the second row contained only the needle extract as a control for microbial growth not specific to the media. The following ten rows (3–12) were a one in ten dilution series with the inoculum per tube ranging from 0.1 to 1.0×10^{-10}. Based on the number of wells that showed positive growth at each dilution (*see* below), the number of colony forming units (CFUs) for each fungal functional group (NO_2^--N + NO_3^--N-, NH_4^+-N-, or N_{org}-using fungi) could be estimated. Another 100 ml of needle extract was plated onto agar plates with nitrite, ammonium, and N_{org} as sole N sources to culture fungal colonies for molecular analysis. All plates were incubated at 25 °C for six weeks in an incubator.

The 96-well plates were used to calculate relative counts of fungi using or transforming NH_4^+-N, NO_2^--N + NO_3^--N, and N_{org} in each of the upper- and mid-canopy layers. For the nitrite plates, the absence of nitrite was used as a positive count for the presence of nitrite- or nitrate-using fungi, and was detected using the Griess Reagent (Korner 1999, Woods 2008). For the ammonium plates, the absence of ammonium was used as a positive count for fungi using ammonium (Solorzano 1969). For the N_{org} plates, the presence of both ammonium and

nitrite was used as positive counts for fungi using N_{org} (Woods 2008). To accurately measure the appearance or disappearance of N, all plates were read on an absorbance microplate reader (BioTek ELx800, BioTek Instruments Inc., Winooski, VT) at 630 nm and 590 nm for ammonium and nitrite, respectively. Counts for each fungi plate reading were calculated using the most probable number (MPN) method for Microsoft Excel (Briones and Reichardt 1999). One gram of needles was used to determine the gravimetric water content in the needles; microbial counts in CFUs were made per g of needle dry weight.

Endophytic fungi were not examined in this study as few fungal endophytes were extracted from this plantation in the preliminary study (C. Woods unpubl. data), despite their prevalence in natural black-spruce stands (Johnson and Whitney 1992, Sokolski *et al*. 2007). This may be because we focused our sampling on current-year needles, which have been shown to have a lower rate of colonization of endophytic fungi than older needles (4% in current year needles and 90% in 3-year-old needles, Johnson and Whitney 1992), or it could be due to an effect of intensive forest management on endophytic microbes, something that could be addressed in future studies.

Needle biomass for the entire tree was estimated using pre-established allometric equations for black spruce (Czapowskyj *et al*. 1985). Upper-canopy and mid-canopy needle biomass were estimated using proportions of total needle biomass in the upper and mid canopy measured in the field (i.e., based on the height of placement of the throughfall collectors, the upper canopy was estimated to be 2/3 and the mid canopy was estimated to be 1/3 of the live canopy). Estimates of fungal abundance for the upper and mid canopy of each tree was made by multiplying the number of CFUs per g of needle dry weight by the needle biomass of the upper and mid canopy (with the understanding that CFU counts do not represent standing crop of fungi, but are often used as a relative measure of abundance; e.g., Stadler and Müller 2000).

During a real-time rain event in July 2007, throughfall from three trees and bulk precipitation were collected in sterile bags for 60 min to determine the presence of live microbes in throughfall. The sterile bags were hanging from the branch so as not to affect the rate of canopy drip off the needles. The samples were stored at 4 °C until microbial analysis (within 24 h) whereby 100 μl of each sample was plated onto agar plates for fungi using the same media as the needle extracts above. The bulk precipitation and live throughfall plates were examined qualitatively.

Fungal DNA extraction and PCR amplification and analysis

The goal of this study was not to describe completely the composition of the microbial community in the black spruce canopy, but to elucidate its function. Therefore, only 13 fungi were chosen for molecular identification using the ITS region based on their abundance in the agar plates under the assumption that the most abundant fungal colonies would have the greatest impact on N transformations in the canopy.

