Nitrophobic ectomycorrhizal fungi are associated with enhanced hydrophobicity of soil organic matter in a Norway spruce forest.

[1]Juan Pablo Almeida, [1]Nicholas P. Rosenstock, [2]Susanne K. Woche, [2]Georg Guggenberger and [1]Håkan Wallander

[1]Department of Biology, MEMEG, Lund University, 22362 Lund, Sweden

[2]Institute of Soil Science, Leibniz Universität Hannover, Herrenhäuser Str. 2, 30419 Hannover, Germany

Correspondence to: Juan Pablo Almeida (jpalmeidava@gmail.com)

Abstract

In boreal forests an important part of the photo assimilates are allocated belowground to support ectomycorrhizal fungal (EMF) symbiosis. The production of EMF extramatrical mycelium can contribute to carbon (C) sequestration in soils but the extent of this contribution depends on the composition of the EMF community. Some species can decrease soil C stocks by degrading soil organic matter (SOM) and certain species may enhance soil C stocks by producing hydrophobic mycelia which can reduce the rate of SOM decomposition. To test how EMF communities contribute to the development of hydrophobicity in SOM we incubated sand-filled fungal-ingrowth meshbags amended with maize compost for one, two or three growing seasons in non-fertilized and fertilized plots in a young Norway spruce (*Picea abies*) forest. We measured hydrophobicity as determined by the contact angle, the C/N ratios in the meshbags contents along with the amount of new C entering the meshbags from outside (determined by C3 input to C4 substrate), and related that to the fungal community composition. The proportion of EMF species increased over time to become the dominant fungal guild after three growing seasons. Fertilization significantly reduced fungal growth and altered EMF communities. In the control plots the most abundant EMF species was *Piloderma oliviceum*, which was absent in the fertilized plots. The hydrophobicity of the meshbag contents reached the highest values after three growing seasons only in the unfertilized controls plots and was positively related to the abundance of *P. olivaceum,* the C/N ratios of the meshbag contents, and the amount of new C in the meshbags. These results suggest that some EMF species are associated with higher hydrophobicity of SOM and that EMF community shifts induced by fertilization may result in reduced hydrophobicity of soil organic matter which in turn may reduce C sequestration rates.

Key words: Ectomycorrhizal fungi, Contact angle, hydrophobicity, fertilization, fungal communities.

1 Introduction

Fertilization of forests has been suggested as a way to increase C sequestration to mitigate climate change (Jörgenssen et al., 2021). In support for this, Bergh et al. (2008) found more than doubling of aboveground growth of young Norway spruce forests in response to yearly additions of a complete fertilizer in experimental sites in Sweden. A major part of gross primary production, between 25% and 63% according to Litton et al. (2007), is however allocated belowground to roots and associated ectomycorrhizal fungi, and this portion usually declines in response to fertilization (Högberg, 2010). In support for this, reduced growth of EMF mycelium was found in the young fertilized Norway spruce stands studied by Bergh et al. (2008) (Wallander et al., 2011).

EMF form extensive mycelial networks, which efficiently distribute C in the soil (Smith and Read, 2008), and this mycelium turns into necromass when the mycelium dies. Necromass from different EMF species decomposes at different rates (Koide et al., 2009). Melanin content appears to have a negative influence for necromass decomposition, but physical protection is also an important factor to reduce decomposition according to Fernadez et al. (2016). SOM can be protected from decomposition in aggregates where hydrophobic coatings of mineral particles change the physical properties of the particles, reduce water films around them and limit water penetration inside the aggregates. This affects the mobility of microbial decomposers and extracellular enzymes from the soil solution and reduces organic matter decomposition (Leelamanie et al., 2016 ; Goebel et al., 2011 ; von Lützow et al., 2006), and hydrophobic SOM generally decomposes slower than hydrophilic SOM (Nguyen and Harvey, 2003; 2001) . Since some EMF species form hydrophobic, while others form hydrophilic mycelia (Unestam and Sun, 1995), the composition of the EMF community may thus have fundamental importance for the SOM properties and subsequently for carbon sequestration rates in the soil.

In contrast to carbon accumulating activities by EMF, certain species may also reduce soil C stocks by oxidizing organic matter to release nitrogen and phosphorus. Some EMF species use 'brown-rot' Fenton chemistry and some use 'white-rot' peroxidases to do decompose SOM (Shah et al., 2016; Lindahl and Tunlid, 2015; Bödecker et al., 2014). This can result in 30% decrease in SOM according to Lindahl et al (2021). Ectomycorrhizal fungi may thus have opposing effects on the amount of SOM, and differences in community composition was proposed as one explanation for different C accumulation rates in boreal forests in northern Sweden (Clemmensen et al., 2015 ; 2013); later successional stages that accumulated more C were dominated by ericoid mycorrhizal fungi with recalcitrant necromass, while younger successional stages that accumulated less C were dominated by EMF of long distance exploration types with a high capacity to degrade soil organic matter. Certain species of EMF may have exceptional importance for organic matter degradation as the presence of *Cortinarius acutus* (which has retained the enzymatic capability to breakdown SOM to access nutrients) was linked to 33% lower C storage in the organic topsoils in 359 investigated stands in boreal forests in Sweden (Lindahl et al., 2021).

