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Chapter 4

Long-term increase in snow depth leads to compositional changes in arctic ectomycorrhizal fungal communities

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FUNGI IN THE GREENING ARCTIC
Abstract

Arctic ecological processes are mainly regulated by air and soil temperature, snow cover distribution and persistence. Recently, various climate-induced changes have been observed in arctic tundra ecosystems, e.g. shrub expansion, resulting in reduction in albedo and greater C fixation in aboveground vegetation as well as increased rates of soil C mobilization by microbes. Importantly, the net effects of these shifts are unknown, in part because our understanding of belowground processes is limited. Here, we focus on the effects of increased snow depth, and as a consequence, increased winter soil temperature on ectomycorrhizal (ECM) fungal communities in dry and moist tundra. We analyzed deep DNA sequence data from soil samples taken at a long-term snow fence experiment in Northern Alaska. Our results indicate that, in contrast to previously observed responses of plants to increased snow depth at the same experimental site, the ECM fungal community of the dry tundra was more affected by deeper snow than the moist tundra community. In the dry tundra, both community richness and composition were significantly altered while in the moist tundra, only community composition changed significantly while richness did not. We observed a decrease in richness of *Tomentella*, *Inocybe* and other taxa adapted to scavenge the soil for labile N forms. On the other hand, richness of *Cortinarius*, and species with the ability to scavenge the soil for recalcitrant N forms, did not change. We further link ECM traits with C soil pools. If future warmer atmospheric conditions lead to greater winter snow fall, changes in the ECM fungal functional ecology will likely influence C emissions and C fixation through altering N plant availability, fungal biomass and soil-plant C-N dynamics, ultimately determining important future interactions between the tundra biosphere and atmosphere.
**Introduction**

Artic ecosystems are beginning to exhibit significant shifts in ecosystem structure and function induced by changes in climatic conditions (Tape *et al*., 2012; Ellmendorff *et al*., 2012). Despite interannual and regional variability, global mean surface temperature have consistently increased since the late 19th century (Collins *et al*., 2013). In the Arctic, temperatures have risen between 0.06 to 0.1 °C per year, while the global average increase has been ca. 0.017 °C per year during the past 30 years (Comiso & Hall, 2014). These temperature increases have already had major consequences, including accelerated summer ice loss, extended periods of open water in the Arctic Ocean and delayed autumn freeze up (Stroeve *et al*., 2014). At the same time, precipitation in the Arctic has increased (greatly exceeding the global average increase), especially during the cold season, where most precipitation falls as snow (Kattsov & Walsh, 2000; Screen & Simmonds, 2012). Additionally, state of the art models predict further increases, possibly by more than 50% of the current precipitation, leading to thicker snow cover (Colins *et al*., 2013; Bintanja & Selten, 2014). Deeper snow would have a suite of consequences for tundra ecosystems. These include protection from the abrasive wind (Liston *et al*., 2002; Sturm *et al*., 2005; Blok *et al*., 2015), warmer winter soil temperatures and increased soil moisture with subsequent effects on thaw depth and C storage (Natali *et al*., 2012; 2014), N turnover (Schimel *et al*., 2004; DeMarco *et al*., 2011), plant phenology and mineral nutrition (Borner *et al*., 2008; Leffler & Welker, 2013; Pattison & Welker 2014), vegetation composition (Wahren *et al*., 2005; Welker *et al*., 2005) and soil microbial respiration (Aanderud *et al*., 2012; Natali *et al*., 2014). However, with the exception of Buckeridge & Grogan (2008) that compared bacterial and fungal biomass growth responses, how arctic soil fungi communities may respond to changes in winter snow depth conditions is still largely unknown.

Microbial activity in the Arctic has been shown to increase due to higher winter soil temperatures inducing changes in the nitrogen (N) cycle dynamics, particularly in moist tussock tundra and less so in dry heath tundra in Arctic Alaska (Schimel *et al*., 2004; DeMarco *et al*., 2011; Natali *et al*., 2014; Pattison & Welker, 2014). In the Arctic, fungi are considered to constitute the bulk of soil microorganisms biomass (Callaghan *et al*., 2005) and Hobbie & Hobbie (2006) estimated that up to 86% of the N obtained by tundra plants is via mycorrhizal fungi. In exchange, plants can allocate between 10 to 20% of their photosyntheate-derived C to their fungal partners (Harley, 1971; Hobbie, 2006), constituting an important pool of soil C. Additionally, these exchanges might be positively correlated, i.e., increased allocation of plant C to the mycorrhizal partner might lead to increased uptake of N from the soil pool and subsequent delivery to the plant host (Talbot & Treseder, 2010). The limiting step in soil N cycling is the breakdown of macromolecular organic compounds, particularly the depolymerization of proteins (Schimel & Bennet, 2004; Jones & Kielland, 2012) that has been correlated with fungal
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biomass in high-latitude ecosystems (Wild et al., 2013), and is particularly attributed to ectomycorrhizal (ECM) and ericoid mycorrhizal fungi (Read & Perez-Moreno, 2003). Recently, several studies reported major changes in the arctic fungal mycorrhizal communities in response to summer warming (Deslippe et al., 2011; Geml et al., 2015; Morgado et al., 2015; Semenova et al., 2015), with the fungal community of moist tussock tundra typically showing more pronounced response than the dry heath tundra, including potential shifts in functional traits and the subsequent ecosystem functional processes. However, possible effects of increased winter soil temperatures on the richness and compositional structure of soil fungal communities have not yet been investigated.

Tundra plant community responses to increased winter snow depth include a combination of shifts in community composition as well as increases in net plant productivity (Borner et al., 2008; Natali et al., 2014) and plant N tissue concentrations (Leffler & Welker, 2013). At the community level, the general trends are increases in shrub coverage and litter layer, decrease in lichens, bryophytes, and in leaf C:N ratio (Welker et al., 2005; Wipf & Rixen, 2010; Pattison & Welker, 2014). Wahren et al. (2005) and Mercado-Díaz (2011) reported (from the same experimental plots that we used in our study) an increased coverage of several species of deciduous and evergreen shrubs, and a sedge species. Although most of these plants are highly dependent on root-associated fungi, especially ECM fungi, to acquire soil nutrients, how soil fungi community changes in response to deeper snow remains uncertain. Here we focus on ECM fungal community responses to long-term increased snow depth and the associated warming soil temperatures across the dry heath and moist acidic tussock tundra.