Pure cultures were obtained for each colony and DNA was extracted using an UltraClean Soil DNA Isolation Kit (MoBio Laboratories, Carlsbad, CA; Woods 2008). Fungal DNA was amplified using the highly conserved fungal rRNA gene primer ITS1F previously described (Gardes and Bruns 1993, Manter and Vivanco 2007). The reaction mix had a total volume of 20 ml containing 1.5 ml dNTP, 0.5 ml of primer (forward and reverse), 0.4 ml TITANIUM™ Taq (BD Biosciences, San Jose, CA), 2.0 ml manufacturer's reaction buffer, and 1 ml DNA template. DNA concentration was not determined. PCR was performed using a 96-Well GeneAmp® PCR System 9700 (Applied Biosystems, Foster City, CA). The PCR cycle was programmed as follows: initial denaturation at 95 °C for 3 min, 40 cycles of denaturation at 95 °C for 30 s, annealing at 59 °C for 45 s, and extension at 72 °C for 2 min, followed by final extension at 72 °C for 10 min. Aliquots of 1 ml (with 1 ml loading buffer and 4 ml of sterile high performance liquid chromatography [HPLC] water) were run on a 1% (w/v) agarose gel and stained with ethidium bromide to confirm the amplification of products. Positive PCR products (9 samples)

were sent to Laboratory Services (University of Guelph) for bidirectional sequencing using both forward and reverse primers. Nucleotide sequences for each isolate were obtained and aligned using SeqMan Pro (DNAStar Lasergene 7, Madison, WI). The consensus sequences were then compared to fungal ITS sequences in GenBank (NCBI website) with the BLASTn algorithm (Dong and Xiang 2007).

Statistical analysis

Analysis of variance (ANOVA) was performed for all of the throughfall parameters: volume (analyzed as depth [mm]), concentration (mg l^{-1}) and flux (i.e., absolute amount; mg) of NO_2^--N + NO_3^--N, NH_4^+-N, and dissolved organic nitrogen (DON) using the PROC GLM procedure in SAS ver. 9.1. The experimental units were the individual trees in the stand (nine in total). The total variance was partitioned into the following sources: Position (position of throughfall collector in each tree), Tree (replicate trees in the stand), Position × Tree interaction, and experimental error. Orthogonal contrasts were used to determine differences in N concentration between bulk precipitation and throughfall, between the live canopy and the lichen-covered dead branches, and between the upper and mid portion of the live canopy. Shapiro-Wilk's test for normality and a plot of residuals against predicted values was used to test for homogeneity. Values of absolute amounts of N_{inorg} and N_{org} (mg) had to be log-transformed to meet the assumption of homogeneity of the model. One value of NO_2^--N + NO_3^--N and three values of NH_4^+-N were defined as outliers according to a Cook's D statistical test and removed from the analysis.

ANOVA was performed for the number of CFUs of epiphytic fungi for all tests that showed N use (epiphytic fungi for each N source: NO_2^--N, NH_4^+-N, and N_{org}) using the same GLM model as for the throughfall analysis. To meet the assumptions of the model, the numbers of CFUs for epiphytic fungi that used nitrite as a sole N source, and epiphytic fungi that use N_{org} as a sole N source had to be log-transformed. No CFU counts of fungi grown in 96-well plates with ammonium as a sole N source were made as there was no depletion of ammonium from these plates.

Finally, correlation analyses were conducted on the change in concentration of NO_2^--N + NO_3^--N with the total number of CFUs (i.e., estimated CFUs per canopy level) of nitrite-using fungi, and the change in concentration of DON with the total number of CFUs of first, N_{org}-using fungi and, second, all fungi. The product-moment correlation coefficients were computed. We used correlation analyses as both variables were assumed to be measured with error and we had no *a priori* assumptions about the cause and effect relationship between fungal abundance and N concentrations in throughfall.

Results

Throughfall

The concentrations of NO_2^--N + NO_3^--N (ANOVA: $F_{1,46} = 125.1$, $p < 0.0001$) and NH_4^+-N ($F_{1,45} = 70.5$, $p < 0.0001$) in rainwater (bulk precipitation) were significantly reduced by the canopy (Fig. 1), while DON ($F_{1,45} = 30.6$, $p < 0.0001$) concentrations increased. The live canopy (i.e., foliage and its associated microbial community) substantially depleted NO_2^--N + NO_3^--N (by 72%) and NH_4^+-N concentrations (by 63%), as shown by the decrease in N_{inorg} concentration by the upper and mid canopy (Fig. 1). Concentrations of NO_2^--N + NO_3^--N were not further altered as throughfall passed through the dead, lichen-covered branches of the lower canopy ($F_{1,46} = 0.1$, $p = 0.7$); however, NH_4^+-N concentrations increased significantly (by 40%) by the lower canopy relative to throughfall leaving the live canopy ($F_{1,45} = 5.84$, $p = 0.02$) (Fig. 1). These results suggest that the live canopy and its associated microbes are actively scavenging N_{inorg} from rainwater, while the dead, lichen-covered branches are not. The lichen-covered branches of the lower canopy enriched rainwater with N_{org} (by 55%) significantly more than did the live canopy ($F_{1,45} = 34.3$, $p < 0.0001$; Fig. 1).

