Dramatic changes in ectomycorrhizal community composition, root tip abundance and mycelial production along a stand-scale nitrogen deposition gradient

Rasmus Kjøller1, Lars-Ola Nilsson2, Karin Hansen3, Inger Kappel Schmidt2, Lars Vesterdal2 and Per Gundersen2

1Department of Biology, University of Copenhagen, Øster Farimagsgade 2D, 1353 Copenhagen K, Denmark; 2Forest & Landscape Denmark, University of Copenhagen, Rolighedsvej 23, 1958 Frederiksberg C, Denmark; 3IVL Swedish Environmental Research Institute, SE-100 31 Stockholm, Sweden

Summary

• Nitrogen (N) availability is known to influence ectomycorrhizal fungal components, such as fungal community composition, biomass of root tips and production of mycelia, but effects have never been demonstrated within the same forest.
• We measured concurrently the abundance of ectomycorrhizal root tips and the production of external mycelia, and explored the changes in the ectomycorrhizal community composition, across a stand-scale N deposition gradient (from 27 to 43 kg N ha\(^{-1}\) yr\(^{-1}\)) at the edge of a spruce forest. The N status was affected along the gradient as shown by a range of N availability indices.
• Ectomycorrhizal root tip abundance and mycelial production decreased five and 10-fold, respectively, with increasing N deposition. In addition, the ectomycorrhizal fungal community changed and the species richness decreased. The changes were correlated with the measured indices of N status, in particular N deposition and N leaching.
• The relationship between the altered ectomycorrhizal community, root tip abundance and mycelial production is discussed in the context of the N parameters. We suggest that increased N deposition to forests will cause large changes in ectomycorrhizal fungal community structure and functioning, which, in turn, may result in reduced N uptake by roots and fungi, and increased losses of N by leaching.

Introduction

Since the 1960s, the rate of reactive nitrogen (N) released to the environment by human activities has increased immensely (Galloway et al., 2003). This has led to increased productivity in forest ecosystems, but is also of major environmental concern in terms of the leaching of nitrate into ground and surface water reserves and negative impacts on biodiversity in natural habitats, including reduced production of fruit bodies of ectomycorrhizal (EcM) species (Arnolds, 1991).

EcM fungi form symbioses with tree roots and feed host trees with N taken up from the soil in exchange for photosynthetically derived carbon (C) (Smith & Read, 2008). Normally, > 90% of the nutrient-absorbing roots of EcM trees are colonized by their symbiont fungi (Taylor et al., 2000), and most nutrients, including N, therefore enter the plant via the fungal pathway. Nitrogen may be taken up from the same pool as accessed directly by plant roots, that is, ammonia, nitrate and, to some extent, amino acids (Persson & Nasholm, 2001), but, complementarily, many EcM fungi can also mobilize N from sources not directly available to plant roots, for example peptides, proteins or other organic N sources, such as chitin (Lindahl & Taylor, 2004). As N often is the major limiting nutrient in temperate and boreal forests (Tamm, 1991; Hyvönen et al., 2008), this contribution by the fungal symbionts may be crucial for tree growth under such conditions.

In the field, elevated N deposition or fertilization affects the biomass and community composition of EcM fungi. It has been recognized that the production of fruit bodies of EcM fungi is depressed by increased N deposition (Arnolds, 1991; Termorshuizen, 1993; Wallenda & Kortke, 1998). Below ground, species richness on root tips is also affected, although typically not as dramatically as above ground (Taylor et al., 2000; Lilleshov et al., 2002a,b; Avis et al., 2003; Toljander et al., 2006). Significant changes in fungal community composition with increased N availability seem to be the rule (Fransson et al., 2001; Lilleshov et al., 2002a,b; Avis et al., 2003; Toljander et al., 2006; Parrent & Vilgalys, 2007). Indeed, N availability has been shown to be a major determinant of EcM communities across north-western Europe (Cox et al., 2010). In a series of studies, Lilleshov et al. (2001, 2002a,b) demonstrated how the community changed along an N deposition gradient. In short, the results showed that,
the higher the N deposition, the higher the dominance of species presumably using inorganic N sources successfully (nitrophilic species), and vice versa. Similar results were found by Taylor et al. (2000) with fungi isolated along a long-distance north–south European transect. The general trends among studies have been a decrease in Cortinarius spp. (Lilleskov et al., 2002a,b; Avis et al., 2003; Toljander et al., 2006), whereas specific Lactarius or Russula species increased with increased N loads (Lilleskov et al., 2002a,b; Avis et al., 2003).