It is well known that fertilization with N has a strong impact on growth and composition of EMF (Lilleskov et al., 2011; Wallenda and Kottke 1988). Lilleskov et al. (2011) demonstrated that EMF sensitive to N (e.g. *Cortinarius, Tricholoma, Suillus, and Piloderma*) usually produce hydrophobic mycelia while N tolerant species often produce hydrophilic mycelia (e.g. *Laccaria*). Loss of hydrophobic EMF species at high N input could thus have consequences for SOM formation and C sequestration rates, but it is not well known to what extent EMF abundance has a significant effect on the overall hydrophobicity of SOM.

In our study with young Norway spruce forests reported above (Wallander et al., 2011), we used mesh bags amended with maize compost (C4 plant material enriched in ^{13}C)

to estimate EMF fungal growth in control and fertilized plots. In the present study we analysed the fungal communities as well as the hydrophobicity of the same mesh bag contents. The mesh bags were harvested after one, two or three growing seasons in order to follow fungal succession and development of hydrophobicity over time. All samples were subjected to 454-sequencing in order to characterize the fungal communities. We expected community composition to be influenced by fertilization, and hydrophobicity to increase over time when EMF biomass and necromass accumulates. We also expected more N to be removed by EMF from the mesh bags in the control than in the fertilized treatment. In addition, we expected higher hydrophobicity in control versus fertilized plots due to a higher proportion of hydrophobic species.

2 Material and Method

2.1 Study site

The experimental forest was located close to Ebbegärde in south-eastern Sweden (56°53'N 16°15E') in a 10 year old Norway spruce forest at time of sampling. The soil is a podzol on coarse sandy glacial till (site index G29), and the depth of the humus layer varied between 3 and 8 cm.

The treatments were designed in randomized block design with 3 fertilization treatments and 3 blocks per treatment (n=3). The plot size was 40 x 40 m. The fertilization treatments were: the unfertilized Control plots and 2 Fertilization regimes. In the fertilization treatments specific amounts of nitrogen (N) (ammonium and nitrate) were applied to optimize plant growth without inducing leaching. The amount of N additions was based on needle N determinations and monitoring of N in soil water (Bergh et al 2008). Thus, the fertilization was applied by hand as 50-100 kg N ha^{-1} every year for the first fertilization regime and as 100-150 kg N ha^{-1} every second year in the second fertilization regime (fertilization begun in

101 2002). To avoid nutrient imbalance caused by fertilization, the amount of micronutrients was
102 adjusted to optimum nutrient proportions for *Picea abies* (as calculated by Ingestad 1978).
103 For a more detailed description of the fertilization regime see Linder (1995) and Bergh et al.
104 (2008). For this study both fertilization regimes were treated as one fertilization treatment.

105

106 **2.2 Experimental design**

107 We used triangular shaped ingrowth bags made of nylon mesh (50 µm mesh size, 10 cm
108 side,~1 cm thick) to capture fungi growing in the soil. This mesh size allows the ingrowth of
109 fungal hyphae, but not roots (Wallander et al., 2001). The mesh bags were filled with 30 g
110 acid-washed quartz sand 0.36-2.0 mm, 99.6% SiO_2, Ahlsell AB, Sweden) heated to a
111 temperature of 600 °C overnight to remove all organic carbon. The sand was then mixed with
112 0.8% (w/w) maize compost. Maize compost was used since it has a unique C isotopic
113 signature, which makes it possible to estimate C influx into the mesh bags. Results from these
114 measurements are presented in Wallander et al. (2011), Maize compost was produced by
115 cutting maize leaves into small pieces and compositing in an isolated plastic compost bin for
116 12 months. After that the compost was kept at +4 °C. Fresh compost was forced through a 2
117 mm mesh and then mixed with dry sand to make a uniform mixture. The sand maize mixture
118 had a carbon content of 0.4%. The bags were buried at approximately 5 cm depth in the
119 interface between the organic horizon and the mineral soil where EM fungi are abundant
120 (Lindahl et al., 2007). First harvest was done in November 2007, after 8 months incubation.
121 The second harvest was done in November 2008 and the third harvest was done in November
122 2009. Four meshbags were pooled to make 1 composite sample for each block, year and
123 treatment. In the laboratory the mesh bags were opened and the contents from the four
124 replicate mesh bags from each experimental plot were carefully pooled and mixed.

Subsamples were taken for subsequent analyses (ergosterol, hydrophobicity, C and N content, fungal community) and immediately frozen.

The abundance of $\delta^{13}C$ as well as total C and N content were analyzed using an elemental analyzer (model EuroEA3024; Eurovector, Milan, Italy) connected to an Isoprime isotope-ratio mass spectrometer (Isoprime, Manchester, UK) as described by Wallander et al. (2011). The isotopic shift that occurred when ^{13}C depleted C (mainly EMF mycelia) entered the bags from outside was used to calculate the amount of new C in the mesh bags. To estimate ectomycorrhizal growth, the fungal cell membrane compound ergosterol was measured as a biomarker of fungal biomass. Ergosterol was extracted from 5 g of the pooled sand-maize mixture from the meshbag. Briefly the sample was subjected to saponification using a solution of 10 % KOH in methanol and the non-polar phase (where the ergosterol is present) was separated using cyclohexane. The ergosterol was quantified by high-performance liquid chromatograph (Hitachi model L2130), a UV detector (Hitachi model L2400). For more detailed regarding the protocol see Wallander et al. (2011).