The live canopy (upper and mid) had a significantly lower rainfall interception rate compared with the lichen-covered branches ($F_{1,44} = 13.6$, $p = 0.0006$) which intercepted 60% of the

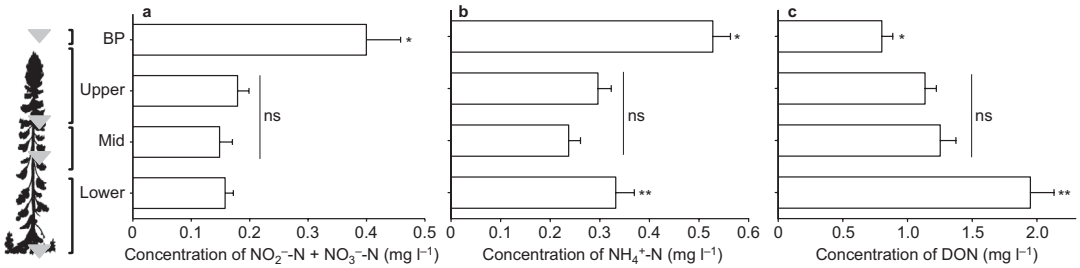

Fig. 1. Concentrations (mean + SE) (mg l^{-1}) of (**a**) NO_2^--N + NO_3^--N, (**b**) NH_4^+, and (**c**) DON (dissolved organic nitrogen) in bulk precipitation (BP) and throughfall from three canopy levels: upper canopy (Upper) and mid canopy (Mid), which is made up of the live canopy and their epiphytic microbes, and the lower canopy (Lower), which is made up of epiphytic lichen on dead branches in a black spruce plantation in northern Ontario, Canada. The grey triangles on the tree show the locations of BP and throughfall collectors. An asterisk (*) indicates significant differences between BP and all canopy levels ($p < 0.05$). Two asterisks (**) indicate significant differences between the epiphytic lichen and the live foliage ($p < 0.05$). Upper- and mid-canopy levels are not significantly (ns) different from each other at $p < 0.05$.

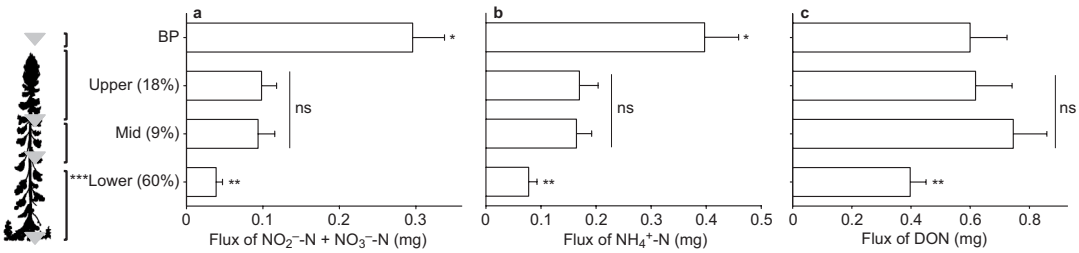

Fig. 2. Fluxes (mean + SE) (absolute amounts; mg) of (**a**) NO_2^--N + NO_3^--N, (**b**) NH_4^+, and (**c**) DON (dissolved organic nitrogen) in bulk precipitation (BP) and throughfall from three canopy levels: upper canopy (Upper) and mid canopy (Mid), which is made up of the live canopy and their epiphytic microbes, and the lower canopy (Lower), which is made up of epiphytic lichen on dead branches in a black spruce plantation in northern Ontario, Canada. The grey triangles on the tree show the locations of BP and throughfall collectors. An asterisk (*) indicates significant differences between BP and all canopy levels ($p < 0.05$). Two asterisks (**) indicate significant differences between the epiphytic lichen and the live foliage ($p < 0.05$). Upper- and mid-canopy levels are not significantly (ns) different from each other at $p < 0.05$. The percent of precipitation intercepted by each canopy level is in brackets beside the canopy level. *** Lower canopy interception was significantly higher than that of the live canopy.