Nitrogen availability influences the production of EcM root tips and external mycelia. Under extreme N-limited conditions, fungal biomass may increase as a response to N addition (Clemmensen et al., 2008), but, in general, EcM biomasses of both roots tips and, especially, external mycelia are reduced (Wallander & Nylund, 1992; Arnebrant, 1994; Wallenda & Kottke, 1998; Fransson et al., 2001; Nilsson & Wallander, 2003; Nilsson et al., 2005, 2007; Ostonen et al., 2011).

The objective of this study was to concurrently monitor the effects of N deposition on EcM fungi – such as changes in mycelial production, root tip abundance and community composition – along a stand-scale N deposition gradient. We took advantage of a forest edge-generated N gradient from high N deposition at the edge to two to three times lower N deposition in the interior forest. Deposition effects at forest edges are well described (e.g. Beier & Gundersen, 1989; Wuyts et al., 2008), and these serve as excellent experimental systems because the gradients are short (within 100 m) and steep, thereby limiting the variation in other site-related parameters. The study was performed as part of a larger research program monitoring N deposition effects on C and N storage and dynamics in forests. This enabled a unique possibility to co-analyze changes in mycorrhizal abundance and community structure with a wide range of environmental parameters.

We expected that increased N availability driven by the N deposition gradient would decrease EcM mycelial production, EcM root tip abundance and EcM fungal diversity, as well as change the EcM fungal community structure and functioning.

Materials and Methods

Study site

Thyregod (55°54′05″N, 9°16′42″E) is located in Jutland, Denmark, 25 km north-west of Vejle, 105 m above sea level. The soil is a sandy, nutrient-poor and relatively dry Haplic Arenosol (WRB). The climate is temperate, with a mean annual temperature of 7.5°C and a mean annual precipitation of 875 mm.

A Norway spruce (Picea abies (L.) Karst.) stand was established in 1970s and was expanded to its current size in c. 1990. The local emission from the farm has therefore persisted for at least 30 yr.

Sampling and analysis of N fluxes and pools

From July 2005 to July 2007, throughfall and soil solution were collected monthly in two transects perpendicular to the forest edge and 20 m apart. Measuring stations were established in each transect at 0, 10, 25, 50, 75 and 90 m from the forest edge, with 0 m denoting the first stems of the Norway spruce stand behind the few oak trees at the edge. The spruce stand was very dense; therefore, we did not expect a legacy directly from the oak belt to be pronounced beyond the first (0 m) measuring station, but there was oak regeneration present throughout the forest and therefore some potential for oak roots to be present together with spruce roots at all distances. Throughfall was sampled by three funnels per station placed 1 m above the ground. Soil solution from the mineral soil at 0.9 m depth was sampled by three suction cup lysimeters per station. Mineral soil C and N stocks were estimated by sampling the upper mineral soil in different layers (0–0.05, 0.05–0.15 and 0.15–0.30 m). Mineral soil was sampled in April 2006 at three points at each distance from the edge using a 5-cm soil corer. In July 2006, inorganic N concentrations (NO3− and NH4+) were measured in the top 0–5 cm of mineral soil from three transects between the two throughfall monitoring transects. Fresh soil was hand sorted to remove roots, and subsamples were extracted in 1 M KCl for inorganic N.

Root and mycelial samples

Roots and mycelia were sampled between the two transects from eight points at the same distances from the forest edge as the N status parameters (0, 10, 25, 50, 75 and 90 m from the forest edge). The distance between sampling points was 2 m, that is, 14 m between the outermost points. The distance of 2 m was chosen to reduce autocorrelation between samples (Lilleskov et al., 2004), but to still keep sampling points within the range of the two transects. On 9 May 2006, 2-cm soil cores were removed to a depth of 10 cm at each sampling point and in-growth mesh bags (Wallander et al., 2001) were inserted into the holes. Loose litter of the forest floor was gently removed before soil coring; thus, the cores spanned the FH layer and the top of the mineral soil. Mesh bags were made of nylon with a mesh size of 37 μm and were filled with 50 g of acid-washed coarse sand (size, 0.5–2 mm) and sealed with a plastic sealer. The bags were inserted into the soil such that the tops of the bags were visible when removing the litter layer of the forest floor. After 244 d (8 November 2006), the bags were retrieved. First, the bags were pulled out and then a similar sized soil sample (2 × 10 cm) was taken adjacent to the mesh bag hole to record the EcM root tip community. Mesh bags and soil/root samples were kept cool after sampling and stored at 4°C.

