



## Review

## Evaluation of methods to estimate production, biomass and turnover of ectomycorrhizal mycelium in forests soils – A review

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## ABSTRACT

Mycorrhizal fungi constitute a considerable sink for carbon in most ecosystems. This carbon is used for building extensive mycelial networks in the soil as well as for metabolic activity related to nutrient uptake. A number of methods have been developed recently to quantify production, standing biomass and turnover of extramatrix mycorrhizal mycelia (EMM) in the field. These methods include minirhizotrons, in-growth mesh bags and cores, and indirect measurements of EMM based on classification of ectomycorrhizal fungi into exploration types. Here we review the state of the art of this methodology and discuss how it can be developed and applied most effectively in the field. Furthermore, we also discuss different ways to quantify fungal biomass based on biomarkers such as chitin, ergosterol and PLFAs, as well as molecular methods, such as qPCR. The evidence thus far indicates that mycorrhizal fungi are key components of microbial biomass in many ecosystems. We highlight the need to extend the application of current methods to focus on a greater range of habitats and mycorrhizal types enabling incorporation of mycorrhizal fungal biomass and turnover into biogeochemical cycling models.

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## 1. Introduction

A better understanding of below ground carbon (C) flux is of fundamental importance to predict how changing climate will influence the C balance of forest (and other) ecosystems (Litton and Giardina, 2008). Litton et al. (2007) reported below ground C allocation in forest ecosystems can represent 25–63% of GPP on a global scale, and this C has a large influence on the physical, chemical and biological properties of soils. The below ground allocation of C links

activity in the forest canopy to the activity in the soil, and provides a flow of organic C from shoots to soil via fine roots and mycorrhizal hyphae. The pathways by which this organic C can enter soils are complex, involving both biomass turnover (Godbold et al., 2003), biomass grazing (Setälä et al., 1999) and turnover of low molecular weight exudates from roots and fungal hyphae (van Hees et al., 2005). The fate of C entering soil systems is also complex. Much of this C is lost as respiration (Janssens et al., 2001) and a small but significant fraction enters the soil organic matter (SOM) pool. Determination of the pools and fluxes of biomass inputs in isolation from fine roots and mycorrhiza provides a major scientific challenge. Some studies (e.g. Wallander et al., 2004) suggest that biomass pools and inputs from fine roots and mycorrhizal hyphae are in the same order of magnitude. However, estimates of fungal inputs rely on

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methods and conversion factors that contain a certain degree of inaccuracy that needs to be considered.

Precise measurements of production, standing biomass and turnover of extramatrical mycelium (EMM) of mycorrhizal fungi are essential in order to accurately describe the C cycle of terrestrial ecosystems. Although several techniques are available for this, they all have limitations that need to be taken into consideration.

Existing biogeochemical models often treat the uptake apparatus as a single organ, meaning that there is no distinction between roots and mycorrhizal hyphae. It is possible, and probably necessary, to amend this by allocating carbon and nutrients specifically for the fine roots and mycorrhizal hyphae respectively. This would require the development of dynamic allocation routines responsive to carbon, nutrients and water availability (Jönsson, 2006), and would allow the models to simulate nutrient uptake and carbon flux dynamically. In this review, we will discuss and compare available methods to estimate production, standing biomass and turnover of mycorrhizal mycelia (summarized in Tables 1–3). We focus on temperate and boreal forests, in which the dominant plants associate with ectomycorrhizal (ECM) fungi. From a methodological perspective, greatest progress has been made in quantification of production, biomass and turnover of ECM fungi compared to the other main mycorrhizal types (arbuscular and ericoid mycorrhizas). This progress has been driven partly by technical reasons but more importantly because of the recognition of the key roles boreal and temperate forests play in the global C cycle. However, we emphasise from the outset that greater effort must be applied to other ecosystems in which plants are primarily colonised by arbuscular and ericoid mycorrhizal fungi, which also have important roles in regulating biogeochemical cycles. We will also highlight knowledge gaps that need to be filled in order to incorporate mycorrhizal mycelia in models of biogeochemical cycles, which will enable us to better describe the C cycle in forests. Firstly methods to estimate EMM production are described and discussed, since the methodology in this field has developed rapidly over the last decades. We then discuss the advantages and disadvantages of different methods to estimate fungal biomass. Finally we discuss how we can

assess the turnover of fungal hyphae. This area needs clearly to be developed in future research as it is a key process in C sequestration of forest soils. We also include aspects of sampling strategies and indirect estimates of EMM production that have great potential for the future. The mechanisms through which EMM regulate C cycling in terrestrial ecosystems have been considered recently in another review (Cairney, 2012).

## 2. Measurements of mycorrhizal hyphal production

A key problem in the determination of mycorrhizal hyphal production is lack of methods to distinguish growth of mycorrhizal hyphae from that of saprotrophic fungi. As ECM fungi do not form a monophyletic clade (Hibbett et al., 2000; Tedersoo et al., 2010) no single biochemical or DNA based marker can be found to quantify this group from the complex soil environment. Therefore various methods are needed to distinguish the biomass of EMM from that of other fungal mycelia. Mycelial growth can be estimated by direct observation in minirhizotrons (Treseder et al., 2005; Pritchard et al., 2008; Vargas and Allen, 2008a) and by the use of root free in-growth bags or cores, which is the most commonly applied method to measure EMM production in forests (Wallander et al., 2001; Godbold et al., 2006; Hendricks et al., 2006; Kjoller, 2006; Korkama et al., 2007; Parrent and Vilgalys, 2007; Hedh et al., 2008; Majdi et al., 2008).

### 2.1. Observational methods

The first observational studies used plastic sheets placed at the litter/soil interface above root clusters where individual ECM tips were observed by pulling back and replacing the litter at different times (Orlov, 1957, 1960). Lussenhop and Fogel (1999) used a method developed by Waid and Woodman (1957) to estimate hyphal production of the ECM fungus *Cenococcum geophilum* by burying nylon mesh in the soil and harvesting them at two week intervals. Rygiiewicz et al. (1997) introduced the minirhizotron technique, commonly used to study fine roots, to measure temporal occurrence and lifetime of mycorrhizal root tips. However, the use

**Table 1**  
Strengths and weaknesses of currently used methods to estimate production of ECM extramatrical mycelium (EMM).

Methods	Strengths	Weaknesses	Comments
Production of ECM mycelium	<ul style="list-style-type: none"> <li>• Repeated non-destructive sampling possible.</li> <li>• Not dependent on conversion factors.</li> </ul>	<ul style="list-style-type: none"> <li>• Cannot differentiate between saprotrophic or mycorrhizal hyphae.</li> <li>• High resolution needed to observe individual hyphae.</li> <li>• Growth might be different in observation chamber compared to soil.</li> <li>• Difficult to transfer to biomass per land area.</li> </ul>	<ul style="list-style-type: none"> <li>• Changes in rhizomorph production, which are easier to observe, does not automatically imply similar changes in total EMM production.</li> </ul>
Root free in-growth mesh-bags or cores	<ul style="list-style-type: none"> <li>• Easy and relatively cheap method that can be applied in large scales.</li> <li>• Substrates that have no background of old mycelium, chemical markers, DNA etc. can be used.</li> <li>• Substrates can be 'spiked' with isotopic labelled materials, minerals etc.</li> <li>• Relative comparisons may be more reliable than estimates of absolute amounts.</li> </ul>	<ul style="list-style-type: none"> <li>• Growth, standing biomass and turnover may be different in mesh bags compared to soil, and this needs to be further studied.</li> <li>• May select for early colonizers of fungus free space.</li> <li>• Disturbance at installation &amp; harvest. Interactions with soil animals are restricted.</li> <li>• The way the mycelial biomass is assessed may give different results.</li> </ul>	<ul style="list-style-type: none"> <li>• When bags are left in the soil over years or more, the mycelial mass is possibly a reflection of the standing biomass rather than production?</li> <li>• Disturbance is probably larger for larger bags or cores.</li> <li>• Mycelial biomass can be assessed with: dry weight, loss on ignition or with chemical markers.</li> </ul>
Assessment of exploration types	<ul style="list-style-type: none"> <li>• Definition of exploration types is based on EMM production.</li> <li>• ECM communities have been studied in a number of forest ecosystems.</li> <li>• Possible to combine with molecular methods to indirectly non-destructively estimate EMM production.</li> </ul>	<ul style="list-style-type: none"> <li>• Estimation of EMM production is based on observations from (simplified) laboratory conditions – growth might be different in soil due to nutrient conditions and season etc.</li> </ul>	<ul style="list-style-type: none"> <li>• Only 5–10% of all ECM fungi have been characterized and are assigned into exploration types.</li> </ul>

**Table 2**  
Strengths and weaknesses of currently used methods to estimate the biomass of ECM extramatrical mycelium (EMM).