ECM fungi represent the most taxonomically diverse fungal guild in the Arctic tundra (Gardes & Dahlberg, 1996; Geml et al., 2012; Timling & Taylor, 2012), and provide crucial roles in soil-root interaction, particularly in plant N uptake (Read et al., 2004; Ekblad et al., 2013) and in soil C dynamics (Clemmensen et al., 2013; Averill et al., 2014). Recently, an increasing amount of studies on fungal functional traits are amassing valuable insights into the potential in-depth role of the community structure in potential ecosystem functions (e.g. reviewed in Fernandez & Koide, 2014; Treseder & Lennon, 2015). For example, Hobbie & Agerer (2010), gathered evidences from δ^{15}N patterns and argued that ECM fungi have two main strategies for growth and nitrogen acquisition that match the extramatrical mycelium (EMM) characteristics of the ectomycorrhizae. ECM fungi with low abundance of EMM and hydrophilic mycorrhizae with contact, short-distance and medium-distance smooth hyphal exploration types (ETs) were argued to focus on uptake of labile nitrogen (N) forms, such as amino acids, ammonium and nitrate. Supporting this hypothesis, many taxa composing this group showed limited protein growth in culture conditions (Lilleskov et al., 2011). On the other hand, the ECM fungi with higher abundance of EMM with medium-distance fringe, medium-distance mat and long-distance ETs with hydrophobic rhizomorphs (or mycelial
cords), likely focus on widely dispersed and spatially concentrated soil resources requiring efficient long-distance translocation. Such investment in exploratory hyphae should not rely on labile substrates under low nutrient availability; therefore, this group of taxa would require hydrolytic capabilities in order to access non-labile N forms, such as proteins (Hobbie & Agerer, 2010). Supporting this hypothesis some studies pointed to increased exoenzyme activity in ECM fungi with abundant EMM (Tedersoo et al., 2012; Talbot et al., 2013). Another example of a fungal trait and a potential ecosystem function is the presence of melanin in hyphal cell walls, which was thoroughly discussed by Koide et al. (2014). Given these evidences it seems reasonable to use certain fungal traits as a response to environmental conditions which in turn might also induce changes in the ecosystem.

This research focuses on the effects of long-term increased snow depth on ECM basidiomycete communities. Based on the evidence previously stated, we hypothesize that: 1) ECM fungal community composition is strongly affected by increased snow depth, and that the response will be more pronounced on the moist tundra ECM community; and 2) changes in ECM fungal community composition will reflect altered patterns in vegetation, soil nutrient pools and moisture, induced by the increased snow depth. Therefore, we expect altered patterns in the ECM fungal community functional traits.

Material and Methods

Study site and experimental design

This International Tundra Experiment (ITEX) (Henry & Molau, 1997, Welker et al., 1997) study site is located on the northern foothills of the Brooks Range, at the Toolik Lake Field Station. The area lies in the Arctic tundra biome within the bioclimatic subzone E, which covers approximately 36% of the Arctic dry land surface (Walker et al., 2005). The mean air annual temperature is -7 °C and annual precipitation ranges between 200 mm and 400 mm with approximately 50% falling as snow. The average snow depth is 50 cm (DeMarco et al., 2011). The snow fence experiment was established in the summer of 1994 in moist tussock and dry tundra (Jones et al., 1998; Walker et al., 1999; Welker et al., 2000). The snow fences are 2.8 m high and 60 m long, inducing leeward drifts of ca. 60 m long (Walker et al., 1999; Pattison & Welker, 2014). Our sampling was focused on the intermediate zone near the center of the experimental setup, corresponding to ca. 1-1.5 m winter snow depth. Although the deeper snow slightly shortens the growing season by ca. 5-8 days, this does not affect plant phenology significantly (Borner et al., 2008). The average winter soil temperatures at 2 cm depth were -2.9 °C (±0.2) and -4.7 °C (±0.2) in the increased snow depth plots and in the control plots, respectively (Pattison & Welker, 2014).
The vegetation of the dry heath tundra is characterized by *Dryas octopetala*, *Salix polaris*, *Vaccinium* spp. and fruticose-lichens, while the moist tussock tundra is dominated by *Betula nana*, *Salix pulchra* and the sedge *Eriophorum vaginatum*. Detailed descriptions of the plant communities can be found in Walker *et al.* (1999) and Kade *et al.* (2005), and their detailed response to the altered snow depths in Walker *et al.* (1999), Wahren *et al.* (2005), Welker *et al.* (2005), Mercado-Díaz (2011), Pattison & Welker (2014).

We sampled soil at the end of July 2012 from two tundra types, the dry heath and moist tussock tundra experimental sites. In each tundra type, we sampled five plots/replicates with increased snow depth and five plots with ambient snow depth (“control plots”). Each replicate consisted of five soil cores of 2 cm diameter and 20 cm depth each. For each replicate the soil cores were thoroughly mixed and kept frozen until lyophilization. In total we sampled 100 soil cores across 20 plots of ca. 1 m² each.