2.3 DNA extraction, PCR and 454 sequencing

Ten grams of the sand/maize mixture from the composite samples was homogenized using a ball mill without a ball (Retsch, Haan, Germany). DNA was extracted from the homogenized samples by adding CTAB buffer (2 % cetyltrimetylammoniumbromid, 2 mM EDTA, 150 mM Tris-HCl, pH 8), vortexing, and then incubating at 65 °C for 1.5 h, followed by chloroform addition, vortexing, supernatant removal, and isopropanol and ethanol precipitation. The pellet was resuspended in 50 µl of MiliQ-water (Millipore) and further cleaned using Wizard DNA clean-up kit (Promega, Madison, WI, USA).

PCR was carried out for each sample in 3 triplicate 25 µl reactions, using the fungal-specific primers ITS1-F (Gardes and Bruns 1993) and ITS4 (White et al. 1990). Each primer

was elongated with adaptors required for 454 pyrosequencing (ITS1-F/A adaptor and ITS4/B adaptor). The ITS4 also contained a sample specific tag consisting of 8 bases; ITS1-F/A : 5′-CCTATCCCCTGTGTGCCTTGGCAGTCTCAGCTTGGTCATTTAGAGGAAGTAA-3′; ITS4/B :5′-CCATCTCATCCCTGCGTGTCTCCGACTCAG*XXXXXXXX*TCCTCCGCTTATTGATATGC-3′. PCR products were purified with Agencourt AMPure kit (Agencourt Bioscence Corporation, Beverly, MA, USA) in order to remove residual salts, primers and primer dimers. The concentration of the purified PCR products was measured with the PicoGreen ds DNA Quantification Kit (Molecular Probes, Eugene, OR, USA) on a FLUOstar OPTIMA (BMG LABTECH Gmbh, Ortenberg, Germany). Equal amounts of DNA from each sample were pooled into one single pool and submitted for 454 pyrosequencing. Sequencing was performed on a FLX 454 (Roche Applied Biosystems, City, Country) using the Lib-L chemistry at the Pyrosequencing facility at Lund University, Lund, Sweden.

2.4 Bioinformatic analysis

After sequencing sequences were trimmed and filtered using Mothur v1.34 (Schloss et al., 2009). The trim seqs operation was run with the following exclusion parameters: all sequences that mismatched the sample ID barcode at more than one position, mismatched the primers at more than 2 positions, had homopolymers longer than 10 bp, were shorter than 150 bp, or had an average base call quality score below 20 over a moveable window of 40 bases. Sequences outside the *ITS2* region and chimeric sequences were removed using ITSx extractor v1.5.0 (Bengtsson-Palme et al., 2013). After filtering, a Bayesian clustering was applied to the sequences using the Gaussian Mixture model CROP (Hao et al., 2011) at 97% sequence similarity, and a set of operational taxonomic units (OTUs) was thus obtained. Clusters that were only found in one mesh bag sample (one PCR reaction) were excluded,

175 further reducing the possibility that any chimeric sequences were used in our analysis. Search
176 for sequence identities were performed by iteratively BLASTing (Basic Local Alignment
177 Search Tool) against 2 different sequence databases, the first was the UNITE (Koljalg et al.,
178 2005, http://unite.ut.ee/index.php) reference/representative sequence database (21,000 seqs,
179 dynamic taxa threshold, release date 2014-02-09), and the second was the full UNITE+INSD
180 sequence database (377,000 seqs, dynamic threshold, release date 2014-02-15) (Karsch-
181 Mizrachi et al., (2012). The UNITE and INSD databases were purged of all sequences, nearly
182 25% of the total, that did not have any taxonomic information, primarily environmental
183 samples from soils and roots using boolean terms (ex. Environmental, uncultured, root
184 endophyte, unidentified). Sequences were assigned to species when there was at least 97 %
185 similarity between query sequence and top hit. Sequences that failed to match at this threshold
186 were excluded. Separate clusters that matched the same database sequence were subsequently
187 lumped into one OTU.
188 Using names and taxonomy associated with the OTU's, the total fungal community was
189 divided by both phylum (Basidiomycota, Ascomycota, Mucoromycota, Zoopagomycota, and
190 Chytridiomycota) and function (known ectomycorrhizal fungi, unknown ectomycorrhizal
191 status, saprotrophic fungi); OTUs were considered known ectomycorrhizal fungi based on the
192 knowledge of the ecology of known close relatives (genera or below) according to Tedersoo
193 et al. (2010).
194 After filtering, each sample was rarified to the median number of reads using the
195 "rrarefy" function in the VEGAN package (Oksanen et al., 2013) in R (R Core Team, 2013).
196 For community comparison (total, or for ectomycorrhizal fungi), all read abundances were
197 converted to relative abundance, such that the read abundances for all OTUs for each sample
198 totaled to 1.
199

200 **2.5 Hydrophobicity**

201 The hydrophobicity was evaluated in terms of contact angle (CA) with the sessile drop
202 method (Bachmann et al., 2003), using a CCD-equipped CA microscope (OCA 15,
203 DataPhysics, Filderstadt, Germany). Here the angle a drop of water forms at the <solid-liquid-
204 vapor interphase is measured. This contact angle is used to describe the wettability of the
205 surface; a CA≥90 indicates a hydrophobic and a zero CA a hydrophilic surface. A CA>0° and
206 <90° indicates subcritical water repellency.