Methods	Strengths	Weaknesses	Comments	
Biomass of ECM mycelium	Direct measurement of mycelium length in the soil	<ul style="list-style-type: none"> <li>Not dependent on chemical conversion factors.</li> </ul>	<ul style="list-style-type: none"> <li>Difficult to separate mycelium of mycorrhizal and decomposing fungi and living biomass from necromass.</li> <li>Dependent on correct conversion factors from length to biomass.</li> </ul>	
	Root free in-growth mesh-bags or cores	<ul style="list-style-type: none"> <li>See above for mycelium production using bags.</li> </ul>	<ul style="list-style-type: none"> <li>See above for mycelium production using bags.</li> <li>See comments on production estimates using in-growth bags above.</li> </ul>	
	Chemical markers (chitin, ergosterol, PLFAs) combined with incubation	<ul style="list-style-type: none"> <li>Highly sensitive, small amounts can be estimated.</li> </ul>	<ul style="list-style-type: none"> <li>Dependent on conversion factors which can vary between species and growth conditions.</li> </ul>	<ul style="list-style-type: none"> <li>Field studies on variation in conversion factors are lacking.</li> </ul>
	Molecular DNA and RNA methods	<ul style="list-style-type: none"> <li>Possible to estimate biomass of individual species.</li> <li>Targeted especially to dominant species in ECM communities.</li> <li>Techniques under fast development.</li> </ul>	<ul style="list-style-type: none"> <li>Suitable primers depend on fungal species, a number yet to be developed.</li> <li>High costs of next generation sequencing.</li> </ul>	<ul style="list-style-type: none"> <li>Techniques are under development</li> </ul>
Assessment of exploration types	<ul style="list-style-type: none"> <li>Data from ECM Communities on root tips can be extrapolated to EMM.</li> <li>Non-destructive estimation of EMM production possible based on ECM community composition.</li> </ul>	<ul style="list-style-type: none"> <li>EMM biomass of individual exploration types is based on a combination of previously defined estimations.</li> <li>Few ECM types have been grown in cultures, therefore species-specific fungal diameter and conversion of volume into biomass needs further studies.</li> </ul>		

of observational methods to estimate production, biomass and turnover of EMM in the field has been limited. It has mostly been used to study mycorrhizal roots tips (e.g. Rygielwicz et al., 1997; Majdi et al., 2001; Tingey et al., 2005), but few attempts have been made to estimate the length and longevity of rhizomorphs and hyphae (Treseder et al., 2005; Pritchard et al., 2008; Vargas and Allen, 2008a,b). Similar observations may also be possible using root observation windows (Stober et al., 2000). However, none of the direct techniques can distinguish between the mycelium of ECM and saprotroph mycelia. Two types of minirhizotron cameras are commonly used, which also give different image sizes; BTC 100× microvideo camera (Bartz Technologies, Santa Barbara, CA, USA) that provides image sizes of 1.9 × 1.3 cm, and a CI-600 (CID Bio-Science Inc., Camas, WA, USA) that provides a 360-degree image (21.59 × 19.56 cm). The advantage of the minirhizotron techniques, unlike other methods that rely on excavation which can

disrupt extraradical hyphae, is the potential to make repeated, non-destructive observations *in situ* of the same specimen. This allows the specimen to be followed from its emergence (birth) to its disappearance (death). Although the technique has been found useful to monitor the formation and death of mycorrhizal root tips as well as rhizomorphs, several shortcomings exist. For instance, the minirhizotron technique is limited by the resolution and quality of the images (although the technology in this area is progressing rapidly, see for instance Rundel et al., 2009) and the time required for processing (which also restricts sampling intensity, depth and the number of tubes used). Since the technique cannot yet capture the production and turnover of diffuse mycelium it does not enable calculation of overall mycelium production and turnover rates. Furthermore, there is uncertainty in determining when a rhizomorph is dead, leading to the use of different criteria. For instance, Treseder et al. (2005) classified the time of death as the first visual

**Table 3**  
Strengths and weaknesses of currently used methods to estimate the turnover of ECM extramatrical mycelium (EMM).

Methods	Strengths	Weaknesses	Comments	
Turnover of ECM mycelium	Direct minirhizotron	<ul style="list-style-type: none"> <li>Birth and death of individual hyphae can be followed.</li> </ul>	<ul style="list-style-type: none"> <li>Risk of missing the exact birth or death of the hyphae (recording frequency dependent).</li> <li>May target the fast turnover pool since the length of the study period is limited.</li> </ul>	<ul style="list-style-type: none"> <li>The problem with lag-time can possibly be solved if small vertically installed bags are used. But this needs to be evaluated.</li> </ul>
	Direct measurements in growth mesh-bags	<ul style="list-style-type: none"> <li>In areas with rapid EMM growth and insignificant lag times for mesh bag colonization, sequential harvests at different incubation times could be a way to estimate turnover times.</li> </ul>	<ul style="list-style-type: none"> <li>Lag-times to colonize the mesh bags may be too high for this method to give reliable results (see Fig. 1).</li> <li>Turnover may be different in sand than in soil.</li> </ul>	<ul style="list-style-type: none"> <li>The problem with lag-time can possibly be solved if small vertically installed bags are used. But this needs to be evaluated.</li> </ul>
	Isotopic techniques	<ul style="list-style-type: none"> <li>Pulse labelling via the plant is possible.</li> <li>Mesh bags amended with C<sub>4</sub> substrates can be used to continuously measure C input.</li> </ul>	<ul style="list-style-type: none"> <li>Analyses of bulk mycelial materials may give false impression of a fast turnover. Analyses of isotopes in structural components would solve that problem.</li> <li>The method to use C<sub>4</sub> materials is not very sensitive, large fluxes are needed for reliable results.</li> </ul>	

appearance of fragmentation of the rhizomorph, whereas some authors also used the disappearance from the image for determining the death of a rhizomorph (e.g. Pritchard et al., 2008). If a rhizomorph disappears, a judgment had to be made as to whether the rhizomorph has truly died or has become obscured from view due to soil or tube movement. Both criteria are often used for estimating turnover, but may give highly variable results when compared (Børja et al., unpublished). Furthermore, it is not possible to know exactly when a rhizomorph may form or disappear from the camera's visual field between any two subsequent recording events (typically a month, but new automated minirhizotrons for recording images at multiple times per day are in progress (Rundel et al., 2009)). The long lifetime of some rhizomorphs makes it difficult to estimate turnover rate since most minirhizotron studies are conducted over a one (or two) year period. Thus, when using minirhizotrons to estimate production and turnover of rhizomorphs, it is important to consider the recording frequency and study length because both of these affect the accuracy of the estimations.

One method, which was not applied in a forest, but is worthy of mentioning is the 'root box' method of Coutts and Nicoll (1990), as it allows detailed investigation of the growth and survival of diffuse mycelium as well as of rhizomorphs over the year. These authors planted pine seedlings in peat in 2 m tall transparent acrylic tubes, placed the tubes outside and followed the growth of mycelia and rhizomorphs in detail daily from March 1987 to April 1988. This technique may be ideal for detailed studies of various ECM symbioses, for example studies of the different exploration types as defined by Agerer (see below Section 6). Although observational methods have limitations, they also have many advantages, which can substantially increase our understanding of mycelia production and turnover.

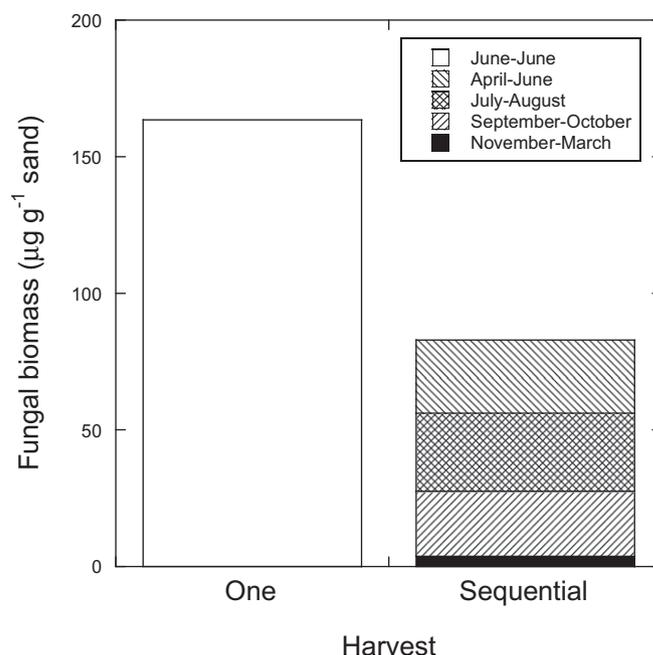
## 2.2. In-growth mesh bags and cores

Mesh bags (e.g. Wallander et al., 2001) are typically made from nylon mesh fine enough to prevent in-growth of roots, but large enough to allow in-growth of fungal hyphae. The fungal communities that colonize the mesh bags are usually dominated by mycorrhizal hyphae as has been verified by trenching experiments (Wallander et al., 2001) and with DNA analyses (Kjøller, 2006; Korkama et al., 2007; Parrent and Vilgalys, 2007; Hedh et al., 2008; Wallander et al., 2010). Mesh sizes between 25 and 50  $\mu\text{m}$  are commonly used. In forest soils with little understorey vegetation, 50  $\mu\text{m}$  prevents in-growth of tree roots, but if understorey Ericaceae or herbs are present, care should be taken so that the fine roots of these do not penetrate the mesh. For example, the fine "hair roots" of ericaceous plants can have diameters of just 20  $\mu\text{m}$  (Bonfante-Fasolo and Gianinazzi-Pearson, 1979). The bags can have different forms and the sides of the nylon mesh can be sealed by sewing, heating and gluing.