**Molecular work and sequence quality control**

The DNA extraction, PCR protocol, Ion Torrent sequencing and data clean-up procedures were described in detail elsewhere (Geml *et al.*, 2014a). For each sample we carried out two independent DNA extractions, using ca. 1 ml of lyophilized soil and pooled them to optimize extraction homogenization. In the PCR we targeted the *ITS2* region of the nuclear ribosomal internal transcribed spacer that is currently accepted as the universal barcode marker for fungi (Schoch *et al.*, 2012). We used primers fITS7 (Ihrmark *et al.*, 2012) and ITS4 (White *et al.*, 1990). The ITS4 primer was labeled with sample-specific Multiplex Identification DNA-tags (MIDs). The amplicon library was sequenced using an Ion 318™ Chip by an Ion Torrent Personal Genome Machine (PGM; Life Technologies, Guilford, CT, U.S.A.) at Naturalis Biodiversity Center. For the initial clean-up of the raw sequence data we used the online platform Galaxy (https://main.g2.bx.psu.edu/root), in which the sequences were sorted according to samples. Primers and adapters were removed. We used a parallel version of MOTHUR v. 1.32.1 (Schloss *et al.*, 2009) for subsequent sequence analyses. Sequences shorter than 150 bp and longer than 400 bp were removed following Blaalid *et al.* (2013), Geml *et al.* (2014ab), Morgado *et al.* (2015) and Semenova *et al.* (2015), with the goal to increase phylogenetic identification potential and quality control while preserving data coverage. The quality-filtered sequences were normalized following Ghihring *et al.* (2012) by random subsampling so that each sample contained equal number of sequences. We then clustered the sequences into operational taxonomic units (OTUs) using OTUpipe (Edgar 2010) with the simultaneous removal of putatively chimeric sequences using *de novo* and reference-based filtering using the curated dataset of fungal *ITS* sequences of Nilsson *et al.* (2011), with the default settings. We used a 97% sequence similarity clustering threshold following many other fungal ecology studies (e.g. O’Brien *et al.*, 2005; Higgins
et al., 2007; Geml et al., 2008; Geml et al., 2009; Amend et al., 2010; Tedersoo et al., 2010; Geml et al., 2012; Kauserud et al., 2012; Brown et al., 2013; Blaalid et al., 2013; Geml et al., 2014b, Davey et al., 2015). Global singletons were discarded from further analysis. The reference database published by Kõljalg et al. (2013) was used to determine the taxonomic affinity of the OTUs using USEARCH v7 (Edgar, 2010). OTUs with less than 80% similarity to any identified fungal sequence were excluded from the final analysis due to unreliable classification, and/or uncertainty regarding their ecological role. A representative sequence of each OTU was deposited in GenBank under the accession numbers KP827673 - KP828017.

**ECM fungal database and EMM determination**

We followed the publication of Tedersoo & Smith (2013) to select the basidiomycete ECM OTUs. For most OTUs we used a ≥90% sequence similarity to determine genera. Because Sebacinales have a diverse ecology we selected ECM OTUs based on their supported phylogenetic placement (with ≥70% bootstrap and/or ≥0.95 posterior probability) among sequences of taxa that were morphologically confirmed as ECM published by Glen et al. (2002), Urban et al. (2003), Ryberg et al. (2009) and Tedersoo & Smith (2013).

To determine the EMM characteristics, we followed the work of Agerer (2006), Tedersoo & Smith (2013) and consulted the DEEMY database (http://deemy.de, accessed in November, 2014 - an information system for the characterization and determination of ectomycorrhizae). In the genus *Russula*, if no EMM information was available for the species of interest, we assumed the EMM characteristics based on the closest species with known characteristics. To determine the closest species, we followed the phylogenetic study by Miller & Buyck (2002). Similarly, for OTUs of the genus *Hebeloma*, we followed the phylogenetic study by Boyle et al. (2006).

**Statistical analysis**

For each replicate, we calculated rarefied OTU accumulation curves using the R package Vegan (Oksanen et al., 2012) and determined the Good‘s coverage (complement of the ratio between the number of local singletons and the total sequence counts). OTU presence was defined as more than 4 sequences on a per sample basis following the suggestion of Lindahl et al. (2013) to minimize false positives (e.g. OTUs that are common in one sample, but may be low-abundant contaminants in others). Due to uncertainty of sequence abundance as indicator of species abundance in the samples (Amend et al., 2010), we carried out analyses with two types of data transformations. First, we transformed the data into presence-absence matrix. Secondly, we used square-root transformed sequence abundance to moderate the influence of OTUs with high sequence counts, while maintaining some approximation of template abundance that may
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reflect ecological significance. We used PC-Ord v. 5.32 (McCune & Grace, 2002) to run non-metric multidimensional scaling (NMDS) on a primary matrix of experimental plots by OTUs and a secondary matrix of plots by OTU richness per taxon, EMM characteristics and sequence counts. The dataset was subjected to 500 iterations per run using the Sørensen similarity (Bray-Curtis index) and a random starting number. We also calculated the Pearson’s correlation coefficient ($r$) values between relative OTU richness per taxon and axes 1 and 2. We tested whether fungal communities were statistically different across the treatments using a multi-response permutation procedure (MRPP) and determined any preferences of individual OTUs for either control or increased snow depth plots in dry and moist tundra using Indicator Species Analyses (Dufrêne & Legendre, 1997) as implemented in PC-Ord v. 5.32. We also tested for significant differences in OTU richness across the dry and moist tundra control and deeper snow plots, per taxa (genera) and EMM characteristics using Student’s $t$-test. Correlation coefficients were calculated as implemented in Microsoft Excel v. 2010 between the most OTU-rich genera and the hyphal exploration types (combined in two functional groups, I – contact, short-distance, medium-distance smooth, and II – medium-distance fringe and long-distance ETs) within plot type, across the dry and moist tundra for the presence-absence and sequence abundance datasets. The Venn diagram for the whole community and genera with higher OTU richness was also calculated, using the online version of the publication by Oliveros (2007).

**Results**

*Through the pipeline: from raw data to taxonomic diversity*

We obtained 3,960,925 sequences with a median length of 268 bp. After quality control and random subsampling we retained 1,161,160 sequences with a mean length and standard deviation of 255.1 ± 52.7 bp. Clustering the sequences at 97% similarity generated 7015 OTUs, excluding global singletons and putative chimeric OTUs, of which 459 ECM basidiomycete OTUs were retained for further analyses. Across all treatments, ECM fungi were represented by 23 genera classified in 7 orders (Table 4.1, Fig. 4.1). Overall, *Cortinarius* and *Tomentella* were the most OTU-rich genera, with 125 (ca. 27%) and 124 OTUs (ca. 27%), respectively, followed by *Inocybe* (79 OTUs, 17%) and *Russula* (40 OTUs, 9%), with the remaining genera having less than 5% of the OTUs per genus. The order Agaricales had by far the highest OTU richness (224 OTUs, ca. 49%), followed by Thelephorales (128 OTUs, ca. 28%), Russulales (57 OTUs, ca. 12%), Cantharellales (33 OTUs, ca. 7%), Sebacinales (11 OTUs, ca. 2%), Boletales (4 OTUs, ca. 1%) and Atheliales (2 OTUs, ca. 1%). The analysis with sequence abundance resulted in similar patterns as the OTU richness analysis (appendix S4.1). The recovered OTU richness was higher than in previous publications that used similar methods to investigate arctic ECM fungal communities, but genera diversity and patterns of genera richness
were in general agreement (Bjorbaekmo et al., 2010; Timling et al. 2012; Geml et al., 2012; Morgado et al., 2015). The asymptotic rarefaction curves (Fig. 4.2a) and estimated Good’s coverage (Fig. 4.2b) indicate that the deep sequencing allowed a very high OTU coverage and that most fungi in the samples were sequenced.