Hyphae were extracted from the sand by floating and decanting. For visual-based quantification of mycelial growth, each clump of mycelium was placed in water in a Petri dish and photographed (Eastman Kodak Company, Scientific Imaging
Systems, Rochester, NY, USA). Pictures were then analyzed with Kodak 1D v 3.6 Software, and the borders of each visual clump of mycelia were drawn using the region of interest (ROI) tool palette. From this, the total mycelial area in pixels can be calculated. After photographing, the mycelia were collected on filter membranes and freeze-dried.

The roots were retrieved from a series of 2-, 1- and 0.5-mm sieves. From each soil sample, healthy-looking mycorrhizal tips were detached from the root system and sorted into morphotypes based on macroscopic features, including color, size, branching pattern and surface texture. For each morphotype, the number of root tips was counted and two replicate root tips were selected for molecular identification and transferred to RLT lysis buffer included in the MagAttract 96 DNA Plant Kit (Qiagen, Hilden, Germany). The rest of the root tips were preserved in 50% ethanol and kept in reserve. All samples were processed within 2 wk after sampling.

In addition to the main sampling in 2006, further root samples were taken in 2008, but only at the distances 0, 50 and 90 m. Three 2-cm soil cores were taken adjacent to each other and pooled. Four such pooled samples were taken at each distance, with at least 2 m between each group of three adjacent samples. In the laboratory, roots were washed as described above and divided into three fractions: EcM root tips, finer roots < 2 mm in diameter and coarse roots > 2 mm in diameter. Each fraction was freeze–dried, ground to a powder and analyzed for C and N content using an isotope ratio mass spectrometer coupled to a Eurovector CN elemental analyzer.

Molecular identification of EcM fungal root tips and mycelia

DNA extraction from root tips, followed by amplification of the fungal internal transcribed spacer (ITS) region, was performed as described in Kjøller & Clemmensen (2009). Restriction fragment length polymorphism (RFLP) analysis of the PCR products, purification and sequencing were performed as described in Kjøller (2006). Sequence chromatograms were analyzed and aligned in BIOEDIT Sequence Alignment Editor Version 6 (Hall, 1999) with sequences of best BLAST matches, considering both the identity and coverage of the matches. The alignments were used to identify sequence types, hereafter referred to as species. There were only relatively few species (see the Results section), and so the species designation proved to be straightforward. Sequences have been deposited in the sequence databank EMBL with accession no: FR877510–FR877529 (see Supporting Information Table S1). Species were also grouped into exploration types based on the definitions made by Agerer (2001) and Agerer & Rambold (2004–2011). In brief, EcM fungi can be grouped into contact, short-distance, medium-distance and long-distance exploration types based on the presence and amount of emanating hyphae and rhizomorphs. In cases in which PCR success from individual samples was poor, PCR products contained double or many bands or RFLP of two replicate root samples showed contrasts, new root tips were taken from the ethanol stock and run through the process as described above. If a specific morphotype still did not result in any PCR amplification or readable sequences, the morphotype was removed from the dataset. In cases in which replicate root tips still produced contrasting RFLP/sequence types, the root tip count of this morphotype was split between the two sequence types. The number of root tips allocated to each sequence type was based on an analysis of at least eight replicate root tips. To complement the root tip community profiling, the fungal community of the mycelia harvested from the in-growth bags was also analyzed. The aim was to identify the dominant types in the mesh bags, but not to exhaustively sample the total fungal diversity colonizing the mesh bags. DNA extraction, PCR, cloning and sequencing of the freeze-dried mycelia were performed as described in Kjøller (2006). From each mesh bag, between 12 and 22 clones were analyzed. In total, 402 cloned sequences were produced from the mycelia extracted. As there were few mycelia produced at 0–25 m, most cloning effort was performed for samples from 50 to 90 m.

In addition to the mycelium area (pixels, see Root and mycelial samples section), mycelial production was also determined as the chitin content left in the pellet after the DNA extraction. Previously, the chitin assay has been applied to pellets from enzyme extractions (Hepper et al., 1988; Kjøller & Rosendahl, 1996). The chitin analysis was based on the colorimetric method for glucosamine (Hepper, 1977). Briefly, chitin is depolymerized and deacetylated in concentrated KOH, producing chitosan. Thereafter, chitosan is deaminated with HNO$_3$ resulting in an aldehyde. The aldehyde reacts with 3-methyl-2-benzothiazolonhydrazine (MBTH) and FeCl$_3$ producing a blue color read at 650 nm.