The mesh bags are usually placed at the interface between mineral and organic horizons. This will maximize fungal in-growth since mycorrhizal fungi are most abundant in this region (Lindahl et al., 2007). However, when the main aim is to estimate EMM production on an area basis, tubular bags that are placed vertically to a desired soil depth have been used (e.g. Kjøller, 2006). This design also allows the comparison of adjacent soil and root samples taken with the same volume, and it is suitable for sequential harvests since the mesh bags can be replaced with minimal disturbance. In addition to bags, cores can be made of plastic tubes with windows made of mesh to allow fungal in-growth. One advantage with such cores is that they can be rotated regularly to detach fungal in-growth in order to function as controls with similar soil physical conditions but no, or little, fungal in-growth

(Johnson et al., 2001, 2002a,b). This is a considerable advantage when the cores are filled with a natural substrate such as soil (see below). Keeping the volume of the in-growth bags (or cores) as small as possible is important when quantifying EMM production because this helps to ensure that soil physical and chemical conditions inside mesh bags are similar to those outside. In addition, small bags may be colonized more rapidly than larger ones.

Mesh bags are usually incubated in the soil during one growing season because this will give the net production for that year. In some cases a prolonged incubation time (two growing seasons) is necessary in order to detect EMM stimulation by specific substrates such as apatite or other minerals (e.g. Hagerberg et al., 2003; Potila et al., 2009). Berner et al. (2012) suggested that this may be an effect of early colonization by fast-growing ECM species, while species stimulated by minerals are more slowly growing. It has been shown that the stimulation of EMM by apatite was dependent on the P status of the forest (Wallander and Thelin, 2008), while other studies showed that large differences in EMM growth occurred after 5 months along a nitrogen deposition gradient (Kjøller et al., 2012) and in a nitrogen fertilized forest (Nilsson and Wallander, 2003). These findings show that effects of forest management on EMM growth can sometimes be detected with shorter incubation periods. The length of the incubation period thus depends on the purpose of the study. If the main goal is to test for differences between treatments (e.g. forest management or effects of substrates amended to the mesh bags), a longer incubation time can be used. But if the main goal is to estimate net annual production from a specific site, one growing season should be used. On the other hand, if quantifying temporal variation in fungal production is the goal, shorter incubation times than one growing season are used (e.g. Nilsson et al., 2005). Regardless of the approach, it should be noted that a lag time exists before EMM enter bags after they have been inserted into the soil. As an illustration of this, twice as much fungal biomass was found in mesh



**Fig. 1.** Fungal in-growth into mesh bags buried in young (10–20 years) Norway spruce forests at Tönnersjöhedens experimental park. Bags were either incubated for 12 months (one harvest) or for 2 (July–August, September–October), 3 (April–June) or 5 (November–March) months periods (sequential harvests). SE for the mean EMM production after one harvest was 37.5, and the SE for the added sequential harvests was 9.3.

bags that were incubated for 12 months, compared to the added amounts in mesh bags that were incubated for 2–5 month periods in 10 young Norway spruce sites in southern Sweden (Fig. 1). Another aspect that complicates the estimate of production is the turnover of the fungal biomass in the mesh bags. A longer incubation period allows more necromass to form and decompose, which results in underestimation of the total production.

There has been concerns raised that the use of pure quartz sand in mesh bags may affect growth of EMM, which can lead to inaccuracies in production rates and biomass estimates (Hendricks et al., 2006). Hendricks et al. (2006) used 10 cm wide cores placed *in situ* for 1 month to demonstrate that mycelial in-growth was greater when natural soil was used as the in-growth substrate rather than pure sand. Whilst for many habitats the use of natural soils as a substrate is desirable, subsequent measurements can be confounded because of the large and variable amounts of background fungal biomass. If more specific methods to quantify ECM fungal biomass are developed (see below Section 3.6), natural soil could be used more reliably. Indeed, growth of arbuscular mycorrhizal fungi has been quantified in mesh bags amended with natural SOM using fatty acids (Labidi et al., 2007; Hammer et al., 2011), which are available for this mycorrhizal group (NLFA 16.1ω5, Section 3.5). Another uncertainty with the mesh bag method is that some ECM fungi appear to show preferences towards certain types of resource. In addition, some species avoid growing in mineral substrates (*Cortinarius*) probably because they are adapted to an environment where they utilize organic nutrients from SOM (Read and Perez-Moreno, 2003). Therefore, despite being abundant on root tips, species within the genera *Cortinarius* may avoid sand filled mesh bags even when they are common on the root tips while the opposite situation is the case for other species (e.g. *Xerocomus*; Kjølner, 2006; Kjølner et al., 2012). The EMM community in mesh bags may thus not represent the community that prevails in the soil, which may be a problem in some studies. An important advantage with the mesh bag method is that the fungi studied are recently formed, while fungi that we can detect in the soil can be old and inactive (see below Section 2.2).

Another important aspect that needs to be considered is that newly placed mesh bags provide a non-exploited area in the soil. Such spaces are probably rare in established forests but may be common in newly planted forests where the EMM from the previous forest can be expected to die back. In a tree age chronosequence, the EMM production was 3 times greater in young forest (10–20 y) compared to older forests (30–130 y) suggesting that young trees are investing more C to establish a mycorrhizal network, while less C is needed to sustain this network in older forests (Wallander et al., 2010). It is possible that mesh bags select for fast-growing species adapted to newly planted forests. For this reason, EMM production may be overestimated when incubating mesh bags over one growing season. As noted previously, such effects may be minimized by reducing as far as possible the volume of mesh bags and cores.

From the discussion above it seems that some factors result in overestimation while other results in underestimation of EMM production using the mesh bag method. As methods to measure fungal biomass and necromass improve (see e.g. Section 3.6), it might be possible to follow the fungal community in mesh bags over several years and quantify the yearly production after the initial empty space has been colonized. A combination of chitin and ergosterol analysis (see below) may give an indication of the ratio between biomass and necromass. Another way to quantify annual production of EMM, including necromass, is to analyse the isotopic change in  $^{13}\text{C}/^{12}\text{C}$  in mesh bags that have been amended with organic material from  $\text{C}_4$  plants, and follow this change through time (Wallander et al., 2011). A similar approach was used by

Godbold et al. (2006) who filled cores with  $\text{C}_4$  soil to estimate the contribution of fungal hyphae to new soil C over a 2.5 year period. Amendment of organic matter in the mesh bags would make the substrate more natural for growth of ECM fungi and probably produce communities more similar to those of the surrounding soil but brings with it greater abundance of saprotrophic fungi. An interesting approach to reduce in-growth by saprotrophic fungi but still use more natural soil was reported by Melanie Jones and co-workers in Canada who used an outer mesh bag with sand, which functioned as a barrier for saprotrophs, and an inner mesh bag with sterilized soil where EMM of ECM fungi proliferated (Lori Phillips and Melanie Jones, pers. comm.).

It is clear that the fungal community colonizing mesh bags may not accurately mirror the mycelial community in natural soil i.e. some species or clades may be over represented and some are underrepresented or even missing in the mesh bags. On the other hand, when working with natural soil it is also difficult to claim that only EMM are in the extracted DNA pool. One needs to be very careful in removing all roots and in reality it will be difficult to state that a soil sample is indeed completely free of ectomycorrhizal root tips or small detached pieces of ectomycorrhizal mantle. Furthermore, extraction of fungal spores in the soil may lead to false positives in the community profile. Extracting DNA or RNA from sand-filled mesh-bags at least ensures that only nucleic acid from actively (or recently active) mycelia is amplified. Another benefit is that the hyphae from the mesh bags are easily extracted from the sand and simple and cheap nucleic acid extraction methods can be applied to produce good quality templates for PCR. Whether extracting nucleic acid from sand-filled mesh bags or directly from soil, primer bias is a confounding factor preventing an accurate description of the fungal community. For each specific primer combination chosen, some groups will be over, and some groups under expressed or even completely missed (Bellemain et al., 2010). As an example of the latter, *Tulasnella* sp. are often completely missed with the standard ITS1-F and ITS4 primer combination (Taylor and McCormick, 2008). In general, careful consideration of primers combinations for the specific study system in question should be made, and the results obtained treated with sound caution.