precipitation (lower canopy; Fig. 2). This large interception of rainwater by the epiphytic lichen resulted in a significant depletion in absolute amounts (i.e. flux) of NO_2^--N + NO_3^--N ($F_{1,69} = 10.4$, $p = 0.0007$), NH_4^+-N ($F_{1,66} = 9.5$, $p = 0.0001$), and DON ($F_{1,69} = 2.9$, $p = 0.02$) relative to the live canopy (Fig. 2). Although epiphytic lichens are not reducing the concentration of N_{inorg} in throughfall (Fig. 1), they have access to the N contained in the large amount of water they intercept (Fig. 2). The largest depletion in N_{inorg} flux occurs within the first 4 m of the canopy (upper canopy, Fig. 2). The live canopy and its associated microbial community are actively scavenging N_{inorg} from rainwater as evidenced by the large depletion in both concentrations (Fig. 1) and absolute amounts of N_{inorg} despite the small amount of rainwater intercepted by the upper canopy (18%, Fig. 2).

Overall, the net uptake of the whole canopy and its epiphytes of NO_2^--N + NO_3^--N, NH_4^+-N, and N_{org} from precipitation was 2.16 kg ha^{-1} yr^{-1}, 2.98 kg ha^{-1} yr^{-1}, and 1.94 kg ha^{-1} yr^{-1}, respectively. Nitrogen fluxes in bulk precipitation were 2.50 kg ha^{-1} yr^{-1} (NO_2^--N + NO_3^--N), 3.65 kg ha^{-1} yr^{-1} (NH_4^+-N), and 5.36 kg ha^{-1} yr^{-1} (DON). Because the throughfall collectors were placed only under branches and not in between trees (i.e., not randomly distributed throughout the stand), these extrapolations to the stand level should be viewed as rough estimates only, and interpreted with caution.

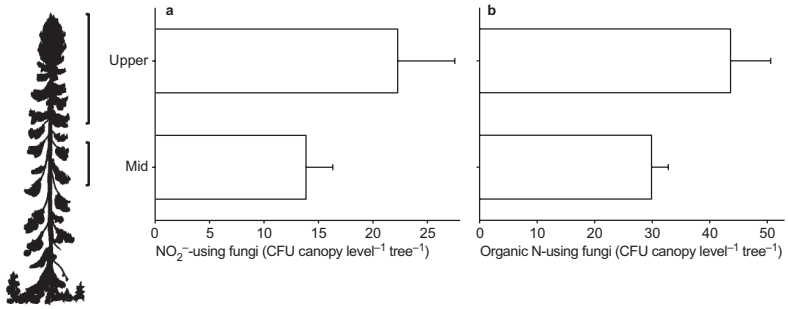

Fig. 3. Estimated total number (+ SE) of CFUs of (**a**) NO_2^--N-using fungi and (**b**) N_{org}-using fungi on live foliage of the upper and mid canopy levels collected from a black spruce plantation in northern Ontario, Canada.

Fungal communities

The total counts of CFUs for NO_2^--N-using fungi (ANOVA: $F_{1,16} = 2.1$, $p = 0.2$) and N_{org}-using fungi ($F_{1,16} = 3.3$, $p = 0.09$) were not significantly different between the upper and mid levels of the live canopy (Fig. 3), likely due to the large amount of variation in fungal counts. No fungi were found to transform ammonium as ammonium was not depleted in the 96-well plates, but the reason for this is unknown as fungal colonies grew successfully on agar plates with ammonium as a sole N source.

Nine fungi were sequenced successfully (Table 1). The dominant fungal species came from the genera *Cladosporium*, *Hormonema*, *Capnodium*, *Taphrina*, *Penicillium*, *Phialophora*, and *Acrodontium*. All of these fungi grew on media with N_{org} and NH_4^+-N as sole N sources, except for *Cladosporium* that grew on media with nitrite as a sole N source. All of the fungi were isolated from both the upper and mid canopy except for *Taphrina* that was isolated from the upper canopy only.

Fungi fell from the live canopy during rain events as the bulk precipitation plates had no fungal growth while the throughfall plates with nitrite, ammonium, and N_{org} had fungal colonies. Of all the throughfall plates, those with nitrite alone had the highest growth of fungi.