207 For measurement, material from the meshbags contents was fixed on a glass slide with
208 double-sided adhesive tape in an ideally one-grain layer. Placement of a water drop is
209 recorded and the initial CA evaluated after ending of mechanical disturbances by drop shape
210 analysis (ellipsoidal fit) and fitting tangents on the left and right side of the drop, using the
211 software SCA 20 (DataPhysics, Filderstadt, Germany; Goebel et al., 2013). CA is given as the
212 mean CA of the left and right side of the drop. As an estimate about CA stability, CA again
213 was evaluated after 1 s (denoted as CA_{1s}) and after 5 s (denoted as CA_{5s}; Bachmann *et al.,*
214 2021).

215 Three replicates from each treatment (Control or Fertilized) and each incubation period (2007,
216 2008, 2009) were used in the measurements. One slide per replicate was prepared and for
217 each slide six drops were placed and averaged to obtain one CA per replicate (n=6). Two
218 slides containing the non-incubated sand-maize compost mix were also analyzed as a non-
219 treated reference material. Due to the coarse texture of the meshbag material, the drop volume
220 was 6 µL.

221 **2.6 Statistical analysis**

222 The statistical analyses for the fungal communities were performed using the VEGAN
223 package (Oksanen et al., 2013) in R (R Core Team, 2013). Fungal communities were
224 visualized with ordination using non-parametric multidimensional scaling (NMDS) using the

metaMDS function. Differences in community structure were visually compared with centroids and the associated 95 % confidence interval associated with a t-distribution around the standard error of the centroid. To detect if the fungal communities were significantly influenced by the treatments (fertilization and incubation periods), permutational multivariate analysis of variance (PERMANOVA; Anderson, 2014) was performed. Pairwise comparisons between treatments were tested using pairwise Adonis test.

To test for differences in hydrophobicity (contact angle), C/N ratios, new C inside the meshbags and ergosterol ANOVA and two ways ANOVA were performed using the CAR package (Fox & Weisberg, 2019) in R (R Core Team, 2013). To test for differences in the relative abundance of EMF species between the treatments, Dunn's test for non-parametrical samples was performed (Dinno, 2015).

Principal component analysis (PCA) was used to analyze the relationships between the most abundant fungal species and the properties of the meshbag contents (hydrophobicity (contact angle), C/N ratios, new carbon inside the meshbags, ergosterol) using the package FactoMineR (Lê et al., 2008) in R (R Core Team, 2013).

3 Results

3.1 Fungal biomass

The concentration of ergosterol, as an estimate of fungal biomass, in the mesh bags have been reported earlier (Wallander et al., 2011) and is summarized in Table 1. In brief, ergosterol content increased from a starting value of 0.7 (original maize compost) to 2.2 mg g^{-1} in the mesh bags after incubation for one growing season in control plots. After this the concentration did not change significantly over the coming two years. In fertilized plots the concentration was significantly lower than the control plots (ANOVA, $F= 13.4$; $p<0.01$)

3.2 Hydrophobicity, C content and C/N ratio of SOM

Incubation in the field significantly increased hydrophobicity of the meshbag contents in the unfertilized control plots as indicated by CA_{1s} and CA_{5s} (ANOVA, $F= 6.2$; $p<0.05$ and ANOVA, $F=10.2$; $p<0.01$; respectively). CA of the control plots increased with incubation time, but only the CA_{1s} and CA_{5s}were significantly different from the reference material (non-incubated sand-maize compost mix), i.e., stability of CA was increased (Fig 1a).
Incubation in the field also affected hydrophobicity of the meshbag contents in the fertilized plots as indicated by the initial CA and CA_{1s} and CA_{5s} (ANOVA, $F= 5.2$; $p<0.05$; ANOVA, $F= 4.1$; $p=0.06$ and ANOVA, $F=3.9$; $p=0.05$; respectively). The CA stability (CA_{1s} and CA_{5s}) was increased compared to the reference material only in the one-year incubation meshbags. As time of incubation in the soil increased, however, CA decreased. After 3 years of incubation the initial CA became significantly smaller in comparison with the reference material (Fig 1b). There were significant differences in the CA (initial, CA_{1s} and CA_{5s}) between meshbags from the control and fertilized plots in the 3-years incubation bags with smaller CA (initial, CA_{1s} and CA_{5s}) for the fertilized plots compared to the control (2009; Fig 1c, ANOVA, $F= 3.2$; $p<0.05$; $F= 3,1$; $p=0.05$ and $F=2.8$; $p=0.06$; respectively), but not for the first and second incubation year (2007, 2008)

The concentration of C in the mesh bags was not influenced by time or fertilization but the amount of new C (C3-C presumably from EMF) in the mesh bags was significantly affected by fertilization and was higher in the control plots than in the fertilized plots according to the two-ways ANOVA ($F=5.3$; $p<0.05$). The amount of new C tended to increase with incubation time in the control plots (Table 1). The interaction between fertilization and incubation time were not significant.

There was a positive correlation between the amount of new C and the hydrophobicity for both CA_{1s} and CA_{5s} (Pearson, T = 2 , p=0.06, Cor=0.45) (Fig 2a and b respectively). When breaking down the data by fertilization regime, there was a positive correlation between amount of new C and the hydrophobicity for the CA_{5s} in the control plots (Pearson, T = 2.2 , p=0.06, Cor=0.63) (Fig 2c) but the correlation tended to be negative in the fertilized plots (Fig 2d).