Data analysis

Species accumulation curves, which relate the number of detected species to the number of root tips, were calculated using EstimateS (http://viceroy.eeb.uconn.edu/EstimateS). The influence of distance to the forest edge on the N fluxes and pools, the total number of root tips, mycelial production, and the total and relative abundances of the four most abundant species was analyzed by simple linear regression analysis. The relative abundance of a species is the percentage of root tips assigned to that species as a function of the total number of root tips at that distance from the forest edge. Analyses were run using the SAS Enterprise Guide 4.1 interface to the SAS 9.1 package (SAS Institute Inc., 2003). The linear correlations (Pearson’s r) between the environmental variables and the log-transformed total number of root tips, mycelial production, species richness, percentage EcM PCR clones from mycelia and relative abundance of species were also calculated using the SAS Enterprise Guide.

Possible similarities between the fungal communities at the six distances from the forest edge were analyzed by Bray–Curtis ordination using PC-ORD5 software which simultaneously calculates the correlation with environmental parameters (McCune & Grace, 2002). Program parameters in PC-ORD were set to the Sørensen index as the distance measure and subjective selection of 0 and 90 m as the endpoints. Furthermore, runs were performed with variance regression for endpoint selection, which produced similar results to that above. The use of absolute or
relative abundances did not change the overall pattern of the results or the correlations with N status parameters.

Results

Nitrogen gradient variables

Throughfall N and soil solution NO$_3$-N increased significantly towards the forest edge, demonstrating the presence of the expected stand-scale N gradient (Table 1). The same trend was observed for foliar litterfall N, total topsoil N content and topsoil extractable NO$_3$-N. Fine root N concentrations decreased significantly with distance from the forest edge (Table 1). In particular, the change in soil solution nitrate concentration from 25 mg NO$_3$-N L$^{-1}$ at the edge to 0.5 mg NO$_3$-N L$^{-1}$ in the interior forest (90 m) illustrates the existence of an N enrichment gradient from N in excess amounts close to the edge to almost complete N retention inside the forest. Thus, we used distance from the edge as a proxy for N input and N enrichment in our further analysis of mycorrhizal responses. The remaining soil and plant N parameters showed no significant gradient response along the edge gradient, although the lowest values were found inside the forest at 90 m (Table 1).

Root tip numbers

In total, 4157 root tips were sampled and split into morphotypes. Five hundred and twenty-four root tips belonged to morphotypes which proved impossible to amplify or sequence, and were removed from the dataset. Two hundred and twenty-six root tips generated sequences from fungi judged not to form EcM symbioses and these, too, were removed before further analysis. Sequence accession numbers and root tip frequencies are given in Table S2. The final dataset thus included 3389 EcM root tips (Table 2). The number of EcM root tips decreased significantly towards the forest edge ($P = 0.01$, linear regression analysis).

Five-fold fewer root tips were found at 0 m than at 90 m (Table 2, Fig. 1).

Mycelial production

Mycelial production in in-growth mesh bags responded significantly to the N gradient ($P = 0.04$, linear regression analysis), as did the root tips. Mycelia were almost absent at 0–25 m, but abundant from 50 to 90 m (Fig. 1). Despite the apparent drop in mycelial production at 90 m, there were no significant differences between the chitin contents between 50 or 75 m and 90 m (two-sample t-tests). The high values at 50 and 75 m were the result of a few highly productive mesh bags (data not shown). The two methods for estimating mycelial production were highly correlated (Fig. S1).

Fungal root tip community composition

From the combined RFLP and sequence analysis of the root tips, 16 EcM species could be identified (Table 2). The species richness decreased from 10 at 90 m to four at the edge. This decrease was not just attributable to the smaller number of root tips sampled at the edge, as indicated by the asymptotic nature of the species sampling effort curves (Fig. 2). This analysis grouped the species richness according to three distances: 50 and 90 m showed the highest diversity, 0 m the least and 10, 25 and 75 m showed intermediate species richness. The most abundant species was Tylospora fibrillosa, followed by Cenococcum geophilum, Lactarius quietus and Tylospora asterophora (Table 2). These four species colonized 85% of the EcM root tips in the forest stand. Over 90% of the root tips were colonized by species belonging to Atheliales (44%), C. geophilum (23%) or Lactarius spp. (24%).