There was a significant correlation between the number of CFUs of NO_2^--N-using fungi in the upper and mid canopies and the change in concentration of NO_2^--N + NO_3^--N in throughfall from the upper and mid canopies ($r = -0.55$, $p = 0.02$; Fig. 4A); that is, the greater the numbers of CFUs, the greater was the reduction in concentration as rainfall passed through the upper and mid canopy levels. There was a marginally significant positive correlation between the number of CFUs for all fungi and the change in concentration of DON in throughfall ($r = 0.42$, $p = 0.08$; Fig. 4B). There was a positive but non-significant correlation between the number

Table 1. Identification of isolates of epiphytic fungi from the surface of black spruce needles collected from two canopy levels (upper and mid) of three trees in three 50 m^2 plots from May to July, 2007 in a black spruce plantation in northern Ontario. The E value for every isolate was 0.0. All epiphytic fungi were found in both the upper and mid canopy except where denoted otherwise. rRNA = ribosomal RNA.

Accession no.	Description	Nitrogen
EF432298.1	*Cladosporium* sp. B5B 18S rRNA gene	NO_2^-
AF013228.1	*Hormonema dematioides* 18S rRNA gene	Organic
AF013225.1	*Hormonema* sp. GCA 2004 18S rRNA gene	Organic
AY805548.1	*Capnodium* sp. Olrim 506 18S rRNA gene	Organic
AF492088.1*	*Taphrina communis* strain NRRL T-842 ITS 1, 5.8S rRNA gene	NH_4^+, Organic
AJ748692.2	*Penicillium virgatum* 18S rRNA gene (partial)	NH_4^+
EF619868.1	Uncultured ascomycete clone 2S2.21.S04 18S rRNA gene	NH_4^+
AY843112.1	*Acrodontium crateriforme* ITS 1	NH_4^+
AY857542.1	*Phialophora sessilis* strain CBS 243.85 18S rRNA gene	Organic

* found only in the upper canopy.

of CFUs for N_{org}-using fungi and the change in concentration of DON in throughfall ($r = 0.35$, $p = 0.15$; Fig. 4C).

Discussion

Inorganic N

The live canopy and its epiphytic microbes had a significant influence on N_{inorg} transformations in this black spruce plantation. Of the total reduction in N_{inorg} concentration by the spruce canopy, the live foliage and its epiphytic microbes accounted for 72%. The reduction in N_{inorg} as rainfall passed through the live canopy could be due to direct foliar uptake, and/or uptake by epiphytic microbes, or canopy interception of precipitation and subsequent volatilization of dissolved N. Because there was little interception of precipitation by the upper and mid canopy, N_{inorg} must be actively taken up either by the foliage or its associated microbial community, or both. The ability of a plant to take up nutrients through its leaves depends on the thickness of the cuticle such that plants with a thicker cuticle, like conifers, have lower uptake rates than plants with a thinner cuticle (Chalker-Scott 2008). In apple trees that have a much thinner cuticle than spruce trees, for example, the uptake of N by leaves varied between 10% and 48% (Dong *et al*. 2005). These low values for uptake of N by leaves with a relatively thin cuticle suggest that the uptake rates by spruce foliage alone does not explain the large depletion of N_{inorg} we found in our spruce trees.

Although we did not attempt to directly differentiate the effects of live foliage and its epiphytic microbes on N transformations, we found a substantial community of epiphytic fungi on the spruce needles, and provided evidence that these fungi assimilate and transform N_{inorg} and N_{org} sources. Epiphytic microbes on leaf surfaces acquire nutrients from precipitation-derived water droplets or from nutrients deposited onto leaves in gaseous or particulate form (Blakeman 1971). Therefore, these canopy microbes must be accessing N from either the needle surface or N deposited onto the forest canopy through wet or dry deposition. In our study we found a significant relationship between the number of