The C/N ratio of the mesh bag content was 11.9 in the initial material, which increased to an average of 14.6 and 12.2 after 3 years of incubation in the control and fertilized plots respectively (Table 1). According to the two way ANOVA, fertilization had a significant effect on the C/N ratios of the meshbags (ANOVA, F=6.1, $p<0.05$). The impact of incubation time or the interaction between fertilization and incubation was not significant. During the first two incubation years (2007, 2008) there were no differences between the C/N ratios in the control and fertilized samples. During the third incubation year (2009) the C/N ratios in the control samples were significantly higher than the C/N ratios in the fertilized samples.

3.3 Effects of fertilization and incubation time on fungal community composition

After all bioinformatic processing and quality filtering, followed by rarefaction to a maximum of 1200 sequence reads per sample (minimum 612), and elimination of all operation taxonomic units (OTUs) that were only found in one sample, 26943 sequence reads were recovered that were apportioned to 146 OTU's.

The total fungal communities were significantly influenced by incubation time and by fertilization according to the Permanova analysis ($p<0.001$; F=5.4 and $p<0.001$; F = 8.4, respectively) (Fig 3a)

298 Fertilization had no significant effect on the total fungal community during the first year but
299 during the second year and third year the fertilization effect was found to be significant
300 (pairwise Adonis, p = 0.06; F =2 and p = 0.02; F = 5.3, respectively)
301 The proportion of EMF sequences increased significantly over time in the mesh bags (Dunn
302 test, χ^2 =18, p<0.0001), (Fig 3b). 11 % and 7% of the sequences were EMF during the first
303 growing period in the control and fertilized plots respectively. These values increased to 24%
304 and 31% after two years of incubation in the control and fertilized plots respectively, and to
305 78% and 72% after three growing seasons in the control and fertilized plots respectively
306 (Table 1). The number of EMF reads was significantly correlated with the new C in the
307 meshbags (Pearson, T = 2.4, p < 0.05, Cor=0.46).
308
309 The more abundant hydrophobic EMF genera were *Piloderma* and *Amphinema* (Fig 3c and d
310 respectively) while the more abundant hydrophilic genus was *Tylospora* (Fig 3e).
311 The proportion hydrophobic EMF species (the sum of the relative abundance of fungal reads
312 belonging to hydrophobic EMF species) tended to be higher in the control plots (up to 57% of
313 the total fungal reads) in comparison with the fertilized plots (up to 44% of the total fungal
314 reads) in the three-years-incubation bags, but this increase was not significant. Additionally,
315 the proportion of hydrophobic EMF species in relation to hydrophilic EMF species in the
316 control plots tended to be higher than in the fertilized plots in the three-years bags but this
317 was not significant. When both treatments (control and fertilization) where analyzed together,
318 there was no correlation between the proportion of hydrophobic species and the contact angle.
319 The proportion of hydrophobic EMF species was positively correlated with the averaged
320 contact angle (initial C.A, C.A at 1s and C.A at 5s) in the control plots (Pearson, T = 2.9,
321 p<0.04, Cor=0.68) (Fig 3f) but not in the fertilized plots.
322

323 *Piloderma* increased in abundance over time in the control plots to become the dominating
324 genus (up to 47 % of the relative abundance) after three years of incubation (Fig 3c). The
325 most dominant species in the control plots was *Piloderma olivaceum* which was reduced to
326 0% in the fertilized plots independent of incubation time (Fig 3b). *Tylospora fibrillosa* was
327 also reduced in response to fertilization (Dunn test, χ^2 =13.4, p<0.0001), while *T.*
328 *asterophora* showed an opposite trend (Dunn test, χ^2 =4.4, p<0.05) (Fig 3b). *Amphinema* sp
329 5. was the most abundant species in the fertilized plots and was enhanced by fertilization
330 (Dunn test, χ^2 =3.8, p<0.05) (Fig 3b).
331
332 **3. 4 Principal component analysis**
333 The principal component analysis (Fig 4) separated the samples by incubation time along the
334 principal component 1. This component explained 34% of the variance. Samples belonging
335 to the three-years incubation bags were ordinated to the right of the principal component 2
336 Along the principal component 2 the samples were separated by the fertilization treatment.
337 This component explained 25.7 % of the variance. Samples belonging to the unfertilized
338 controls were ordinated above the principal component 1. The linear model showed that the
339 fertilization/incubation treatments were significantly associated with the PC1 (F=8.3, p <
340 0.01) and the PC2 (F=18 , p< 0.0001). The proportion of EMF and Basidiomycota increased
341 over time while the proportion of saprotrophic fungi and Ascomycota decreased with
342 increasing incubation time. The EMF species *Tylospora fibrillosa* and *Piloderma olivaceum*
343 were positively related with the CA (initial CA, CA_{1s}, CA_{5s}), with the C/N ratios and with the
344 amount of new carbon inside the meshbags; and their vectors were directed towards longer
345 incubation time and opposite to the fertilization treatments.
346
347

348

349 **4 Discussion**

350 **4.1 Effect of incubation and fertilization on the total fungal communities**