Of the four most dominant species, T. fibrillosa increased significantly, whereas L. quietus decreased in abundance, with distance from the forest edge (Table 2). Tylospora asterophora and C. geophilum did not change significantly across the gradient.

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Table 1 Nitrogen variables and pH (mean ± SE) at distances from the spruce forest edge at Thyregod (K. Hansen, unpublished; IVL, Stockholm, Sweden)

<table>
<thead>
<tr>
<th>Nitrogen variable</th>
<th>Distance from forest edge</th>
<th>0 m</th>
<th>10 m</th>
<th>25 m</th>
<th>50 m</th>
<th>75 m</th>
<th>90 m</th>
<th>P values $^1$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Throughfall N (kg ha$^{-1}$ yr$^{-1})^2$</td>
<td>42.8</td>
<td>35.5</td>
<td>38.2</td>
<td>36.2</td>
<td>35.5</td>
<td>27.1</td>
<td>0.05</td>
<td></td>
</tr>
<tr>
<td>Soil solution NO$_3$-N (mg L$^{-1})^2$</td>
<td>25</td>
<td>22</td>
<td>10</td>
<td>10</td>
<td>14</td>
<td>0.5</td>
<td>0.05</td>
<td></td>
</tr>
<tr>
<td>Total N 0–5 cm (kg ha$^{-1})^2$</td>
<td>1087</td>
<td>1125</td>
<td>1128</td>
<td>896</td>
<td>914</td>
<td>1005</td>
<td>0.12</td>
<td></td>
</tr>
<tr>
<td>Soil C : N 0–5 cm</td>
<td>16.8</td>
<td>18.9</td>
<td>21.0</td>
<td>19.6</td>
<td>19.1</td>
<td>20.1</td>
<td>0.35</td>
<td></td>
</tr>
<tr>
<td>Foliar litterfall N (mg g$^{-1}$)</td>
<td>2.32</td>
<td>2.04</td>
<td>1.93</td>
<td>1.83</td>
<td>2.01</td>
<td>1.77</td>
<td>0.10</td>
<td></td>
</tr>
<tr>
<td>NH$_4$-N (µg N g$^{-1}$ soil)$^3$</td>
<td>22 ± 8</td>
<td>37 ± 10</td>
<td>128 ± 97</td>
<td>37 ± 8</td>
<td>25 ± 14</td>
<td>12 ± 4</td>
<td>0.53</td>
<td></td>
</tr>
<tr>
<td>NO$_3$-N (µg N g$^{-1}$ soil)$^3$</td>
<td>14 ± 5</td>
<td>23 ± 9</td>
<td>29 ± 16</td>
<td>20 ± 7</td>
<td>11 ± 5</td>
<td>7 ± 4</td>
<td>0.17</td>
<td></td>
</tr>
<tr>
<td>Fine root (&lt; 2 mm) N concentration (% dry weight)$^4$</td>
<td>1.9 ± 0.2</td>
<td>–</td>
<td>–</td>
<td>1.5 ± 0.05</td>
<td>–</td>
<td>1.3 ± 0.05</td>
<td>0.01</td>
<td></td>
</tr>
<tr>
<td>Coarse root (&gt; 2 mm) N concentration (% dry weight)$^4$</td>
<td>1.3 ± 0.1</td>
<td>–</td>
<td>–</td>
<td>1.1 ± 0.1</td>
<td>–</td>
<td>0.7 ± 0.05</td>
<td>0.17</td>
<td></td>
</tr>
<tr>
<td>pH (CaCl$_2$) 0–5 cm$^3$</td>
<td>3.1</td>
<td>3.0</td>
<td>2.8</td>
<td>2.9</td>
<td>3.0</td>
<td>2.9</td>
<td>0.45</td>
<td></td>
</tr>
</tbody>
</table>

$^1$Distance effect based on simple linear regression.

$^2$Mean between the two transects (see the Materials and Methods section).

$^3$Inorganic N was measured in the top 0–5 cm of mineral soil.

$^4$Data from root samples taken in 2008, $n = 4$ (only roots from three distances were taken).
Cenococcum geophilum seemed to be less abundant at 0 and 10 m relative to 25–90 m, but the relationship was nonlinear and rather asymptotic.

In addition to the analysis of the four dominant species, similar comparisons were made for species compiled into mycelial exploration types (cf. Agerer, 2001) (Fig. 3). The proportions of root tips belonging to contact and short-distance exploration types (Table 2) were both significantly related to distance from the forest edge ($P = 0.0004$ and $P = 0.0005$, respectively), but contact types decreased, whereas short-distance exploration types increased, with distance to the edge (Fig. 3).