Figure 4.1. Total ECM fungal OTUs, classified by taxonomic and functional traits, comparing ambient snow with increased snow depth plots. The legend for each pair of graphics is organized by colors and in a clock-wise manner. Abbreviations are C: contact, S: short-distance, MDS: medium-distance smooth, MDF: medium-distance fringe, L: long-distance.

<table>
<thead>
<tr>
<th></th>
<th>Dry heath tundra</th>
<th>Moist acidic tussock tundra</th>
<th></th>
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</thead>
<tbody>
<tr>
<td></td>
<td>DC</td>
<td>DS</td>
<td>p</td>
</tr>
<tr>
<td><strong>Tomentella</strong></td>
<td>21 ± 13.55</td>
<td>3.4 ± 2.07</td>
<td>0.02*</td>
</tr>
<tr>
<td><strong>Cortinarius</strong></td>
<td>9 ± 4.12</td>
<td>9.4 ± 6.80</td>
<td>0.46</td>
</tr>
<tr>
<td><strong>Inocybe</strong></td>
<td>10.4 ± 5.32</td>
<td>5.2 ± 1.92</td>
<td>0.05*</td>
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<tr>
<td><strong>Russula</strong></td>
<td>1.8 ± 2.48</td>
<td>2.8 ± 3.03</td>
<td>0.25</td>
</tr>
<tr>
<td><strong>Alnicola</strong></td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td><strong>Amanita</strong></td>
<td>0 ± 0</td>
<td>0.2 ± 0.45</td>
<td>0.19</td>
</tr>
<tr>
<td><strong>Amphinema</strong></td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td><strong>Boletus</strong></td>
<td>0.6 ± 0.55</td>
<td>0 ± 0</td>
<td>0.04*</td>
</tr>
<tr>
<td><strong>Ceratobasidium</strong></td>
<td>1.6 ± 0.55</td>
<td>1.6 ± 0.89</td>
<td>0.50</td>
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<tr>
<td><strong>Clavicorona</strong></td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td><strong>Clavulina</strong></td>
<td>0.6 ± 1.34</td>
<td>0.4 ± 0.55</td>
<td>0.38</td>
</tr>
<tr>
<td><strong>Hebeloma</strong></td>
<td>1.6 ± 2.61</td>
<td>0.4 ± 0.89</td>
<td>0.19</td>
</tr>
<tr>
<td><strong>Hymenogaster</strong></td>
<td>-</td>
<td>-</td>
<td>-</td>
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<tr>
<td><strong>Laccaria</strong></td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td><strong>Lactarius</strong></td>
<td>0.2 ± 0.44</td>
<td>0.8 ± 0.84</td>
<td>0.10</td>
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<tr>
<td><strong>Leccinum</strong></td>
<td>0.6 ± 0.55</td>
<td>0.8 ± 0.45</td>
<td>0.27</td>
</tr>
<tr>
<td><strong>Membranomyces</strong></td>
<td>0.4 ± 0.55</td>
<td>0 ± 0</td>
<td>0.09</td>
</tr>
<tr>
<td><strong>Piloderma</strong></td>
<td>0.2 ± 0.45</td>
<td>0 ± 0</td>
<td>0.19</td>
</tr>
<tr>
<td><strong>Pseudotomentella</strong></td>
<td>0.8 ± 1.30</td>
<td>0.4 ± 0.55</td>
<td>0.28</td>
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<td><strong>Sebacina</strong></td>
<td>0.8 ± 1.79</td>
<td>0.4 ± 0.55</td>
<td>0.33</td>
</tr>
<tr>
<td><strong>Sistotrema</strong></td>
<td>1.4 ± 1.34</td>
<td>0.6 ± 1.34</td>
<td>0.19</td>
</tr>
<tr>
<td><strong>Tomentellipsoid</strong></td>
<td>0.2 ± 0.45</td>
<td>0 ± 0</td>
<td>0.19</td>
</tr>
<tr>
<td><strong>Tulasnella</strong></td>
<td>0.8 ± 0.84</td>
<td>0.2 ± 0.45</td>
<td>0.10</td>
</tr>
<tr>
<td><strong>C/ S/ MDS</strong></td>
<td>42.2 ± 19.82</td>
<td>15.6 ± 3.78</td>
<td>0.02*</td>
</tr>
<tr>
<td><strong>MDF/ L</strong></td>
<td>11.8 ± 4.82</td>
<td>11 ± 6.63</td>
<td>0.42</td>
</tr>
<tr>
<td><strong>Hi</strong></td>
<td>34.6 ± 20.73</td>
<td>12 ± 4.69</td>
<td>0.04*</td>
</tr>
<tr>
<td><strong>Ho</strong></td>
<td>16.4 ± 8.02</td>
<td>11.6 ± 7.37</td>
<td>0.18</td>
</tr>
<tr>
<td><strong>All OTUs</strong></td>
<td>54 ± 23.29</td>
<td>26.6 ± 8.02</td>
<td>0.03*</td>
</tr>
</tbody>
</table>
Overall results