351 As expected, the fungal communities were influenced by the fertilization and by incubation
352 time and there was a significant increase in the percentage of EMF reads over time. It should,
353 however, be noted that the ingrowth of EMF in relation to other fungal groups was
354 surprisingly low during the first growing season (<12% of the fungal sequences), which is
355 much lower than what has been found in earlier studies (Parrent and Vilgalys, 2007;
356 Wallander et al., 2010). Some of this variation may be due to different weather conditions, the
357 first year was wetter than normal while the third was close to normal in precipitation
358 (Wallander et al., 2011), or due to larger belowground carbon allocation when the trees
359 approach canopy closure during the third year, as discussed in Wallander et al. (2010).
360 Whether shifts in EMF were due to selection of later succession fungal taxa or variation in
361 climatic conditions remains unclear but is ultimately not particularly important in terms of
362 understanding how shifts in EMF relate to soil organic matter cycling. Thus, the EMF
363 abundance was highest during the third year and this increase was associated with higher C/N
364 ratios and hydrophobicity in the control plots and higher input of new C in the control and
365 fertilized plots. This suggests a strong relation between EMF and the changes in the properties
366 of the organic material in the meshbags.

367 The most dominant EMF genera in our study were *Amphinema, Piloderma* and
368 *Tylsopora* which also are common in other studies of EMF communities in coniferous forests
369 (Almeida et al., 2019; Walker et al., 2014; Tedersoo et al., 2008). In the control plots, the
370 most dominant species was *P. olivaceum* which did not colonize the meshbags collected from
371 fertilized plots. *Piloderma* is a common genus in boreal forests and is reported to be more
372 abundant in soils rich in organic N (Heinonsalo et al., 2015; Lilleskov et al., 2002), and to

decline in response to inorganic N fertilization (Teste et al., 2012), and elevated N deposition (Kjöller et al., 2012; Lilleskov et al., 2011; Lilleskov et al., 2002a ; Taylor et al., 2000). The decline of *Piloderma* in the fertilized plots in the present study is not surprising since this genus produces abundant hydrophobic rhizomorphs that might constitute a large C cost for the host (Defrenne et al., 2019), which is not economical for the symbiosis at high mineral N concentrations. Other more direct effects of the fertilizer on the growth of *Piloderma* mycelium are also possible. The increase in the C/N ratios of the meshbag substrates from the control treatment might be thus an effect of biomass accumulation of *Piloderma* species, since EMF fungi in general have a higher C/N ratio than maize compost (Wallander et al., 2003). Additionally, it has been shown that *P. olivaceum* produces proteases that improve the ability of the host trees to utilize N from organic compounds (Heinonsalo et al., 2015). Therefore, N released from the maize compost by this fungus could have been transferred to the host plants, which would contribute to the increase in C/N ratios in the control plots in comparison with the fertilized plots. This explanation is consistent with results described by Nicolas et al. (2017), who used FTIR and NEXAFS to analyze chemical changes of similar maize compost incubated in mesh bags over one growing season in a Norway spruce forest in southwestern Sweden. They found that heterocyclic-N compounds declined in mesh bags in comparison with non-incubated reference material, which was interpreted as an effect of removal by EMF and transfer to the host trees. This decline was higher in the unfertilized control plots compared with fertilized plots. In the fertilized plots of the present study, the amount of new C tended to increase in the three-year incubation bags where the C/N ratios reached the lowest values, indicating limited N removal by the EMF colonizing these bags.

Amphinema sp. 5 responded positively to fertilization in our study which is supported by a study by Kranabetter (2009) who found strong increase in the abundance of *Amphinema* colonized root tips along productivity gradients in Canada. While a reduced abundance of *T.*

398 *fibrillosa* was observed in the fertilized plots, *T. asterophora* responded positively. Similarly
399 contrasting effects between this two species were found in other studies as well (Teste et al.,
400 2012 ; Kjöller et al., 2012; Toljander et al., 2006). In an N deposition gradient Kjøller et al.,
401 (2012) found increased abundance of *Tylospora asterophora* in areas with high N throughfall
402 while *T. fibrillosa* abundance decreased with higher N deposition. Reduction of *T. fibrillosa*
403 in response to fertilization may be a result of C starvation since it has been shown that this
404 species is more dependent on C transferred from a living host in order to colonize new
405 seedlings on a clear cut compared to *Amphinema* sp. which readily colonized saplings on
406 clear cuts (Walker and Jones, 2013).
407
408 **4.2 Effect of incubation and fertilization on hydrophobicity**
409 As expected, hydrophobicity increased over time in respect to the reference material
410 (non-incubated maize-sand mixture), and this increase occurred only in the unfertilized
411 controls at the last sampling when the fungal communities in the mesh bags were dominated
412 by EMF. This increase in hydrophobicity was expected to be an effect of the accumulation of
413 fungal biomass and necromass over time as it has been shown that organic C (Woche et al.,
414 2017; Mataix-Solera & Doerr, 2004; Chenu et al*.,* 2000) and microbial biomass and
415 necromass contribute to the hydrophobicity of soils (Schurig et al., 2013; Šimon *et al.,* 2009;
416 Capriel, 1997). However, the total amount of C was similar for all the incubation times and
417 was not affected by fertilization indicating that C content alone could not explain the
418 variations in hydrophobicity. Instead, the amount of new C entering the meshbags from
419 outside was found to be significantly correlated with hydrophobicity (CA_{1s} and CA_{5s}). This
420 new C is expected to be of EMF origin as discussed by Wallander et al*.* (2011). Since
421 saprotrophic fungi utilize the maize compost material as their C source, it is expected that new
422 C inputs come from plant photoassimilates and are brought by EMF fungi (Wallander et al.,

2011). Therefore, these results suggest that the accumulation of biomass and necromass of EMF origin over time might contribute to the buildup of hydrophobicity in SOM in the control plots.