The (Bray–Curtis) ordination clearly segregated the fungal community along axis 1 according to distance to the edge (Fig. 4). The ordination indicated how communities along axis 1 correlated with N throughfall, soil solution NO$_3$-N, foliar N, topsoil total N and topsoil NO$_3$-N in one direction, and soil C : N ratio in the opposite direction (Fig. 4).

In the direct correlation analyses, distance from the edge, throughfall N, total topsoil N and C : N ratio, soil solution NO$_3$-N and foliar litterfall N were the most explanatory variables for the EcM changes in species or species groups seen across the gradient (Table 3).

### Table 2 Ectomycorrhizal root tips of each species expressed as a percentage of the number of tips per distance; total numbers of root tips per species and per distance class are also shown

<table>
<thead>
<tr>
<th>Species</th>
<th>Exploration type</th>
<th>Distance from forest edge</th>
<th>Total no. root tips</th>
<th>Number of species</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>0 m</td>
<td>10 m</td>
<td>25 m</td>
</tr>
<tr>
<td>Tylospora fibrillosa</td>
<td>Short</td>
<td>–</td>
<td>1.9</td>
<td>16.6</td>
</tr>
<tr>
<td>Cenococcum geophilum</td>
<td>Short</td>
<td>8.7</td>
<td>–</td>
<td>23.9</td>
</tr>
<tr>
<td>Lactarius quietus</td>
<td>Contact</td>
<td>56.8</td>
<td>55.2</td>
<td>41.1</td>
</tr>
<tr>
<td>Tylospora asterophora</td>
<td>Short</td>
<td>33.3</td>
<td>10.2</td>
<td>5.4</td>
</tr>
<tr>
<td>Meliniomyces bicolor</td>
<td>Short</td>
<td>1.1</td>
<td>10.2</td>
<td>7.5</td>
</tr>
<tr>
<td>Lactarius necator</td>
<td>Contact</td>
<td>–</td>
<td>–</td>
<td>3.6</td>
</tr>
<tr>
<td>Meliniomyces variabilis</td>
<td>Short</td>
<td>–</td>
<td>19.0</td>
<td>–</td>
</tr>
<tr>
<td>Amphinema sp. 1</td>
<td>Medium</td>
<td>–</td>
<td>3.2</td>
<td>1.9</td>
</tr>
<tr>
<td>Xerocomus badius</td>
<td>Long</td>
<td>–</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>Piloderma sp. 1</td>
<td>Medium</td>
<td>–</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>Lactarius rufus</td>
<td>Contact</td>
<td>–</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>Tomentella sp. 1</td>
<td>Short</td>
<td>–</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>Paxillus involutus</td>
<td>Long</td>
<td>–</td>
<td>0.2</td>
<td>–</td>
</tr>
<tr>
<td>Tylophilus felleus</td>
<td>Long</td>
<td>–</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>Cortinarius biformis</td>
<td>Medium</td>
<td>–</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>Tomentella stuposa</td>
<td>Contact</td>
<td>–</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>Total no. root tips</td>
<td>183</td>
<td>431</td>
<td>535</td>
<td>522</td>
</tr>
<tr>
<td>Number of species</td>
<td>4</td>
<td>7</td>
<td>7</td>
<td>10</td>
</tr>
</tbody>
</table>

1See Supporting Information Table S1 for sequence accession numbers.
2Exploration types cf. Agerer (2001) and personal communication with Dr L. Tedersoo, University of Tartu, Estonia.
3Distance effect based on simple linear regression.

**Fig. 1** Ectomycorrhizal (EcM) root tips (open circles) and mycelial production (closed circles) across the nitrogen (N) deposition gradient in a Norway spruce (*Picea abies*) forest at Thyregod. Bars indicate standard error of the mean. Mycelial production was measured as glucosamine content left in the pellets after DNA extraction of the mycelia from mesh bags.

**Fig. 2** Calculated expected number of species as a function of the ectomycorrhizal (EcM) root tips analyzed for each of the six distances from the forest edge.
Fungal mycelial community composition

Few EcM clones were found from the mesh bags at 0–25 m from the forest edge, coinciding with the low abundance of mycelia found in general (Table 4 and Fig. 1). By contrast, most EcM clones were found at 90 m, whereas the distances of 50 and 75 m fell in between these two extremes. Only six EcM species were identified from the sampled mycelia, all of which were also present as root tips within the community (Table 4). *Tylotus fibrillosa* and *T. asterophora*, two of the dominant root tip types, were also abundant as mycelia. By contrast, two of the low-abundance root tip types, *Paxillus involutus* and *Amphinema* sp. 1, were abundant as mycelial clones. Because of the relatively few and unbalanced data, no attempts at statistical analysis were pursued for the mycelial clone data. None of the contact exploration types (*Lactarius* and *Meliniomyces* spp.) were found among the mycelial clones.