The NMDS analysis of the square-root sequence abundance matrix resulted in a 2-dimensional solution with a final stress and instability of 0.1062 and < 0.00001, respectively. The results of the Monte Carlo test indicated that all two dimensional solutions using the real data were significantly better than occurrences by chance ($p < 0.01$). The coefficients of determination for the correlations between ordination distances and distances in the original n-dimensional space were axis 1: $r^2 = 0.599$; axis 2: $r^2 = 0.240$; total $r^2 = 0.839$; orthogonality = 88.3%. The NMDS ordination plot was orthogonally rotated by the treatment to visualize correlations between snow depth effect and fungal community composition in general (Fig. 4.3a). The MRPP analysis indicated a clear distinction between dry and moist ECM community composition ($p < 0.0000001$, $A = 0.14601$). The NMDS and MRPP analysis with the presence-absence matrix results were similar (appendix S4.2a). Across the ambient snow plots of both dry and moist tundra, *Tomentella* was the most OTU rich genus with 103 OTU (ca. 29%), followed by *Cortinarius* with 78 OTUs (ca. 22%), *Inocybe* with 66 OTUs (ca. 19%) and *Russula* with 28 OTUs (ca. 8%). All the other genera had less than 5% of the OTUs per taxa and combined solely represented ca. 22% of the OTUs. On the other hand, across the deeper snow plots, *Cortinarius* was the most OTU rich genus with 78 OTUs (ca. 36% of all OTUS), followed by *Tomentella* with 45 OTUs (ca. 20%), *Inocybe* with 28 OTUs (ca. 13%), *Russula* with 24 OTUs (11%). All the other genera had less than 5% of the OTUs per taxa (Fig. 4.1). Differences between the ambient and deeper snow plots were also evident at the order level. Agaricales and Russulales had an increased OTU richness in deeper snow areas, while Thelephorales and Cantharellales had a decrease. Globally, the majority of the OTUs were only present in the ambient snow plots - ca. 53%, while ca. 24% were solely found in the deeper snow plots and the remainder 23% present in both (data not shown). There was a significant decrease in OTU richness from the ambient to the deeper snow plots ($p = 0.0377$), with the control plots having on average 66.2 ± 24.5 OTUs, while the deep snow plots had 43.8 ± 28.4 OTUs (Table 4.1). Together the contact, short-distance and medium-distance smooth ET represented the most OTU rich
group in the control plots with an average of 46.1 ± 17.9 OTUs per plot, while the medium-distance fringe and long-distance ETs group had an average of 16.6 ± 7.09 OTUs per plot. Comparing with the OTU richness values of the deep snow plots, the first group had a significant decrease ($p = 0.0042$, 24.6 ± 14.05 average OTUs per plot); whilst the later did not change significantly ($p = 0.36$, 15.2 ± 12.71 average OTUs per plot) (appendix S4.3). The overall pattern of changes in functional traits were also depicted when comparing the unique OTUs in the ambient with the deeper snow plots (Fig. 4.1).

Figure 4.3. Non-metric multidimensional scaling (NMDS) ordination plots of basidiomycete ECM fungal communities from the ambient and increased snow depth plots based on OTU sequence square-root abundance in (a) the whole community (dry and moist tundra), (b) the moist tundra, (c) the dry tundra. Vectors with $|r| ≥ 0.5$ are represented on the ordination plot. Abbreviations are C: contact, S: short-distance, MDS: medium-distance smooth, MDF: medium-distance fringe, L: long-distance, Hi: hydrophilic, Seq counts: sequence reads.
**Dry heath tundra**

The NMDS analysis of the square-root sequence abundance matrix resulted in a 2-dimensional solution with a final stress of 0.0763 and a final instability < 0.00001. The results of the Monte Carlo test indicated that all two-dimensional solutions using the real data were significantly better than occurrences by chance ($p < 0.01$). The coefficients of determination for the correlations between ordination distances and distances in the original n-dimensional space were axis 1: $r^2 = 0.667$, axis 2: $r^2 = 0.183$, total $r^2 = 0.849$ and orthogonality = 77.8%. The NMDS ordination plot was orthogonally rotated by the treatment to visualize correlations between snow depth and fungal community composition in general, and the taxonomic groups and EMM characteristics in particular (Fig. 4.3c). The MRPP analysis indicated a clear distinction between control and deep snow ECM community composition ($p = 0.0039$, $A = 0.04940$). The NMDS and MRPP analysis of the presence-absence matrix results yielded similar conclusions (appendix S4.2c). The groups with the strongest negative correlation (Pearson’s correlations) with the increased snow depth were OTUs of the contact, short-distance and medium-distance smooth hyphal ET ($r = -0.950$), *Tomentella* ($r = -0.937$), OTUs with hydrophilic hyphae ($r = -0.935$), *Inocybe* ($r = -0.935$), sequence counts ($r = -0.912$), total OTU richness ($r = -0.896$), *Sebacina* ($r = -0.682$), *Tulasnella* ($r = -0.582$), *Sistotrema* ($r = -0.533$). None of the groups showed a strong positive correlation ($r > 0.5$) with the increased snow depth. The control plots had on average $54 \pm 23.29$ OTUs per plot, while the treatment plots had $26.6 \pm 8.02$ OTUs per plot. This difference was statistically significant ($p = 0.03$). *Tomentella* was the most OTU-rich genus in the control plots with $21 \pm 13.55$ OTUs per plot, followed by *Inocybe* with $10.4 \pm 5.3$. Interestingly, across the deep snow plots, both genera showed a significant decrease on the average OTUs per plot ($p = 0.022$ and 0.047, respectively). On the other hand, OTU richness in *Cortinarius* was nearly unaffected and this genus had the highest mean richness in the increased snow depth plots (Table 4.1). At the order level, there was an abrupt decrease in the proportion of Thelephorales OTUs, from 36% in the control plots to 17% in the deep snow plots, while most other orders had an increase in proportion between the ambient and increased snow depth plots, with Agaricales having 55% of all OTUs (across the deep snow plots) (Fig. 4.1).

Together the contact, short-distance and medium-distance smooth ETs represent by far the most OTU-rich functional group, with an average of $42.2 \pm 19.82$ OTUs per plot, while the long-distance and medium-distance fringed ETs solely had on average $11.8 \pm 4.82$ OTUs per plot, across the ambient snow conditions plots. Interestingly, in the deeper snow plots the first group had a significant decrease ($p = 0.02$) in OTU richness, while the latter group maintained similar OTU richness ($p = 0.42$). The vast majority of OTUs were only present in the control plots (60%), a smaller percentage was present in both the control and the increased snow depth plots (20%), and only a minority (18%) was strictly present in the deeper snow plots (Fig. 4.4). We observed this pattern for the
increased snow depth alters arctic fungal community composition

two most OTU rich genera of the control plots, i.e. *Tomentella* and *Inocybe*. In comparison the percentage of OTUs that are solely present in the control plots in *Cortinarius* and *Russula* is considerably lower (Fig. 4.4). Five (undetermined) *Tomentella* OTUs were indicators of the ambient snow plots, while no OTU was considered indicator of the deeper snow plots (appendix S4.4).