### 3. Quantification of fungal biomass in mesh bags and soil

The examination of mycelia in mesh bags should start with a visual classification under a dissecting microscope. This allows a check for the presence of mycelial strands, whether or not they are hydrophilic, and gives insights in exploration types of mycorrhizal fungi (see below Section 5). The amounts of total hyphae can be estimated either by extracting fungal hyphae and converting estimates of hyphal length to biomass, or by using different chemical markers (chitin, ergosterol, phospholipid fatty acid 18:2ω6,9) as proxies for biomass. These methods are described below and the benefits and disadvantages are discussed (Tables 1–3).

#### 3.1. Direct measurements of fungal weight and hyphal length

One approach to estimate fungal biomass that can be used in mesh bags only, is to extract the mycelium from the sand substrate and determine its weight. In this way conversion factors between biomass and a chemical marker can be avoided, but it assumes that all extractable matter is of fungal origin. This is not the case because bacteria and precipitated SOM can be present in the mesh bags, but they probably contribute very little to the weight of putative fungal material extracted. Since it is difficult to remove all sand grains, it is usually necessary to burn the extracted mycelia and use the loss on

ignition as an estimate of the biomass (Hagerberg et al., 2003; Korkkama et al., 2007). The C concentration of fungal material is approximately constant (around 45%; Taylor et al., 2003) and C content can be used as a proxy for biomass in the mesh bags. When analysed on a mass spectrometer, both the content and isotopic signature of C can be obtained, which makes it possible to calculate the proportion of ECM and saprotrophic mycelia in the mesh bags because these two groups have different isotopic signatures (Wallander et al., 2001). The recovery of mycelium using this method can be tested by analysing the ergosterol content of both the sand (before and after extraction) and the extracted mycelia.

Fungal hyphae can be extracted from the mesh bags and separated from sand particles by centrifugation and collected on a filter paper for estimates of hyphal length. This approach produced similar results as direct estimates of EMM weight as described above (Wallander et al., 2004). Estimates of hyphal lengths can be converted to biomass using conversion factors from Fogel and Hunt (1979). However, a possible problem with this method is to fully account for rhizomorphs, which are multi-hyphal organs produced by many ECM fungi during growth through soil. The rhizomorphs facilitate efficient transport of carbon towards the mycelia front and mineral nutrients towards to mycorrhizal roots (Cairney, 1992). Separate counts must be carried out for rhizomorphs and hyphae, as they differ greatly in weight per unit length.

### 3.2. Chemical markers; chitin

Among the three possible fungal biomarkers, chitin seems the most stable parameter to assess the total fungal contribution to microbial tissue in soil (Joergensen and Wichern, 2008). Recent results (Drigo et al., 2012; Koide et al., 2011) from additions of laboratory cultivated mycelial necromass suggest rapid decomposition of chitin in soil. Indeed, fungal cell walls of all true fungi contain chitin, a structural compound with a similar role as cellulose in higher plants. In soil, other organisms may contribute to chitin contents such as microarthropods that contain chitin in their exoskeleton. However, this contribution is probably minimal as their biomass is typically below 0.5% of the fungal biomass (Beare et al., 1997; Simpson et al., 2004). An average chitin concentration of 5% of dry matter was found in a review of various species of fungi mainly grown *in vitro* and belonging mainly to Basidiomycetes, Ascomycetes and Zygomycetes (Appuhn and Joergensen, 2006). No statistically significant difference between the mean values from the three fungal orders was found, and a conversion factor from glucosamine to fungal C of 9 was proposed (Appuhn and Joergensen, 2006).

Using data from Joergensen and Wichern (2008), we estimate that  $\pm$ one standard deviation around the mean gives a span of around 6–50 of the glucosamine to C conversion factor, which suggests a rather low precision in the conversion. However, it is unknown if the variation in glucosamine content is smaller or larger when in symbiosis. In the one published study known to us, extramatrical mycelium of *Paxillus involutus* in symbiosis with *Pinus sylvestris* had a glucosamine content of 4.5% (Ekblad et al., 1998). A similar value was found in mycelium extracted from mycelial in-growth bags that were installed in the upper-most soil horizon at the tree line in a *Larix decidua* and *Pinus uncinata* stand near Davos. These two values are close to the average for the pure cultures of the Joergensen and Wichern (2008) review.

It is also possible to measure the chitin content from the pellet left after protein, lipid or DNA extraction (Kjøller and Rosendahl, 1996; Kjøller et al., 2012). This then allows measurements of enzyme activities or molecular identity in the exact same samples that are quantified for chitin. Further studies are needed on chitin concentrations in EMM of mycorrhizal fungi when in association

with roots in forest soil. However, this fungal biomarker does not enable us to distinguish between saprotrophic and ECM fungi in soil samples or to separate living and dead mycelium, although this may be possible by combining ergosterol and chitin analysis (see below).

Chitin assay is easily performed using one of two steps; (i) hydrolysis either with KOH (e.g. Frey et al., 1994) that produces deacetylated chitin (chitosan), or with HCl (Appuhn et al., 2004), H<sub>2</sub>SO<sub>4</sub> (e.g. Zamani et al., 2008) or methanesulfonic acid (Olk et al., 2008) that produces glucosamine, and (ii) measurement of the concentrations of hydrolysis products. Chitosan and glucosamine contents can be measured with colorimetric procedures specifically assaying amino sugars (Plassard et al., 1982). Free glucosamine can also be measured with chromatographic techniques (Ekblad et al., 1998).

### 3.3. Chemical markers; ergosterol

The second chemical marker that has been used to estimate fungal biomass is ergosterol (22E)-Ergosta-5,7,22-trien-3 $\beta$ -ol (C<sub>28</sub>H<sub>44</sub>O). This compound is a membrane lipid, found almost exclusively in membranes of living fungal cells, and is the commonest sterol of Ascomycota and Basidiomycota. As ergosterol is generally not synthesized by plants and animals, and only present in low amounts in some microalgae (Grant and West, 1986; Newell et al., 1987; Weete, 1989), it has been frequently used as fungal biomarker in soils (Djajakirana et al., 1996; Möttönen et al., 1999; Bååth, 2001; Wallander et al., 2001; Hagerberg et al., 2003; Zhao et al., 2005; Högberg, 2006; Karliński et al., 2010) and correlations with other methods are usually good (Bermingham et al., 1995; Stahl and Parkin, 1996; Montgomery et al., 2000; Ruzicka et al., 2000; Högberg, 2006). Assay of ergosterol was first employed by Seitz et al. (1977) to quantify fungal infections in stored grain. In mycorrhizal fungi, the analysis of ergosterol was first applied by Salmanowicz and Nylund (1988), but has been used frequently since then (e.g. Nylund and Wallander, 1992; Ekblad et al., 1995, 1998; Laczko et al., 2004; Olsrud et al., 2007). Total ergosterol contents in mycorrhizal roots of *P. sylvestris* plants was correlated to visual estimates of root colonization (Ekblad et al., 1995) as well as to the chitin contents (Ekblad et al., 1998). In contrast, total ergosterol concentration of ericoid hair roots of dwarf shrubs from northern subarctic mires did not correlate with visual estimates of colonization but was instead positively correlated with the colonization of dark septate endophytes, which makes it questionable as a marker for ericoid mycorrhizal fungal colonization (Olsrud et al., 2007). Some studies suggest that ergosterol is a good proxy for active fungal biomass because it was found to degrade shortly after the cells death (Nylund and Wallander, 1992), and ageing mycorrhizal root tips contain low ergosterol concentrations (Ekblad et al., 1998). However, other studies suggest a slow metabolism of ergosterol under certain circumstances, such as disruption of below ground C allocation, increased N loads, addition of toxic compounds like pesticides, or existence of substantial amounts of free ergosterol in soil for considerable periods with little mineralization (Zhao et al., 2005). Soil perturbations, that may negatively influence vitality and growth of soil fungi, resulted in disruption of the proportion between soil ergosterol concentration and soil fungal biomass C (Zhao et al., 2005) and between ergosterol and phospholipid fatty acid (PLFA) 18:2 $\omega$ 6,9 (Högberg, 2006). These contradictory results were further criticized and discussed by Young et al. (2006) and Zhao et al. (2006). Mille-Lindblom et al. (2004) reported very slow degradation of free ergosterol in environmental samples without living mycelium when protected from sunlight and suggested that ergosterol may be stable when connected to dead fungal mycelium.

However, significant degradation of ergosterol was observed by the authors under influence of light.