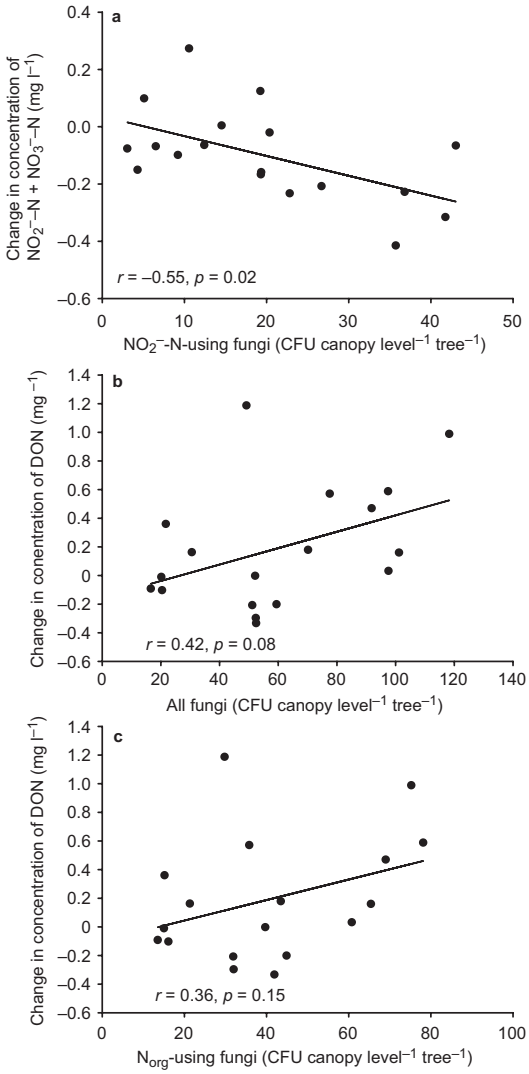

Fig. 4. Relationships between estimated total number of CFUs and change in N concentration (mg l^{-1}) in throughfall collected from the upper and mid portions of the live canopy. Change in concentration at canopy level x was calculated as $TF_x - TF_{x-1}$, where TF_x is the concentration of N in the collector at canopy level x, and TH_{x-1} is the concentration of N in the collector at the canopy level above it with bulk precipitation being the canopy level above the upper canopy. (**a**) CFUs of NO_2^--N-using fungi with the change in concentration of NO_2^--N + NO_3^--N in throughfall (mg l^{-1}); (**b**) CFUs of organic-N-using fungi with the change in concentration of DON (mg l^{-1}); (**c**) CFUs of all fungi with the change in concentration of DON (mg l^{-1}). Pearson's correlation coefficient, p values, and lines of best-fit are shown

CFUs of nitrite-using fungi and the change in concentration of NO_2^--N + NO_3^--N in through-

fall, which suggests that the depletion of N_{inorg} in rainwater is, at least in part, due to the presence of epiphytic fungi.

The epiphytic lichen in this plantation forest affected N_{inorg} transformations as absolute amounts of N_{inorg} were depleted by the epiphytic lichen. Epiphytic lichens had little effect on the concentration of N_{inorg} in throughfall but did intercept large amounts of water and, hence, N. A more focused study examining the impacts of epiphytic lichen cover and composition on N transformations in this black spruce plantation found similar results to the current study; epiphytic lichen had little impact on NO_2^--N + NO_3^--N concentrations but lichens enriched rainwater with NH_4^+-N (Berg 2008). Epiphytic lichens have been shown to take up N from precipitation in other studies (Greenfield 1992, Lang *et al*. 1976), and have been postulated to affect precipitation chemistry (Lang *et al*. 1976). Epiphytic lichens in our study depleted absolute amounts of N from rainwater, thereby affecting precipitation chemistry as predicted by Lang *et al*. (1976). As a result of their poikilohydric nature in which they absorb water and its dissolved nutrients over their entire body surface, epiphytic lichens are passive players in N assimilation from rainwater. Because there were no cyanolichens in this spruce plantation, epiphytic lichens could represent a canopy sink of N_{inorg} because they depleted absolute amounts of N_{inorg} from rainwater, and we found little evidence of dead branches with their epiphytic lichen breaking off of the trees or epiphytic lichen on the forest floor (C. Woods pers. obs.). *Picea* trees in general have low rates of branch pruning with some branches lasting for almost the entire life of the tree (Takahashi 1996). The majority of dead branches in this 50-year-old plantation were still attached to the tree, which may be why lichen cover is so high; the persistent dead branches may give epiphytic lichen a lot of time for successful colonization and establishment. These persistent branches with their high epiphytic lichen load could, therefore, limit the amount of atmospherically-deposited N that reaches the forest floor, particularly in plantations where trees are planted at such high densities relative to naturally regenerated, unmanaged forests.