The correlation coefficient between the taxonomic groups and ETs revealed significant positive and negative correlations among specific groups (S7). Across the ambient snow depth plots, *Inocybe* OTU richness showed a significantly negative correlation with *Russula*, and a significantly positive correlation with *Tomentella*. Interestingly, across the increased snow depth plots, the negative correlations of OTU richness between the previously mention genera decreased sharply to non-significant values.

**Moist tussock tundra**

The NMDS analysis of the square-root sequence abundance matrix resulted in a 2-dimensional solution with a final stress and instability of 0.0759 and < 0.00001, respectively. The results of the Monte Carlo test indicated that all two dimensional solutions using the real data were significantly better than occurrences by chance ($p < 0.01$). The coefficients of determination for the correlations between ordination distances and distances in the original n-dimensional space were axis 1: $r^2 = 0.607$; axis 2: $r^2 = 0.265$; total $r^2 = 0.872$; orthogonality = 93.1%. The NMDS ordination plot was orthogonally rotated by the treatment to visualize correlations between snow depth effect and fungal community composition in general, and the taxonomic groups in particular (Fig. 4.3b). The MRPP analysis indicated a clear distinction between ambient and deeper snow ECM community

Figure 4.4. Percentage of unique and shared OTUs for all the community and most diverse taxa per treatment (ambient snow and increased snow depth) in the dry and moist tundra types. Abbreviations are DC: dry tundra ambient snow, DS: dry tundra increased snow depth, MC: moist tundra ambient snow, MS: moist tundra increased snow depth.
The NMDS and MRPP analysis of the presence-absence matrix yielded similar results (Appendix S4.2b). *Inocybe* and the group of OTUs with short-distance exploration type had a strong negative correlation with the increased snow depth plots, $r = -0.757$ and $-0.775$, respectively, as well as the OTUs with hydrophilic hyphae ($r = -0.540$) and *Lactarius* ($r = -0.536$). On the other hand, *Alnicola* and *Laccaria* OTU richness had the strongest positive correlation with the deeper snow plots with $r = 0.696$ and $r = 0.510$, respectively. The control plots had on average 72.4 ± 22.02 OTUs per plot, while the deep snow plots had 53 ± 31.01 OTUs per plot. Despite the considerable decrease, the difference was not statistically significant ($p = 0.15$). The most OTU rich genus in the ambient snow areas was *Tomentella* with 20.6 ± 7.4 OTUs per plot, followed by *Inocybe* with 10.4 ± 5.3. On the deeper snow areas, while *Tomentella* had only a marginally significant ($p=0.07$) decrease on the average OTUs per plot to 12.6 ± 8.33, *Inocybe* had a significant ($p=0.02$) decrease to 3.2 ± 1.92 OTUs per plot. On the other hand, *Cortinarius* was the genus with higher OTU richness in the deeper snow plots with 9.4 ± 6.8, and showed no significant changes ($p = 0.47$) on OTU richness compared with the ambient snow plots (Table 4.1). Interestingly, in the deeper snow plots, on the order ranking, Thelephorales increased the percentage of OTUs while all the remaining orders decreased (Fig. 4.1), mainly due to the decrease in *Inocybe*, *Russula* and *Lactarius* OTUs richness. Regarding EMM characteristics, in the control plots the contact, short-distance and medium-distance smooth had on average 50 ± 17.13 OTUs per plot, while the long-distance and medium-distance fringed had 22 ± 5 OTUs per plot. In the deeper snow plots the first group had a marginally significant decrease ($p = 0.07$) to 33.6 ± 15.08 OTUs per plot, while the latter group did not change significantly ($p = 0.38 - 19.4 ± 16.59$ OTUs per plot). Most OTUs (45%) were only present across the ambient snow areas, 25% were present in both the control and deeper snow plots and 30% were only present in the deeper snow plots (Fig. 4.4). *Tomentella* and *Russula* followed the overall OTU distribution pattern. Conversely, *Cortinarius* had a contrary pattern, with a higher percentage of OTUs solely recovered from the deep snow plots - 41%, 33% solely present on the control plots and 25% present in both the control and deeper snow areas. On the other hand, *Inocybe* had 63% of OTUs only recovered from the ambient snow plots, 23% recovered solely from the deep snow plots and only 13% were found in both the ambient and deep snow plots (Fig. 4.4). Seven unidentified *Tomentella* OTUs, *Cortinarius huronensis*, *C. cf. flos-paludis*, *Inocybe leiocephala*, *I. nitidiuscula*, *Russula renidens*, and *Lactarius torminosus* were indicator OTUs of the ambient snow areas. On the deeper snow plots, *Tomentella lapida*, one unidentified *Russula*, one unidentified *Laccaria*, one unidentified *Inocybe*, one unidentified *Cortinarius*, and three unidentified *Alnicola* OTUs were indicator OTUs (appendix S4.4).

The correlation coefficient between the taxonomic groups and ETs (appendix S4.5) revealed a significant positive correlation among two groups in the ambient snow plots: *Cortinarius - Russula*, and between the group of OTUs with contact, short-distance
and medium-distance smooth ET, and the group of OTUs with medium-distance fringe and long-distance ET. In the increased snow depth plots, the significant positive correlation, among these groups, was also observed, and further extended to the pairs *Tomentella* - *Cortinarius*, and (marginally significantly) for *Russula* - *Tomentella*. Interestingly none of the groups tested had a negative correlation either at the control or the deeper snow plots.

**Discussion**

The results presented here clearly show that long-term increase in snow depth alters ECM fungal community composition in moist tussock and dry heath tundra, with a considerable portion of OTUs not being resilient to the resulting changes in environmental conditions. These conclusions are based on the significant decrease in average OTU richness in the deeper snow plots and the considerably low percentage of OTUs shared between the two snow depths we studied. Recently it has been shown that ECM fungal community is also sensitive to summer warming with the communities of the moist tundra showing a more evident response altering community composition, trait patterns and OTU richness levels (Geml *et al*., 2015; Morgado *et al*., 2015).