Calculations of conversion factors from ergosterol to fungal biomass have been derived from various fungi and considerable variations in ergosterol concentration in fungal mycelium were reported (Lösel, 1988; Weete, 1989; Nylund and Wallander, 1992; Djajakirana et al., 1996; Montgomery et al., 2000). The average concentration of ergosterol reported thus far for different soil, aquatic and plant inhabiting fungi is  $4.5 \mu\text{g mg}^{-1}$  dry mass of mycelia, and this is used to determine fungal biomass in soil. However, the ergosterol concentration in fungal mycelium extracted from mesh bags is less ( $1.2 \mu\text{g mg}^{-1}$ ; Hagerberg et al., 2003). This may indicate that laboratory-grown mycelia contain more ergosterol than field grown mycelia, or that mycelia from mesh bags are contaminated with non-fungal material. The average relative recovery of ergosterol from soil samples was 62%, ranging from 58 to 88% (Montgomery et al., 2000), and the recovery factor value was 1.61 (1/0.62). The authors concluded that determination of fungal biomass (FB) on the basis of ergosterol analysis requires correcting ergosterol concentrations by the proportion of unextracted mycelial ergosterol according to the following calculation:

$$FB(\mu\text{g g}^{-1} \text{ soil}) = \text{Ergosterol}(\mu\text{g g}^{-1} \text{ soil}) \times f \times Rf,$$

where  $f = 250$  ( $1/4 \times 1000$ , mg biomass  $\mu\text{g}^{-1}$  ergosterol), and  $Rf = 1.61$  (correction factor for average percent recovery, 1/0.62) (Montgomery et al., 2000).

Separation of ergosterol into free and esterified forms might give some additional information of the vitality of the fungal mycelium. Usually total ergosterol is quantified (Nylund and Wallander, 1992), in other cases the free form is used as a biomass marker (Martin et al., 1990). Free ergosterol is a component of the cell membranes, while the esters are found in cytosolic lipid particles. A  $^{14}\text{C}$ -labelling study of *Saccharomyces cerevisiae* indicated that the free sterols and esters are freely inter-changeable and that relatively more esters are formed when the fungus is going into a stationary phase (Taylor and Parks, 1978). Analysis of dried fungal material suggests that the free form can also be converted into the esterified form in this material and that the esterified is more stable than the free form (Yuan et al., 2008). The majority of ergosterol from in-growth bags was found in the free form (90%), while the free ergosterol was below 20% in the mineral soil, supporting the view of increasing proportion of esterified ergosterol in older SOM (Wallander et al., 2010). The relation between free and esterified ergosterol and ergosterol and chitin (Ekblad et al., 1998) could potentially be used as markers for the ratio of active and inactive fungi in soil. This possibility would be very useful but needs to be evaluated further. One problem with analysing free ergosterol in certain soils is to get the extracts clean for chromatographic analysis (Adam Bahr, pers. comm.). Ergosterol can be easily extracted from variable materials and is detectable in low concentrations. The assay comprises of extraction, purification and quantification of the molecule using high-performance liquid chromatography with a UV detector. Young (1995) developed an efficient microwave-assisted method (MAE) to extract ergosterol from a variety of matrices, which has since been applied to soil samples (Montgomery et al., 2000).

### 3.4. Chemical markers; PLFAs

PLFAs are essential components of cell membranes and they decompose quickly after cell death (White et al., 1979) and are commonly used as chemical markers of soil fungi. As eukaryotes and different groups of prokaryotes contain more or less specific

ester-linked lipid fatty acids (Lechevalier and Lechevalier, 1988; Zelles, 1997, 1999), the analysis of PLFA composition and concentrations are useful as a tool for quantitative and qualitative examination of microbial communities in soil (fungi, bacteria, protozoa; e.g. Tunlid and White, 1992; Cavigelli et al., 1995). However, use of PLFAs for biomass estimation has recently been questioned, because the same PLFAs are stated to indicate very different groups of organism (Frostegård et al., 2011). For instance, the PLFAs cy17:0 and cy19:0, usually considered to be indicators of Gram-negative bacteria are also found in large amounts in some Gram-positive bacteria (Schoug et al., 2008). The PLFA 16:1 $\omega$ 5, common in arbuscular mycorrhizal fungi (Graham et al., 1995; Olsson et al., 1995), and sometimes used as a marker of Glomeromycota fungi in soil, plant roots and external mycelium (e.g. Gryndler et al., 2006), is also found in bacteria (Nichols et al., 1986). Moreover, some environmental conditions, such as temperature or toxic soil contaminants may influence the rate of PLFA degradation, independently with the turnover of soil microorganisms (Frostegård et al., 2011).

The PLFA 18:2 $\omega$ 6,9 is the most commonly used PLFA to estimate fungal biomass (Wassef, 1977; Lechevalier and Lechevalier, 1988; Dembitsky et al., 1992). It occurs in all eukaryotes, and is only found in low amounts in bacteria. This PLFA is a dominating fatty acid of fungal fruit bodies (e.g. Dembitsky et al., 1992; Olsson, 1999; Karliński et al., 2007) and spores (Brondz et al., 2004). A strong positive correlation was found between PLFA 18:2 $\omega$ 6,9 and the fungal marker ergosterol in soils from cultivated fields, gardens, grasslands and forests (Frostegård and Bååth, 1996; Kaiser et al., 2010). The PLFA 18:2 $\omega$ 6,9 has been used as a bioindicator of EMM in soil (Högberg et al., 2010), but it is particularly useful in experiments where other soil fungi can be eliminated or reduced, such as when using in-grow mesh bags where ECM mycelium is preferentially trapped (e.g. Wallander et al., 2001; Hagerberg and Wallander, 2002). To convert PLFA 18:2 $\omega$ 6,9 to microbial carbon content, Joergensen and Wichern (2008) reported a weighted conversion factor of  $107 \mu\text{g C nmol PLFA}^{-1}$ , but values between different species grown in culture could vary 17-fold (Klamer and Bååth, 2004). Another PLFA that is common in fungi, especially Zygomycota, is 18:1 $\omega$ 9 (Dembitsky et al., 1992; Ruess et al., 2002; Brondz et al., 2004). The concentration of 18:1 $\omega$ 9 is usually closely correlated to 18:2 $\omega$ 6,9 (Frostegård et al., 2011). This PLFA is, however, also present in some bacteria (Schoug et al., 2008) and has not proven useful as a fungal indicator in agricultural soils (Frostegård et al., 2011).

A faster way to analyse fatty acids in soil samples is to analyse the whole cell fatty acids (WCFAs) without separation of neutral lipid fatty acids (NLFAs) and PLFAs. WCFAs reflect both microbial biomass and energy reserves of eukaryotes and are a relatively reliable method of studying fungi (Larsen et al., 2000; Thygesen et al., 2004; Karliński et al., 2007) and mycorrhiza-associated microorganisms in the field (Brondz et al., 2004; Ruess et al., 2005; Karliński et al., 2007). The analysis of WCFA composition requires 10 times less soil material than the PLFA analysis (Drenovsky et al., 2004). Since much of the WCFA is in the form of neutral lipid fatty acids (NLFAs) in triacylglycerols, a storage compound in eukaryotes, a recorded change in WCFA of NLFAs may be a result of changes in the amount of storage C rather than a change in size of the microbial population in a soil. Incorporation of glucose into fatty acids can be used to demonstrate the high microbial activity in soils. Lundberg et al. (2001) used 'solution state'  $^{13}\text{C}$  NMR and found that the amount of  $^{13}\text{C}$  in fatty acids peaked 3–13 days after glucose addition to a forest soil, and that it had declined by 60% 28 days after the glucose addition. A similar result was found after extraction and analyses of NLFAs and PLFAs at different time intervals after glucose additions to various soils

(Bååth, 2003). Due to the potential for large temporal variation in storage triacylglycerols, NLFAs and WCFAs are probably less suitable than PLFAs as relative measures of the microbial biomass in soils. However, the ratio of neutral lipid fatty acids (NLFAs) and PLFAs was proposed as a method to study the physiological state of the microbial population in the soil (Bååth, 2003).

The analytical procedure for PLFAs and NLFAs comprises four steps: (i) extraction of lipids, (ii) lipid fractionation, (iii) mild alkaline methanolysis, and (iv) GC analyses (White et al., 1979; Frostegård et al., 1991). Recently, the lipid fractionation was modified slightly by Dickson et al. (2009), who reported that the replacement of pure chloroform by the mixture chloroform: acetic acid (100:1, v/v) increased the effectiveness of NLFAs elution from the silica columns and eliminated an interference of NLFAs with glycolipid and phospholipid fractions. Following hydrolysis, their fatty acids (FA) are released and detected using gas chromatography (GC). PLFA analyses should be done as soon as possible after sampling since the composition may change even when stored at low temperatures (Wu et al., 2009). The best strategy is to shock-freeze the samples with liquid nitrogen and further storage at  $-18^{\circ}\text{C}$  until analysis. Homogenization of soil samples using a ball mill to a particle size less than  $10\ \mu\text{m}$  prior to analysis has been recommended to achieve the most reliable results (Wilkinson et al., 2002).