Our results show that fertilization reduced ergosterol concentration in the meshbags in comparison with the control samples (Wallander et al., 2011) and this coincided with a decrease in the hydrophobicity over time in comparison with the unfertilized controls and the non-incubated reference material. It has been shown that fungi may enhance soil water repellency of soil particles since some filamentous fungi produce insoluble substances like ergosterol and hydrophobins (Mao et al., 2019; Rillig et al., 2010). For instance, Hallet et al. (2001) found that soil hydrophobicity decreased when fungi were killed after fungicide additions. Therefore, it is possible that the lower fungal biomass in the fertilized plots in our study led to a decrease in hydrophobicity as incubation time in the soil increased. However the concentration of ergosterol in the meshbags from the control plots did not increase with incubation time and even tended to decline in the last incubation sampling when hydrophobicity increased, indicating that ergosterol alone is not a good predictor of hydrophobicity. It is possible that high ergosterol values after one growing season was an effect of high abundance of yeast like *Guehomyces*, *Cryptococcus*, *Rhodotorula* and *Candida*, which are unlikely to contribute to hydrophobicity but dominated the fungal communities of the mesh bags during the first growing seasons. These fungi decreased drastically in abundance in the three-years incubation bags. The ergosterol content per dry mass of yeasts are much higher than in filamentous fungi (Pasanen et al., 1999), which might explain the high ergosterol values in the first incubation periods. From these results we conclude that hydrophobicity is more associated with EMF fungal colonization (measured as the amount of new C) than with total fungal biomass (measured by ergosterol). It should be also noted that

448 we cannot rule out the possibility that other compounds from the soil entered the meshbags
449 during the underground incubation. In soils, polymeric substances coming from SOM, root or
450 microbial exudates can have hydrophobic properties (Vogelmann et al., 2013). Hence, the
451 hydrophobic changes in the material could be partly explained by other sources than EMF
452 mycelium. However, the significant correlation between the new carbon in the bags and the
453 EMF reads and the negative effect of fertilization on the C.A might suggest that
454 hydrophobicity changes in the meshbag content are caused mainly by EMF.

455 Given the apparent association of EMF colonization with higher hydrophobicity over time,
456 some EMF species may be expected to be more important than others for this process. We
457 expected higher hydrophobicity in the control plots in response to a higher proportion of
458 hydrophobic long-distance exploration types species. Indeed, the proportion of hydrophobic
459 EMF species in the control plots tended to be higher in comparison with the fertilized plots in
460 the meshbags incubated for three years. From the hydrophobic species in the control plots,
461 *Piloderma* spp. constituted the majority of fungal species with up to 47% of the total fungal
462 reads. The presence of *Piloderma* species like *P.olivaceum,* known to form hydrophobic
463 mycelia, (Lilleskov et al., 2011, Agerer, 2001), and that was totally absent in the fertilized
464 plots is likely to contribute significantly to hydrophobicity of SOM. In the fertilized plots
465 there was also an increase over time in the proportion of hydrophobic EMF species
466 (*Amphinema* being the most abundant hydrophobic genus) which was not accompanied by an
467 increase in hydrophobicity. This may suggest that necromass from *Amphinema* do not
468 accumulate to the same extent as for *Piloderma* and is probably not associated with the
469 hydrophobicity in the meshbags. These findings suggest that hydrophobicity of living
470 mycelium might not necessary influence the water retention of the organic material to a large
471 extent. This is consistent with the findings of Zheng et al. (2014) who found that the
472 hydrophobicity of EMF mycelium does not necessary enhance soil water repellency. They

tested how different EMF strains inoculated on *Pinus sylvestris* affected water repellency of sandy loamy soil. The mycelium hydrophobicity of the fungal strains used in their experiment was previously tested by drop immersion on fungal mycelium growing on pure cultures. The authors found that the mycelium from hydrophobic species generally enhanced water repellency but not all hydrophobic isolates had positive effect on soil hydrophobicity. It was suggested that beside mycelium hydrophobicity other species-dependent factors like growth patterns, the degree of soil particles coverage or the amount of hydrophobic substances produced by the fungus might influence soil water repellency. In the present study the difference in hydrophobicity between treatments might not be related only to the exploration types of the abundant species but also by species-dependent features. For example, the characteristic color yellow of *Piloderma* comes from an insoluble pigment called corticrocin (Gray & Kernaghan 2020; Schreiner et al., 1997). Moreover, the hyphae of *Piloderma* is reported to be coated with calcium oxalate crystals (Arocena et al., 2001) probably as a strategy against grazers or repel water to avoid microbial predation (Gray & Kernaghan 2020; Whitney & Arnott 1987). Thus, these particular features of *Piloderma* make it a good candidate to explain the enhanced the hydrophobicity of the material in the control meshbags which is supported by the association between the abundance of this fungus, the new C in the meshbags and the CA.