**Discussion**

This study demonstrates, for the first time, concurrent changes in EcM community composition, mycelial production and root tip number along an N deposition gradient in a spruce forest edge. Previously, such effects have been reported in studies measuring only one or two of the above-mentioned mycorrhizal parameters (Wallenda & Kottke, 1998; Lilleshov et al., 2002a,b; Nilsson & Wallander, 2003; Nilsson et al., 2005; Cox et al., 2010). Nevertheless, the present study is in agreement with previous studies. Mycelial reductions were observed with increasing N availability from deposition or fertilization (Wallander & Nylund, 1992; Nilsson & Wallander, 2003; Nilsson et al., 2005, 2007, 2012), and this was also the case for root tip number (Wallenda & Kottke, 1998). Furthermore, community changes and lower species richness seem to be general with increasing N loads (Lilleskov et al., 2001, 2002a,b; Avis et al., 2003; Cox et al., 2010).

The mycelial response to the increased N deposition near the forest edge was far greater than the accompanying decrease in root tips (Fig. 1). Therefore, the decrease in mycelia produced must be explained, at least partially, by other factors. Changes in community composition with inherently different mycelial production may be one explanation for the low mycelial production up to 25 m from the edge. The observed increase in contact exploration types at the expense of short-distance exploration types towards the forest edge supports this hypothesis (Fig. 3). Alternatively, either the N status of the soil or the host trees regulate the mycelial production. Accumulating evidence indicates that the plant N status is a key controller of below-ground C allocation (Lilleskov et al., 2008; Högborg et al., 2010) and thus the availability of C for investment in the external mycelial network. In theory, proximal variables, such as available soil N for fungal uptake or below-ground C allocated to the fungi, should predict mycorrhizal responses better than more distal variables, such as N deposition (Lilleskov & Parrent, 2007). The production of mycelia seemed to decrease at 90 m in comparison with 50 and 75 m. Although nonsignificant, it is intriguing that a lower mycelial production was found when, at the same time, no leaching was present at 90 m. One explanation could be that the species dominating the community at 90 m had a more effective N uptake capacity than those dominating at the other distances. This emphasizes the need for more information on the performance of individual species in order to tie together changes in community composition and function. However, the mesh bags may not accurately record the true EcM mycelial abundance in the soil, as discussed further below.

Six of the nine measured N parameters correlated with one to seven of the mycorrhizal parameters (Table 4). Throughfall N and soil solution NO$_3$-N correlated with both root tip number and the two richness parameters, and the latter correlated with...
most mycorrhizal parameters. These single correlation analyses are summarized in the ordination analysis in which the same six N parameters correlate with the community data (Fig. 4). Previously, N deposition, soil mineral N, foliar N and root N have been shown to be good predictors of mycorrhizal changes (Lilleskov et al., 2001, 2002a,b, 2008; Toljander et al., 2012). Therefore, other studies found no such responses of *Tylospora* spp. to N availability (Jonsson et al., 2000; Lilleskov et al., 2002a,b). Similarly, contradictory results were reported for *C. geophilum*, which often dominates below-ground EcM communities, including those of spruce forests. Responses to N availability range from an increase (Fransson et al., 2001) to no response (Avis et al., 2003) to indications of preference for low-N sites (Lilleskov et al., 2002a,b; Toljander et al., 2006), the latter being in accordance with this study. An increase in specific *Lactarius* or *Russula* species with increasing N availability is probably the most common species response to N reported in the literature (Lilleskov et al., 2002a,b; Avis et al., 2003; Parrent et al., 2006; Cox et al., 2010). The negative correlation between *L. quietus* and distance to the forest edge therefore corroborates these earlier findings. As *L. quietus* may be associated with roots from occasional oak trees within the spruce stand, this conclusion must be taken with great caution. Unfortunately, it was not possible to amplify plant DNA from the root tips stored in ethanol in spite of repeated efforts using several different primer systems. The identity of the host in the samples identified as *L. quietus* may remain unknown. The oak regeneration was scattered throughout the gradient, and so we may have had mixed spruce and oak roots in our samples. As oak, like spruce, may host all exploration types, we regard it as probable that the oak-associated community would change in parallel with the spruce-associated community. The correlations between the changes in exploration types, mycelial production and N leaching should, however, still hold true even if we have both spruce and oak roots in our samples. The other dominating fungi are all well-known spruce associates and are often among the most frequent in spruce forests (Jonsson et al., 2000; Fransson et al., 2001; Peter et al., 2001; Toljander et al., 2006), but several species, including the two dominants *T. fibrillosa* and *C. geophilum*, are also known from oak.