Organic N

The live canopy and its epiphytic microbes enriched rainwater with N_{org}. The release of N_{org} from spruce canopies has been attributed to leaching from foliage and lichens (Gaige *et al*. 2007, Lovett and Lindberg 1993), and microbes (Morris *et al*. 2003). Because there were no lichens in the upper and mid canopy, any increase in DON in the live canopy must be due to foliar leaching (Lovett and Lindberg 1993) or the canopy microbial community. The epiphytic fungi extracted from our spruce canopy may metabolically increase the concentration of N_{org} in throughfall. Leaf surface microorganisms from Scots pine (*Pinus sylvestris*), for example, were found to contribute to the DON pool by the enzymatic degradation of complex proteins (Muller *et al*. 2004). A study in central Maine added ^{15}N-labeled fertilizer to the canopy of red spruce (*Picea rubens*) and eastern hemlock (*Tsuga canadensis*) and found N_{inorg} to be converted to DON in throughfall at a speed that suggested microbial activity as the cause for the transformations and not the live foliage (Gaige *et al*. 2007).

Many of the fungi extracted from the leaf surface in this study have been found to be chemoheterotrophs (i.e., organisms that use organic compounds as sources of energy, hydrogen, and carbon for biosynthesis, Prescott *et al*. 1999) such as *Cladosporium* (Solomon and Oliver 2001), *Hormonema dematioides* (Pirttila *et al*. 2003), and *Taphrina communis* (Rodrigues and Fonseca 2003), and some have been found in natural black spruce stands such as *Hormonema dematioides* and *Cladosporium* (Sokolski *et al*. 2007). These fungi were found to use N_{org} as a sole source of N suggesting that they are accessing organic forms of N from the needle surface. This does raise the question: If the fungi extracted from the needle surface in this black spruce plantation were accessing N_{org} from their environment, why then does the concentration of N_{org} increase under the live canopy? It is possible that the increase in N_{org} in throughfall is due at least partially to the presence of the microbes themselves in throughfall pathways. Rain is a known vector by which epiphytic microbes emigrate from the leaf surface, and rainwater has

been shown to wash off up to 50% of microbial cells from a leaf (Lindow 1996). From our microbial analysis of throughfall and incident precipitation, we found fungi in throughfall but not in incident precipitation confirming the needle surface as a habitat for fungi and a source of N_{org} in throughfall. We also found the concentration of DON in throughfall to increase with the number of CFUs of all fungi extracted from the needle surface providing further support for microbes adding to the DON pool. The amount of N_{org} used by the microbial community may have been offset by the N_{org} washed off in microbial biomass thereby masking any significant uptake of N_{org} by the microbial community. This is evident when looking at the near zero changes in absolute amounts of N_{org} in the live canopy and the lack of a relationship between the number of CFUs of DON-using fungi and the change in concentration of DON.

Lovett and Lindberg (1993) suggested that if microbial action on the canopy surface is primarily responsible for the transformation of N in throughfall, then there should be a direct correlation between N_{inorg} uptake and N_{org} release. This correlation was found in black spruce forests northwest of the current study location and was thought to be the result of N_{inorg} being incorporated into microbial biomass and an equivalent amount of N_{org} being released as microbial necromass (Morris *et al*. 2003). In this study, there is a clear trend of N_{inorg} uptake and DON release in the upper and mid canopy as evidenced by the decrease in N_{inorg} concentration along with an increase in DON concentration by the live canopy, suggesting that the canopy microbial community is having an effect on the transformation of N in rainwater. Epiphytic microbes may, therefore, represent a holding tank of atmospherically-deposited N as they deplete N_{inorg} forms from rainwater, but enrich rainwater with N_{org} either through their metabolism of N_{inorg} or washing off needles into throughfall.

Epiphytic lichen affected the concentration of DON in throughfall. The lichen-covered branches had the greatest depletion in the absolute values of DON, which is due to the large water interception by the lower canopy as the concentration of DON actually increased under the lower canopy. The increase in DON concentration by the lichen-covered branches could be attributed to the leaching of organic acids by lichen (Lovett and Lindberg 1993). Because lichens are known to assimilate organic forms of N (Dahlman *et al*. 2004), the N_{org} in throughfall from the live canopy could be in a form that is inaccessible to the lichen, such as in the form of microbial biomass.