4.3 Ecological significance

The effect of fertilization on fungal communities and its significance for C sequestration has been largely discussed (see Jörgenssen et al., 2021; Almeida et al., 2019; Högberg et al., 2010; Janssens et al., 2010; Treseder, 2004). Additions of inorganic N may have a strong positive effect on plant net primary production (Binkley & Högberg, 2016) but have also been shown to decrease belowground C allocation (Högberg et al., 2010) and consequently

498 decrease EMF biomass (Almeida et al., 2019; Bahr et al., 2015; Högberg et al., 2007, 2010;
499 Nilsson & Wallander, 2003), which will reduce the input of C to the soils and may reduce C
500 sequestration. However, Bödeker et al. (2014) for example, showed that addition of inorganic
501 N significantly decreased the abundance of *Cortinarius acutus*, a species that can enhance
502 SOM decomposition in order to uptake N (Lindahl et al., 2021). The decrease of *Cortinarius*
503 sp was accompanied by a decrease in the enzymatic oxidation in the humus layer of the soil.
504 Therefore, it has been suggested that fertilization might improve C sequestration by
505 suppressing SOM decomposition by some key species EMF like *Cortinarius* (Lindahl &
506 Tunlid, 2015 ; Bödeker et al., 2014). In the current study we show that *Piloderma*, another
507 common species from northern-forested ecosystems, is negatively affected by fertilization and
508 that its decrease might be associated with a decrease in the organic material hydrophobicity.
509 These findings suggest that even if fertilization could reduce the abundance of EMF with
510 decomposer capabilities it may also reduce the accumulation of hydrophobic fungal mycelium
511 that could enhance SOM formation and C sequestration rates. Therefore, the role of different
512 abundant EMF genera like *Piloderma* and *Cortinarius* in boreal forests for establishment and
513 destruction of hydrophobicity and the effect of fertilization on them warrants further research.

Author contributions:

JPA: Conceptualization of the research goals and aims. Data curation and analysis.

Manuscript writing.

NR: Data acquisition, curation and analysis.

SW: Data acquisition, curation and analysis.

GG: Conceptualization and development of the methodology.

HW: Conceptualization and development of the methodology, research goals and aims.

Manuscript writing.

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Table 1:
Average and standard error of the ergosterol concentrations, total C%, C/N ratio, amount of new carbon (C3 mainly from EMF), % of EMF DNA reads, and contact angle determined 5 seconds after placement of water droplets placed on mesh bags material amended with maize compost (CA_{5S}; estimation of contact angle stability). **Low scores letters refer to statistical differences according to posthoc Tukey test and pairwise Dunn test. Asterisks correspond to statistic differences for the C.A after 5 (s) between the meshbag contents and the non-incubated reference material.**

Treatment	Incubation time (years)	Ergosterol µg g^{-1}	C (%)	C/N	Amount of new C mg g^{-1}	% of EMF reads	CA_{5s} °/*SD*
Non-incubated reference material		0.7		11.9			37.3±0.1
Control	1	2.2±0.5 a	0.38±0.02 a	13.2±0.5 ab	0.6 ± 0.2 a	11.3±2.2 a	62±2.8 ab
Control	2	2.3±0.3 a	0.43±0.11 a	14.3±0.4 ab	0.9 ± 0.2 a	24,4±2.3 ab	67±4.4 ab *
Control	3	1.8±0.1 a	0.42±0.07 a	14.6±0.3 a	1± 0.4 a	78.3±1.4 b	78±7 a *
Fertilized	1	1.1±0.5 b	0.42±0.02 a	13 ±0.5 ab	0.5±0.2 a	7 ±3.6 a	62±3.6 ab
Fertilized	2	1.6±0.6 b	0.40±0.04 a	13 ±0.6 ab	0.3±0.2 a	31.3±11.9 ab	57±1.4 ab
Fertilized	3	1.1±0.2 b	0.42±0.04 a	12.4±0.2 b	1 ± 0.3 a	71.8±6.3 b	53±7.5 b

Figure 1: Contact angle (CA) comparisons between a) control treatment and reference material b) fertilization treatment and reference material and c) control and fertilized treatments. Shown is the intial CA (ini), determined directly after placement of the water drop and CA determined 1 second (1s) and 5 seconds (5s) after placement of the water drop. Bars represent standard deviation (n=3).

Figure 2: Correlation between the amount of new C and the hydrophobicity of the meshbag contents measured as the contact angle (CA). The C.A was determined directly after placement of the water drop at a)1 second (1s) and b) 5 seconds (5s). c) Correlation between the amount of new C and the hydrophobicity of the meshbag at 5 (s) in the control plots. d) Correlation between the amount of new C and the hydrophobicity of the meshbag at 5 (s) in the fertilized plots.

Figure 3: Response of the fungal communities in the meshbags to the fertilization treatment and incubation time. a) NMDS ordination analysis of the fungal communities b) Relative abundance of the different fungal species and their corresponding mycelium exploration type and hydrophobic (Ho) or hydrophilic (Hi) properties c) Relative abundance of the genus *Piloderma* d) Relative abundance of the genus *Amphinema* d) Relative abundance of the genus *Tylospora* 3) Correlation between the proportion hydrophobic EMF species and the averaged contact angle (initial C.A, C.A at 1s and C.A at 5s) in the control plots.

Figure 4: Principal component analysis of the most abundant fungal species and the properties of the organic material inside the meshbags.