As exemplified by *C. geophilum*, there are sometimes contradictory results between studies with regard to the response of single species to N availability. The problem may be caused by differences in the quantitative and qualitative N availability.
between forest and soil types. This underlines the importance of recording appropriate environmental data in ways that make these numbers available together with the species abundance data for cross-study meta-comparisons (Lilleskov & Parrent, 2007). In addition, the full dose–response to N availability of each species is rarely known and, hypothetically, a species may increase in one study and decrease in another. The use of multi-level experiments or replicated gradient designs is needed to address this, as discussed further by Lilleskov & Parrent (2007) and Cox et al. (2010). Furthermore, Toljander et al. (2006) discussed the problem in using short gradients for community studies, where samples within a certain distance may correlate with each other.

We tried to reduce the impact of re-sampling individual fungal mycelia between cores by sampling with 2-m intervals (Lilleskov et al., 2004). Nevertheless, stand replication, as used by Cox et al. (2010), is needed to better unravel the environmental drivers for the EcM community composition observed.

The species recorded from the cloned mycelia were a subset of the root tip dataset. Only a few clones were analyzed and the mycelial production from 0 to 25 m from the forest edge was low. In addition, the mycelial community within the mesh bags may not accurately mirror the actual soil mycelial community. In particular, for example, Cortinarius spp. do not tend to grow into mesh bags even though these species produce abundant external mycelia (Kjøller, 2006). If the mycelial community data were to be analyzed more thoroughly, more clones should have been selected from each mesh bag or, ideally, 454 sequencing technology should have been applied (Wallander et al., 2010).

However, this community is strikingly species poor compared with other EcM communities; therefore, less cloning or generation of 454 reads is actually needed to exhaustively sample the species richness. The inclusion of more clones or 454 reads would probably not have changed the percentages of EcM clones obtained along the gradient. An interesting result was that P. involutus, which was only found at low abundance (on root tips) in two soil cores (Table S2), was detected in 16 mesh bags from the entire sampled area (data not shown). Paxillus involutus is a long-distance exploration type within the Boletales, and a similar skewed relation between root tips and mycelia has been seen previously for other Boletales species (Kjøller, 2006). The higher frequency of non-EcM species from the clones of the present study, in comparison with a previous study (Kjøller, 2006), may be explained by the extraction method used. In the former study, mycelia were collected after wet sieving with forces from the edge of the sieve, whereas, in the current study, mycelia were captured on 1.2-μm filter membranes. The latter method also captures some organic particles that probably will add to the subsequent detection of nonmycorrhizal clones. At the high N deposition of the forest edge, the high frequency of non-EcM species probably reflects the increase in saprotrophic fungi at the expense of EcM fungi.

In conclusion, this study has documented a large negative impact of N deposition on EcM biodiversity and biomass in soil and on roots. In particular, the almost entire loss of external mycelia under high-N conditions should be of concern. The supply of nutrients other than N to the tree may be impaired and, at the same time, N retention and uptake by mycelia in the topsoil are reduced. A lower abundance of N-retaining mycorrhizal mycelia under high-N deposition may thus further exacerbate the risk of N losses from forest ecosystems by leaching (Nilsson et al., 2012). Moreover, the mycorrhizal and mycelial changes, via altered carbohydrate flux from above ground, must feed back on important processes, such as soil C sequestration and food web structure. The response of EcM fungi to N should therefore be incorporated into process-based soil C models that aim to incorporate N deposition effects on C dynamics in forest ecosystems.

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Supporting Information

Additional supporting information may be found in the online version of this article.

Fig. S1 Correlation between the two measures of mycelial production.

Table S1 Number of root tips assigned to sequence types representing ectomycorrhizal species, fungal sequence types representing species with unknown tropic status and the number of nonamplifiable root tips; the table also includes EMBL accession numbers for sequence types.

Table S2 Number of ectomycorrhizal root tips in the 48 2 × 10-cm2 soil cores sampled

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