### 3.5. Comparison of chemical markers

It is clear that each of the chemical markers described will bring different information about the fungal biomass, whether total or active. Each of them has advantages and limitations (Tables 1–3). Chitin and ergosterol assays are easier to carry-out than fatty acid (PLFAs or WCFAs) extraction, but fatty acid profiles will bring more information about microbial communities than chitin and ergosterol. On the other hand, the PLFA method is more rapid and less expensive than methods based on nucleic acids (Ramsey et al., 2006; Frostegård et al., 2011). However, none of these chemical markers will enable us to distinguish between fungal types (ECM versus non-mycorrhizal fungi) that can be present in forest soil samples. To distinguish between these “functional” types, molecular analysis (see below) should be used. Biomass estimates when using biomarkers, such as ergosterol, chitin and PLFAs, are highly dependent on the use of conversion factors. Different fungal species vary in concentrations of such biomarkers, but the biomarker to biomass ratio is probably more stable in a more complex community. The concentration of ergosterol in pure cultures of ECM fungi ranged between 1.8 and  $17.6\ \text{mg g}^{-1}$  d.wt. (Nylund and Wallander, 1992; Olsson et al., 1995) and concentration of PLFA 18:2 $\omega$ 6,9 ranged from 0.45 to  $12\ \mu\text{mol g}^{-1}$  d.wt. (Olsson et al., 2003). The content of the WCFAs 18:2 $\omega$ 6,9 was reported as 17–75% of total WCFAs in fruit bodies and as 53–71% of total WCFAs in axenic cultures of ECM fungi (Karliński et al., 2007). Biomarker concentrations may reflect both the biomass and community composition of fungi. In addition, concentrations in a single species can change due to different environmental conditions as were reported for wood-rotting basidiomycete isolates grown in different soils (Tornberg et al., 2003) and ageing, as shown for ergosterol concentrations in the basidiomycete *Hebeloma cylindrosporum* (Plassard et al., 2000), and for ergosterol and fatty acids in pure culture of ECM fungus *Pisolithus tinctorius* (Laczko et al., 2004).

### 3.6. Potential of qPCR for the quantification of EMM biomass

In addition to the lipidic or polysaccharidic markers to quantify the biomass of fungi, the developments of quantitative PCR (qPCR) seem to offer a possible taxon-based alternative. The strength of

DNA (or RNA) based methods is that potentially any phylogenetic level from genotypes to large groups or even total (true) fungi can be targeted (Fierer et al., 2005; Šnajdr et al., 2011). Indeed, methods to quantify general fungi or basidiomycetes have been proposed and tested (Fierer et al., 2005; Manter and Vivanco, 2007; Feinstein et al., 2009). A single species laboratory study comparing quantification of *Trametes versicolor* in wood based on chitin content, ergosterol, wood mass loss, and qPCR, showed reasonable correlations with more discrepancies occurring only with older cultures (Eikenes et al., 2005). There are currently two main limitations of the methodology: nucleic acid extraction bias and the differences in target occurrences per unit DNA or biomass. Different methods of nucleic acid extraction yield not only different quality of DNA and RNA but also different proportions of microbial taxa in the extracts (Feinstein et al., 2009). The success of qPCR rapidly decreases with fragmentation of nucleic acids, resulting in lower counts of target sequences per unit DNA. If a treatment is imposed that alters the extractability of nucleic acid or if different soil types are to be compared, this may influence the qPCR success. For the most frequently used target sequence of fungi-specific qPCR – the rDNA cassette – significant differences in copy number per genome were recorded, ranging from 10 to 200 in different species (Garber et al., 1988; Maleszka and Clark-Walker, 1990; Corradi et al., 2007; Amend et al., 2010), which adds another important source of bias. With the advance of fungal population genomics (five ECM species sequenced to date; see the website of JGI (<http://genome.jgi-psf.org/>) and Martin et al., 2008, 2010) in the future it may be possible to identify a universal single copy gene with adequate sequence variation for counting fungal genomes rather than rDNA copies or for delimitation of certain fungal taxa. Population genomics also brings even greater potential to test hypotheses concerning the contribution of particular genotypes to ECM fungal biomass and turnover (Johnson et al., 2012).

When qPCR specifically targets individual species of fungi, PCR-based abundance estimates represent a plausible proxy of fungal biomass content because the numbers of rDNA copies do not show high variation within a species (Amend et al., 2010). Analyses of individual fungi including *Suillus bovinus*, *P. involutus* and *Hypholoma fasciculare* in the DNA from complex samples showed that it is possible to use qPCR to specifically quantify the biomass of fungi at the species level within a community. Such data are comparable to the much more laborious or expensive approaches like cloning, pyrosequencing or DGGE approaches (Landeweert et al., 2003; Parladé et al., 2007; Šnajdr et al., 2011). Competitive PCR (a variant of qPCR) was used to demonstrate that *Hebeloma cylindrosporum* biomass in bulk soil is greatest near fruit bodies (Guidot et al., 2002). A conversion factor between qPCR-based copy number and fungal biomass and hyphal length was obtained for laboratory cultures of the ECM fungus *Piloderma croceum* showing its potential to quantify the biomass of particular species (Raidl et al., 2005). Unfortunately, due to the appearance of ECM fungi in multiple phylogenetic lineages, the finding of suitable primers to specifically amplify ECM fungal DNA and to distinguish it from non-ECM fungi is highly improbable. However, if combined with the cloning approaches or next generation sequencing, qPCR may provide estimates of ECM fungal biomass in soils. Contemporary next generation sequencing results showed that, at least in certain forest soils, fungal communities are dominated by relatively few species (Buée et al., 2009; Baldrian et al., 2012). These findings suggest that qPCR can be used to target specifically the identified dominant members of the community as an estimate of ECM fungal biomass. Recently, qPCR used for analysis of environmental samples has been expanded from the quantification of DNA towards the quantification of RNA, typically the rRNA, representing microbial ribosomes or ITS sequences in unspliced transcripts of the rDNA

operon. Although it is unknown whether the DNA or the RNA content better corresponds with the quantity of fungal biomass, it is clear that the analysis of ITS sequences in the non-spliced rDNA transcripts (indicating fungal taxa synthesizing their ribosomes) is more suitable to quantify the active part of the fungal community (Anderson and Parkin, 2007). Indeed, decomposers in spruce logs or fungi active in soil in winter with limited photosynthate allocation have been specifically identified by combining DNA and RNA analysis (Rajala et al., 2011; Baldrian et al., 2012).

#### 4. Indirect estimation of length, space occupation and biomass of extramatrical mycelium of ectomycorrhizal fungi

Agerer (2001) proposed a classification of ECM mycelial systems into five exploration types. Accurate determination of EMM production and abundance of different exploration types within ECM communities may be used to estimate the overall production of EMM. The exploration types are described according to their pattern of differentiation, indicating their different ecology: contact type (CT), short distance (SD), medium distance (MD), long distance (LD) and pick-a-back (PB) exploration type. The exploration types have been differentiated based on about 400 different morphotypes of ectomycorrhiza, which have been identified as belonging to different fungal species on several host plant roots based on their morphological and anatomical characteristics (Agerer and Rambold, 2004–2011). The characterized ECM morphotypes represent about 5% of known fungi that can form ectomycorrhiza (Taylor and Alexander, 2005), the number of which is estimated to be 5000–6000 fungal species (Agerer, 2006). From this limited database, it appears that in many genera all known species produce only one exploration type (Agerer, 2001; Hobbie and Agerer, 2010), although some genera (i.e. *Russula* spp.) need species-based classification into an exploration type (Table 4).

An estimation of EMM of ECM fungi in natural soils could be deduced from semi-quantitative estimations of the EMM formed by SD and MD exploration types grown in rhizotrons in symbiosis with

Norway spruce (Agerer and Raidl, 2004). The observations in rhizotrons have lately included other MD subtypes and LD exploration types (Weigt et al., 2011), and indices of specific space occupation, mycelial length and biomass were proposed for each exploration type. Mycelial biomass was estimated based on length measurements using calculations described in Weigt et al. (2011); the standard values for the selected exploration types are presented in Table 5. These standards for the most frequent exploration types, expressed as biomass and occupied space of EMM per unit of ECM system, are suggested as basic factors for characterizing mycelial production costs and space occupation in ecological field studies without any extraction of mycelium and for fungal communities in the soils. Since different exploration types show not only differences in distance of EMM from the root tip, space occupation, biomass and energy (C) inputs, but also in other functional relationships within the ecosystem, the ECM fungal community structure and function can be extrapolated. The differentiation into exploration types can be extrapolated from morphotype characterization based on outer morphology of ectomycorrhiza, rhizotron photographs, and fungal species identifications, using molecular based methods of fungal community composition (Grebenc and Kraigher, 2009), in which fungal species identity is linked to growth characteristics and assigned to a certain exploration type.