The strongest response to increased snow depth was observed in the dry heath tundra, where besides community composition, there was also an overall significant decrease in richness of the ECM community in response to increased snow depth. The ECM fungal richness of the moist tundra also showed a decreasing trend but not in a significant manner. This was surprising, as we anticipated that ECM fungal community in the moist tussock tundra would have a stronger response to deeper snow, because the plant community and N dynamics had been reported to be more strongly affected by the increased snow depth in the moist tundra (Schimel *et al*., 2004; Wahren *et al*., 2005; Mercado-Díaz, 2011). Together with our previous results (Morgado *et al*., 2015), we argue that the dry tundra ECM fungal community likely is more sensitive to changes in snow depth than to summer warming, and that the ECM fungal community in the dry tundra may be more sensitive to increased winter snow depth than the plant community. Our correlation analysis of OTU richness and sequence abundance with the different snow depths showed different patterns among the tundra types. For example, in the moist tundra OTU richness and sequence abundance between *Cortinarius* and *Tomentella* showed a significant positive correlation in the increased snow depth plots, while no significant correlation was observed in the control plots. On the other hand, in the dry tundra no correlation was observed between these two groups in either snow depth. This suggests that in our study the interaction between OTU richness and potential species abundance likely are habitat-specific, and are in agreement with studies that addressed species-species interactions (Kennedy *et al*., 2007; Simard *et al*., 2012; Fransson *et al*., 2013). Collectively, our findings reflect the complexity of arctic tundra responses to
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predicted changes in summer and winter climate and the need to undertake comparative studies that include multiple ecosystem types even at a local spatial scales (Welker et al., 2000; Walker et al., 2008; Sullivan et al., 2008; Sullivan et al., 2010, Rogers et al., 2011; Christensen et al., 2013; Leffler & Welker, 2013; Sharp et al., 2013; Lupascu et al., 2014).

_Tomentella_, the genus with higher richness and sequence counts in the control plots of both tundra types, showed a sharp negative response to increased snow depth, with a significant six fold decrease in average OTU richness and a majority of the OTUs disappearing in the dry tundra, as well as an overall decrease in proportional sequence counts. In the moist tundra, _Tomentella_ richness also showed a decreasing trend, but in a less striking manner than in the dry tundra. The elevated number of indicator OTUs associated with the control plots (5 in the dry and 7 in the moist tundra) also point to the sensitivity of this group to altered conditions. Moreover, two of these OTUs were very closely related with a _Tomentella_ OTU (KJ792685) that had a strong negative effect by increased summer temperatures in the dry tundra (Morgado et al., 2015), further indicating that besides the general trends for the genus, at least one species of _Tomentella_, which is potentially widespread across the dry tundra, is very sensitive to summer and winter warming. Potential explanations to the observed patterns in our study may be linked with their functional traits and potential ecological roles. _Tomentella_ and closely related genera (e.g. _Pseudotomentella_ and _Tomentellopsis_) have melanized cell walls (Agerer, 1987-2002; Agerer et al., 2006), which is not a common feature in ECM basidiomycetes (Kõljalg et al., 2000). Melanins can be produced by fungi, plants and animals, and are dark macromolecules composed of phenolic and indolic monomers, often coupled with protein and carbohydrates (Butler & Day, 1998). They usually constitute a considerable portion of total fungal cell weight and likely require a considerable energetic investment (Rast & Hollenstein, 1977; Butler and Day, 1998). This feature has been extensively argued and was recently shown in physiological experiments (Fernandez & Koide, 2013) to increase tolerance to several environmental stressors, such as freezing (Robinson, 2001) and hydric stress (Singaravelan et al., 2008; Fernandez & Koide, 2013). The increased snow depth not only elevates winter soil temperature, but also increases soil moisture content as well (Wipft & Rixen, 2010), the effect of which likely is greater in the dry tundra. These altered conditions might reduce the competitive advantages of melanin-producing _Tomentella_ adapted to the above-mentioned stress factors (e.g., drought, and very low temperatures). Additionally, _Tomentella_ has either contact, short or medium-distance smooth hyphal ETs, which have been argued to be adapted to labile N soil pools (Hobbie & Agerer, 2010). The plant community responded to increased snow depth with a significant increase in the shrubs and litter layer (Wahren et al., 2005; Mercado-Díaz, 2011), indicating a potential change in soil organic matter input and shifts in C:N ratio, that have been argued to be important regulators of arctic N dynamics (DeMarco et al., 2011). Therefore, it is possible that in
the altered environmental conditions the combination of traits presented by *Tomentella* might not constitute an advantage in scavenging for soil nutrients, which might lead to a detrimental allocation of photosynthates by the ECM host and/or to competitive exclusion by other ECM fungi better suited to the altered conditions.

The decomposition and turnover of ECM fungal biomass likely has a significant role in C and other nutrient dynamics (Wallander *et al*., 2001; Clemmensen *et al*., 2013; Ekblad *et al*., 2013). Melanized hyphae have been argued to be relatively long lived, slow growing (Robinson, 2001) and to have increased cell wall resistance to decomposition (Coelho *et al*., 1997; Butler & Day, 1998; Butler *et al*., 2005), potentially representing a stable and recalcitrant component in the fungal biomass (Treseder & Lennon, 2015). In two laboratory experiments, Fernandez and Koide (2014) showed that the decomposition rate of ECM fungi was inversely correlated with the concentration of melanin and that the inhibition of melanin biosynthesis in an ECM fungi induced faster rates of decomposition. Moreover, Clemmensen *et al.* (2015), observed a correlation between the abundance of taxa with melanized hyphal content and higher carbon storage in the soil. If future climatic conditions lead to increased snow depth in the arctic tundra, the decreasing richness and relative abundance of *Tomentella* might contribute to soil C loss. Several other groups of root-associated fungi, also have melanized hyphae, such as ericoid mycorrhizal fungi and dark septated endophytes. We therefore highlight the need to address responses of those root-associated fungi to increased snow depth to better understand if the above-mentioned warming-induced trend of decreasing richness is common in melanized fungi or is specific to certain phylogenetic lineages. Despite the uncertainties, our evidence and grounded speculations are in line with the results by Natali *et al.* (2014) that addressed winter warming effects on C cycle dynamics and indicated a net soil C loss due to winter warming.