Impacts of intensive forest management

Management practices that alter the stand density, and hence light patterns in the understory, could alter the composition and abundance of the lichen community, which could affect the unidirectional inputs of nitrogen into the stand by affecting rates of water interception. During the sampling period, the area experienced several rainfall events with the plantation receiving 101.6 mm of rainfall of which 60% was intercepted by the sample tree crowns. This is considerably higher than other values reported for black spruce (18.5%, Mahendrappa and Ogden 1973; 24.4%, Chourmouzis 1995; 18.6%, Morris *et al*. 2003; 23%, Price *et al*. 1997). The higher interception rate in our study may be due to the high density of trees (3400 stems ha^{-1}) (Hunt *et al*. 2010) that often accompanies plantation forests resulting in a dense canopy layer, or to a higher load of epiphytic lichen. Chourmouzis (1995) found an interception rate of 24.4% in a black spruce plantation with a stand density slightly lower than the current study of 2200 stems ha^{-1} and almost no lichen on the branches (C. Chourmouzis pers. comm.). Their interception rate is similar to that of the live canopy in our study (27%) suggesting that the interception of precipitation in their black spruce plantation was due primarily to the live foliage (Chourmouzis 1995), and our high interception rate is due to both a higher tree density as well as a higher abundance of epiphytic lichen.

Epiphytic lichen depleted substantial amounts of inorganic and N_{org} from rainwater in our black spruce plantation. The reduced light levels in the lower canopy caused by a high density of trees (3400 stems ha^{-1}) have resulted in a large number of dead branches that have been colonized by epiphytic lichen. Epiphytic lichen

may intercept more rainwater and its dissolved nutrients in trees that have less stemflow than throughfall, such as black spruce (Mahendrappa and Ogden 1973, Price *et al*. 1997), because lichens colonize branches more often than tree trunks (Lang *et al*. 1980, Liu *et al*. 2000). Intensive forest management practices, therefore, could result in the canopy being a net sink for N as a result of higher lichen biomass, which, as previously stated, may limit the availability of atmospherically-derived N to the forest floor. In forest ecosystems with a low N deposition rate, this could limit productivity, while in forests with a higher N deposition rate, this could reduce N saturation, which often leads to N leaching in forest floor soils (Fleischer 2003).

Conclusions

Epiphytic microbes and epiphytic lichens were found to influence N cycling in this black spruce plantation. Using a stratified throughfall sampling design, we were able to separate the effects of live foliage with its epiphytic microbial community from the effects of epiphytic lichens on N transformations in throughfall. Although we did not attempt to differentiate the effects of live foliage from its epiphytic microbial community on N transformations, this study has shown that there is an abundance of fungi on black spruce needles, and their presence and their preferences for different N forms contributes to the transformation of N as it passes through the canopy in rainwater. Historically, it has been thought that the enrichment of N in precipitation passing through the tree canopy was due primarily to leaching from the foliage and epiphytic lichens, and that the depletion of N was due to direct uptake by foliage and epiphytic lichens (Lang *et al*. 1976, Lovett and Lindberg 1993, Tukey 1970). Most studies, however, did not consider the potential effects of epiphytic microbes in the canopy on N transformations. By linking quantitative estimates of N transformations in throughfall to known functions of microbes isolated from the needle surface, and by differentiating the effects of the live canopy and dead, lichen-covered branches in our black spruce plantation, we have found support for our hypothesis that epiphytic microbes influence the transformation of N in forest canopies and should, therefore, be considered in future studies on N cycling in forest ecosystems. We also found epiphytic lichens to substantially deplete throughfall of N, which could impact N cycling in plantation forests where epiphytic lichen may be in higher abundance.

Acknowledgements: The authors would like to thank K. Berg for lichen identification and help with data collection; A. Bermingham and R. Davies for help in the field; Dr. L. Hutchison for the use of his lab at Lakehead University to conduct microbial analyses; and M-A. Renaud for help with molecular analyses. We thank two anonymous reviewers for helpful comments on the manuscript. Funding was provided by a Natural Sciences and Engineering Reseach Council of Canada (NSERC) grant awarded to AMG (Reference number RG25096-01) and a University of Guelph Registrar's Award for Graduate Students to CLW.

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