Indices, such as specific potential mycelial space occupation ( $\text{mm}^2 \text{cm}^{-1} \text{ECM tip}^{-1}$ ), specific EMM length ( $\text{m cm}^{-1} \text{ECM tip}^{-1}$ ), specific EMM biomass ( $\mu\text{g cm}^{-1} \text{ECM tip}^{-1}$ ) can be developed for each exploration type. The specific contribution to EMM by exploration types can be achieved for cultivable and non-cultivable species, and up-scaling of cost–benefit relations is possible (Weigt et al., 2011). The method provides an estimation based on ECM fungi synthesized in experimental laboratory conditions, i.e. on prepared soil substrates, which can influence EMM growth in different exploration types. Therefore, for the calculations presented in Table 5 a number of assumptions had to be made, including i) that growth conditions concerning mycelial growth and space occupation in experimental substrates was similar to

**Table 4**

Representative fungal genera belonging to different exploration types (summarized from Agerer, 2001; Agerer and Rambold, 2004–2011; Agerer, 2006).

Exploration type	Morphology/anatomy	Fungal genus <sup>a</sup>
Contact	Smooth mantle, only few emanating hyphae, ECM tips in close contact with substrates	<i>Arcangiella</i> , <i>Balsamia</i> , <i>Chroogomphus</i> , <i>Craterellus</i> , <sup>b</sup> <i>Lactarius</i> , <sup>c</sup> <i>Leucangium</i> , <i>Russula</i> , <i>Tomentella</i>
Short distance	Voluminous envelope of emanating hyphae, no rhizomorphs	<i>Acephala</i> , <i>Byssocorticium</i> , <i>Cenococcum</i> , <i>Coltricia</i> , <i>Coltriciella</i> , <i>Craterellus</i> , <sup>b</sup> <i>Descolea</i> , <i>Descomycetes</i> , <i>Elaphomyces</i> , <i>Genea</i> , <i>Hebeloma</i> , <i>Humaria</i> , <i>Hygrophorus</i> , <i>Inocybe</i> , <i>Pseudotomentella</i> , <i>Rhodocollybia</i> , <i>Rozites</i> , <i>Russula</i> , <i>Sebacina</i> , <i>Sphaerosporella</i> , <i>Sphaerozone</i> , <i>Tomentella</i> , <i>Tricharina</i> , <i>Tuber</i> , <i>Tylospora</i>
Medium distance: fringe subtype	Fans of emanating hyphae and rhizomorphs, frequent ramifications and anastomoses, rhizomorph surfaces hairy, extended contact to the soil; rhizomorphs type A, <sup>e</sup> exceptionally C, <sup>e</sup> D <sup>e</sup>	<i>Amphinema</i> , <i>Cortinarius</i> , <i>Dermocybe</i> , <i>Hydnum</i> , <i>Lyophyllum</i> , <i>Piloderma</i> , <i>Sistotrema</i> , <i>Stephanopus</i> , <i>Thaxterogaster</i> , <i>Tricholoma</i>
Medium distance: mat subtype	Limited range, rhizomorphs undifferentiated or slightly differentiated type A, <sup>e</sup> C, <sup>e</sup> exceptionally D <sup>e</sup>	<i>Bankera</i> , <i>Boletopsis</i> , <i>Clavariadelphus</i> , <i>Cortinarius</i> , <i>Gautieria</i> , <i>Geastrum</i> , <i>Gomphus</i> , <i>Hydnellum</i> , <i>Hysterangium</i> , <i>Phellodon</i> , <i>Ramaria</i> , <i>Sarcodon</i>
Medium distance: smooth subtype	Rhizomorphs internally undifferentiated, slightly differentiated or with a central core of thick hyphae. Mantles smooth with no or only a few emanating hyphae. Rhizomorphs type B, <sup>e</sup> C <sup>e</sup> and D, <sup>e</sup> exceptionally E <sup>e</sup>	<i>Albatrellus</i> , <i>Amanita</i> , <sup>d</sup> <i>Byssosporia</i> , <i>Cantharellus</i> , <i>Entoloma</i> , <i>Gomphidius</i> , <i>Hygrophorus</i> , <i>Laccaria</i> , <i>Lactarius</i> , <i>Naucoria</i> , <i>Polyporoletus</i> , <i>Pseudotomentella</i> , <i>Russula</i> , <i>Thelephora</i> , <i>Tomentella</i> , <i>Tomentellopsis</i>
Long distance	Smooth mantle with few but highly differentiated rhizomorphs type F. <sup>e</sup> ECM sparsely monopodially branched, coralloid and tuberculate.	<i>Alpova</i> , <i>Amanita</i> , <sup>d</sup> <i>Austropaxillus</i> , <i>Boletinus</i> , <i>Boletus</i> , <i>Chamonixia</i> , <i>Gyrodon</i> , <i>Gyroporus</i> , <i>Leccinum</i> , <i>Melanogaster</i> , <i>Paxillus</i> , <i>Pisolithus</i> , <i>Porphyrellus</i> , <i>Rhizopogon</i> , <i>Scleroderma</i> , <i>Suillus</i> , <i>Truncocolumella</i> , <i>Tricholoma</i> , <i>Tylopilus</i> , <i>Xerocomus</i>
Pick-a-back	Grow within F <sup>e</sup> -type rhizomorphs or mantels, can produce haustoria, can become ectendomycorrhizal. Can form contact, or smooth medium distance type.	<i>Gomphidiaceae</i> ( <i>Gomphidius</i> , <i>Chroogomphus</i> ) growing within <i>Suillus</i> or <i>Rhizopogon</i> ; <i>Boletopsis leucomelaena</i> within unknown ECM; <i>Xerocomus parasiticus</i> within <i>Scleroderma citrinum</i>

<sup>a</sup> In case of controversial issues genus was categorized to exploration types according to Agerer and Rambold (2004–2011).

<sup>b</sup> *Craterellus tubaeformis* forms contact exploration types on *Quercus* but short distance exploration types on *Pinus*.

<sup>c</sup> Underlined genera have representatives in more than one exploration types.

<sup>d</sup> *Amanita citrina* on *Pinus* can form medium distance and long distance exploration types.

<sup>e</sup> The type of rhizomorphs according to Agerer (1987–1998).

**Table 5**  
Characteristics of EMM length, space occupation and biomass for different exploration types (modified after Weigt et al., 2012a,b).

Exploration type	No. of analysed mycelia	Max. distance from root tip (cm)	Projected area per mycelial system (mm <sup>2</sup> )	Mycelial coverage per occupied space (mm <sup>2</sup> mm <sup>-2</sup> )	Specific EMM length (m cm <sup>-1</sup> ECM tip <sup>-1</sup> )	Specific EMM biomass <sup>a</sup> (μg cm <sup>-1</sup> ECM tip <sup>-1</sup> )
Short distance	7	1.2	33 ± 9	0.39 ± 0.07	3.72 ± 1.19	3.24 ± 1.03
Medium distance	14	1.9	84 ± 5	0.58 ± 0.05	6.91 ± 0.54	6.02 ± 0.47
Long distance	3	9.6	630 ± 181	0.28 ± 0.03	55.91 ± 20.25	48.67 ± 17.62

<sup>a</sup> Mycelial biomass was estimated based on length measurements using calculations described in Weigt et al. (2011) combining the formula  $B = r^2\pi*L*D*M$  (Frankland et al., 1978), where  $B$  = fungal biomass,  $r$  = hyphal radius,  $L$  = hyphal length,  $D$  = relative hyphal density,  $M$  = % dry mass = (100 – mycelial moisture content as % of fresh weight)/100.  $r^2\pi*L$  = hyphal biovolume (assuming hyphae to be perfect cylinders) with  $r$  based on the species-specific hyphal diameter (in their study it was 2.2 μm for *Piloderma croceum*, deduced from Brand, 1991; Raidl, 1997).  $L$  was measured using WinRhizo.  $D = 1.09$  g/cm<sup>-3</sup> and  $M = 21\%$  following (Bakken and Olsen, 1983) conversion of hyphal volume into biomass with  $D*M = 0.2289$  g dry mass cm<sup>-3</sup>.

natural soils, ii) no competition or facilitation among mycelia of different fungi has been included, and iii) no site-related growth conditions have been addressed, and several calculation-based assumptions had to be defined (see the explanation at Table 5). However, the proposed exploration type specific standard values may provide a suitable tool for quantification of space occupation, biomass and energy trade-offs of EMM in natural soils. A combination of a further development of the database with descriptions of ECM fungi (Agerer and Rambold, 2004–2011) and functional relationships of different exploration types, grown, observed and assessed in different growth conditions, will contribute to an increasing understanding of the complex belowground mycelial interactions, cost–benefit relations and trade-offs in belowground competition or facilitation.