We observed an abrupt decrease in mean OTU richness of *Inocybe* from the control to the increased snow depth plots in both tundra types. However, a considerable proportion of *Inocybe* OTUs that were found in the increased snow depth plots were also found in the control plots, particularly in the moist tundra. These results suggest that although arctic *Inocybe* spp. seems to be very sensitive to climate changes, a resistant subset of the species seem to be able to withstand changes in the climatic conditions. *Inocybe* spp. were previously argued to be sensitive to altered environmental conditions, such as soil compaction (Hartmann *et al*., 2014) and summer warming (Morgado *et al*., 2015). Additionally, there is evidences that in mature plant stands the rate of root-infection by *Inocybe* might decrease in sites with increased soil moisture (Fleming, 1984). It is possible that in the increased snow depth conditions, the lack of rizhomorphs and hydrophilic ectomycorrhizae of *Inocybe* (Agerer, 2006), a set of characteristics hypothesized to be adapted to labile N uptake (Hobbie and Agerer, 2010), might constitute detrimental traits in relation to other groups of ECM fungi. However, the
increase in shrubs and litter layer might lead to potential patchiness of nutrient soil pools allowing for some species with hydrophilic hyphae to thrive in the increased snow depth. The lack of significant changes in *Cortinarius* richness and the relative increase in their overall sequence counts (a potential surrogate for relative abundance) between the control and increased snow depth plots indicate that this group might be more resistant and/or resilient to altered conditions, and could become more dominant in the warming Arctic. A similar trend for this group was also observed in our previous work that reported ECM fungal responses to long-term summer warming (Morgado et al., 2015). However, in contrast with our previous work, in the present study most OTUs are not shared between the two treatments, potentially indicating that, although average OTU richness does not change, there is a considerable turnover in species composition. This suggests that only a subset of the OTUs present in the control plots are resistant to increased snow depth and that there is substantial functional variation within the genus that allows for the exploitation of new niches created in the altered environment by incoming species. Species of *Cortinarius* form a dense mycelium with medium-distance fringe ET and hydrophobic rhizomorphs (differentiated mycelial cords) (Agerer, 2001). This foraging strategy is adapted for efficient N absorption and nutrient translocation (Hobbie and Agerer, 2010). *Cortinarius* were also reported to have the capability to assimilate organically bounded N (Hobbie & Agerer, 2010), and to transcribe Mn-peroxidase genes (which are involved in the production of exoenzymes) in field conditions and linked through co-localization of DNA abundance with exoenzyme activity that interacts in complex organic matter breakdown (Bödeker et al., 2014). Although little is known about ECM fungal physiological responses to extreme cold, Ma et al. (2011) compared growth responses to very low temperatures (between -40 and +4 °C) and freeze-thaw events of four ECM species from distinct lineages. Their results indicated that *Cortinarius* had the lowest tolerance to freeze-thaw events and the fastest growth when temperatures reach near 0 °C. Because reduced temperatures, hydric stress and freeze-thaw events inhibit the rate of chemical and microbial activity (Robinson, 2001), and given the characteristics and potential ecological role of *Cortinarius* spp., it seems feasible to argue that long-term increased winter soil temperatures, summer moisture and reduced fluctuations in soil temperatures might convey advantages to this group over other ECM fungal groups.

The increasing dominance of fast growing ECM species with high EMM production and fast N mobilization might lead to increased C storage in this soil pool, however, this will be determined by biomass turnover. In an interesting work relating fungal traits, community structure and nutrient soil pools, Clemmensen et al. (2015) found a link between abundance of species with similar ETs similar to that of *Cortinarius* and low accumulation of root-derived soil C. Briefly, they hypothesized that the exploratory hyphae of already explored areas in the soil could be recycled in an autolytic
process, leaving behind just the long-living rhizomorphs connecting the exploratory forefront of the EMM and the ectomycorrhizae. This strategy would enhance their nutrient acquisition and maintain or reduce their biomass, potentially reducing also energetic costs. Due to the potential high turnover of this mycelium biomass this theory also implies a potential reduction in stable soil pools, due to the potential high turnover of this mycelium biomass, and long-term C and N sequestration.

*Russula* did not show any significant change in richness with increased snow depth, and a considerable portion of the OTUs were found both in the control and the treatment plots, indicating that many OTUs were resistant to altered conditions. While most *Russula* spp. lineages have an hydrophilic ectomycorrhizae and contact or short-distance ETs that lack rhizomorphs, other lineages in *Russula* have a medium-distance smooth ET and hydrophobic hyphae (Agerer, 2006). In our dataset, the *Russula* OTUs with short or contact ET did not show change in richness with increased snow depth. However, one OTU with short or contact ET was indicator of the moist control plots while another was indicator of increased snow depth, suggesting species-specific responses to altered conditions. On the other hand, in the increased snow depth plots in the moist tundra, we observed a significant decrease in richness of OTUs that matched *Russula* species with hydrophobic and medium-distance smooth ET. These results indicate that even within a closely related group of species the functional diversity can differ. While some *Russula* species seem to have a considerable fast growth rate at low temperatures (Ma *et al.*, 2011), others are considered to have a slow growth rate (Nygren *et al.*, 2008). Moreover, there is evidence of intrageneric variability in N usage as well (Avis, 2012). It is possible that in some species the hydrophilic ectomycorrhizae allows for the rapid intake of labile N forms by the plants, without mineralization, via diffusion through the mantle of the ectomycorrhizae directly to the plant-host root via the apoplast. This process would avoid energetic costs and the necessity of C allocation to the ECM fungi (Nygren *et al.*, 2008). This may influence the competitive interactions between species with hydrophilic and hydrophobic mycelia.

In conclusion, our data provide first insights into the taxon-specific effects of increased snow depth on the ECM fungal community of Arctic tundra in Northern Alaska. We discovered major shifts in ECM fungal community composition and its potential functional traits by coupling changes on fine scale taxonomic groups with their extramatrical characteristics. We postulate that ECM fungal community shifts induced by long-term increased snow depth likely stimulate C and N mobilization. However, the final balance induced by arctic ECM basidiomycete community in these nutrient pools will likely depend on the changes in the biomass of specific groups, particularly *Tomentella* and *Cortinarius*. Our results also highlight how the fundamental differences in tundra ecosystem control the nature of the existing fungal communities and their responses to deeper snow.
Landscape at Toolik Lake, Alaska