## 5. Assessment of turnover rates

Accurate estimates of the turnover of EMM are essential in order to evaluate the role of mycorrhizal fungi in the C cycle. This requires understanding of both the rate of production and decomposition of mycorrhizal mycelium. Sequential harvesting of EMM in mesh bags may be one way to estimate turnover rates, but there seems to be a lag-phase before EMM enter the mesh bags (Fig. 1). However, the lag-phase is probably dependent on the level of disturbance caused by the installation and further tests with bags of different sizes and sampling frequency are needed to evaluate the applicability of this method. Pulse labelling with <sup>13</sup>C or <sup>14</sup>C has been applied to estimate turnover in arbuscular mycorrhizal mycelium (Staddon et al., 2003; Olsson and Johnson, 2005) but not for estimates of ECM mycelium. One of the problems with isotope techniques is the risk of differences in labelling of different chemical components of the fungal biomass, some of them having a high turnover rate (respiratory substrates), while the heavy isotope will have longer residence time in structural cell materials (Dawson et al., 2002). Analyses of the turnover rate of specific components, such as chitin, may be a way to overcome this. Another issue concerns the collection of sufficient and representative amounts of ECM mycelium for isotopic analysis.

The difference in natural abundance  $\delta^{13}\text{C}$  between C<sub>3</sub> and C<sub>4</sub> organic matter has been used in studies of the turnover of plant and microbial substances in soils. In these analyses a combination of pyrolysis–gas chromatography and isotope ratio mass spectrometry was used (Gleixner et al., 1999, 2002). The combination of in-growth cores with a mesh allowing only hyphae or allowing both roots and hyphae filled with C<sub>4</sub> dominated soils have been used to estimate the contribution of mycelia and roots to the formation of stable SOM (Godbold et al., 2006; Wallander et al., 2011). A critical factor when using differences in natural abundance of <sup>13</sup>C is to have reliable  $\delta^{13}\text{C}$  values of the end-members, e.g. mycelial and plant residues. Similar to pulse labelling techniques, differences in isotopic signature between various components within the plant and fungal materials may be a potential problem that should be

considered. For example, chitin is depleted in both <sup>13</sup>C and <sup>15</sup>N compared to the total fungal biomass (Dijkstra et al., 2006). The EMM in the top soil was depleted in <sup>15</sup>N with around 5‰ compared to mycorrhizal fruit bodies in a Norway spruce site (Wallander et al., 2004), possibly reflecting lower chitin and higher protein contents in fruit bodies compared to mycelia.

We are not aware of any study that has exploited the possibility to estimate the production and turnover of mycelium in FACE-experiments (Free Air Carbon dioxide Enrichment). Sequential installation and harvest of in-growth bags in connection with the initiation or termination of CO<sub>2</sub> treatments should offer ideal periods to estimate the production and turnover of mycelia biomass. During these periods there are drastic shifts in the <sup>13</sup>C signal of the photosynthates (given that the CO<sub>2</sub> that is used to treat the plants has a different  $\delta^{13}\text{C}$  than the atmosphere, which is the case if fossil C has been used to produce the CO<sub>2</sub>). However, these experiments do not have corresponding plots that are isotopically-enriched at ambient CO<sub>2</sub> concentrations, and so exploiting FACE facilities would only be useful to estimate turnover under elevated CO<sub>2</sub> conditions.

In contrast to other types of organic inputs to soils, surprisingly little is known about the decay rate of mycorrhizal mycelium. Mesh bags of the type normally used to assess leaf litter decomposition have been used recently to demonstrate that the N concentration of hyphae explained a large part of the mass loss during the initial 4 weeks of decay (Koide and Malcolm, 2009). An alternative method is to capture and quantify CO<sub>2</sub> produced when hyphae are added to micro-respirometers. This approach was used to show that ECM fungal hyphae rapidly stimulated CO<sub>2</sub> efflux but that the effect was dependent on the species richness of the hyphae entering soil (Wilkinson et al., 2011a). Thus species richness of ECM fungi can be important both for maintaining productivity (Wilkinson et al., 2011b) and in regulating their own decomposition. These findings indicate that decomposition of ECM hyphae may be a key pathway by which C rapidly enters the saprotrophical microbial biomass in soil. The application of stable isotope probing (Radajewski et al., 2000) in which ECM fungal hyphae is enriched in <sup>13</sup>C has recently been used to demonstrate the rapidity of C incorporation into free-living soil fungi via this pathway (Drigo et al., 2012). Despite these recent advances, there is scope for considerably more research quantifying the rate of decay of different genotypes, species and morphologies of ECM fungal hyphae under a range of environmental conditions.

## 6. Importance of sampling design

Regardless of the effort placed in developing reliable methods to quantify production, biomass and turnover of ECM fungi, the utility of the resulting data is often dependent on the sampling design used to obtain the data in the first place. Moreover, it is often desirable to obtain similar datasets from a wide-range of different

ecosystems and habitats, particularly from a modelling perspective. This requires sampling approaches that have similar ability to quantify spatial variation in EMM abundance and biomass. Yet very few investigations employ spatially-explicit sampling strategies designed to deal with the often vast heterogeneity of EMM production in forest systems. This is in part because variation is likely to occur at a wide range of spatial scales; recent work in Douglas fir stands have demonstrated that genets of *Rhizopogon* spp. could form common mycelial networks connecting individual trees within a 30 × 30 m area (Beiler et al., 2010). In contrast, there is also clear evidence that ectomycorrhizas and their associated mycelium can form patchy clusters at scales of just a few cm (Guidot et al., 2002), perhaps due to their plasticity in responding to inputs of nutrient-rich substrates (Bending and Read, 1995). Moreover, spatial variation occurs in three dimensions. Only rarely is quantification of abundance and biomass of either ectomycorrhizal roots or EMM undertaken at multiple depths. Among surface soil horizons in a Swedish boreal forest, ECM fungi tended to be associated with slightly older partially-decomposed organic matter (Lindahl et al., 2007). In the UK, detailed analyses of the vertical distribution of 7 species of ectomycorrhizas and their EMM in a Scots pine stand showed contrasting vertical distribution patterns from 0 to 20 cm (Genney et al., 2006). The EMM of some species like *Cadophora finlandia* was distributed quite evenly with depth while the EMM of *Cortinarius* spp. was concentrated in the upper 10 cm (Genney et al., 2006). This study also demonstrated unequal distribution of the EMM of many species at 2 cm intervals. Geostatistical techniques (Legendre and Legendre, 1998) have recently been applied to provide rigorous analysis of the temporal and spatial patterns of ectomycorrhizas (Lilleskov et al., 2004; Pickles, 2007). For example, Pickles (2007) sampled 48 cores at increasing distances in a 20 × 20 m area to determine when the abundance of key common species showed spatial autocorrelation. Subsequent more intense sampling events (217 cores) in the same location exploited this information and used regular distances of either 1 or 2 m as the primary separation distance to avoid issues with spatial autocorrelation, and to provide detailed interpolated maps of species' abundance (Pickles et al., 2010). The use of geostatistical tools therefore requires an initial high investment in sampling units, but can reap benefits later once optimum sampling distances are identified. Moreover, obtaining data on spatial autocorrelation enables more meaningful inter-site comparisons and so this is an approach we advocate in future studies.

## 7. Conclusions

Although significant progress has been made over the last ten years in our understanding of the importance of the ECM fungal mycelium in C cycling in ecosystems, our understanding is still highly fragmented. In this paper we have summarized the state of the art in this subject as well as the strengths and weaknesses in the methods and techniques applied. Our aim is that this information will ultimately enable researchers to obtain valuable data on the production, biomass and turnover of mycorrhizal mycelium in all biomes, and modify the approaches outlined here for arbuscular and ericoid mycorrhizal systems. Such data are likely to be essential for improving process-based models of terrestrial biogeochemical cycles that currently ignore the distinct role played by mycorrhizal fungi. This may improve their potential to predict nutrient leaching and carbon sequestration. Moreover, these data could also be incorporated into spatially-explicit modelling frameworks of population dynamics.

All of the applied methods and techniques have their own sets of limitations which the users of these methods should consider before applying them (Tables 1–3). To combine several techniques

in the same study, e.g. chemical markers and isotope labelling, may be a way to overcome some of these limitations. An issue that needs more attention is the turnover of EMM, especially the turnover of diffuse mycelium versus rhizomorphs. The ratio between free and total ergosterol, and the ratio between chitin and ergosterol as an indicator of the necromass/biomass ratio may be useful in such experiments and deserves further studies. Also, it could be useful to develop methods enabling us to quantify specifically the level of <sup>13</sup>C enrichment of C in glucosamine residues. Combined to environmental variation of carbon sources available to the ECM fungi (e.g. in FACE experiment using enriched or depleted <sup>13</sup>C–CO<sub>2</sub> sources), such a method could fill the gap regarding the actual rate the turnover of ECM fungi in forest soils.

Indices, such as specific EMM length or specific EMM biomass, developed for different exploration types, can be used for indirect estimations of the C costs of growth and storage in ECM fungal mycelium. The utility of such indirect measures are greatest providing the ECM fungal community structure is known, that the identified species belong to different exploration types, and these show different space occupation, mycelial length and biomass.

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