Field-flow fractionation and gel permeation methods for total soil fungal mass determination

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Abstract

Fungi are critical components of the soil food web. Fungi are important as organic matter decomposers, nutrient recyclers, and fungal hyphae play a role on the formation of soil aggregates that can increase water infiltration, improve water holding capacity, and sequester soil carbon (C). Ergosterol is a sterol found ubiquitously in cell membranes of filamentous fungi and is commonly used as a marker compound for fungal biomass. In contrast, the analysis of chitin is a potentially effective way to monitor changes in total fungal mass (biomass and necromass) under different environmental conditions, because chitin is a more stable compound than ergosterol. By combining this analysis with our previously developed ergosterol method, we can determine both live fungal biomass and by subtraction, fungal necromass, thus providing useful information on the turnover dynamics of total fungal mass. We developed a sample preparation method based on extraction and conversion of chitin to chitosan, after which chitosan is measured by Asymmetrical Flow Field-Flow Fractionation (AF4). Our results show that this analytical method is a simple, fast, and cost-effective technique for the quantitative analysis of chitin from field-collected soils. The detection limit of this method is 6 μg g⁻¹ chitin (chitosan) in dry soil. The final method linear range is 20–500 μg g⁻¹ for chitin and 0.4–10 mg g⁻¹ for fungal biomass. The developed chitin based and existing ergosterol based fungal estimation methods were used for the soil analysis. These methods can combine to determine the living and total fungal biomass.

Keywords

AF4
Chitin
Fungal biomass and necromass
Gel permeation

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1. Introduction

The role of fungi is extremely diverse in the biosphere, especially in soils. In addition to groups of plant pathogens, there are many species of fungi that help plants grow (i.e. mycorrhizae as fungal symbionts), and other groups play an important role in nutrient cycling, decomposition, water retention and infiltration, and soil aggregate formation (Boer et al., 2005; Weil and Brady, 2017). Soil microbes, including fungi, can be considered the main determinants of soil C pools and biochemical composition (Fekete et al., 2008; Voříšková et al., 2014; Frąc et al., 2018). Saprotrophic fungi (and mycorrhizal fungi in some cases), for example, decompose dead organic matter and play the primary role in regulating the flow of C (Steffen et al., 2007; Kotrocžová et al., 2009; Baldrian et al., 2011). These fungi (i.e. brown rot, soft rot, and white rot fungi (Goodell et al., 2008) efficiently decompose the recalcitrant lignocellulose matrix that other organisms are unable to decompose (Boer et al., 2005). Recent research has highlighted the role of other fungi in both decomposition forming and maintaining stable soil organic matter (SOM) pools due in part to their ability to produce a wide variety of extracellular enzymes (Sun et al., 2005; Brzostek et al., 2015; Averill, 2016; Looby and Treseder, 2018). Because fungi play an important role in organic matter degradation in soil, change in fungal biomass is a sensitive indicator of the biogeochemistry of soil function, and thus there is a need for simple and accurate tools to estimate fungal total mass and within that biomass, necromass and their turnover rates. According to Mayer et al. (2021), there is a positive feedback mechanism between soil organic matter turnover and fertility, which is significantly mediated by soil fungi. These findings emphasize the importance of fungi for soil fertility and plant growth, and emphasize the dependence of the soil carbon turnover on underground fungal communities (Kyaschenko et al., 2017; Fernandez et al., 2020; Hansson et al., 2020).

The biomass of fungi in soil ecosystems can be estimated via molecular biomarkers (e.g. ergosterol), metagenomic and
metatranscriptomic tools, measurements of mycelium length, or other similar tools (Wallander et al., 2013). One relatively simple way to quantify fungi is to measure the amount of specific marker compounds present in the fungal cell (primarily the cell wall and cell membrane) (Beni et al., 2014). Ergosterol and chitin are the most commonly used markers for this purpose (Axelsson et al., 1995; Medina et al., 2003; Baldrian et al., 2013; Teste et al., 2016). Of these, chitin is the most abundant in soils and does not degrade as rapidly as ergosterol or PLFAs after cell death. Chitin and ergosterol assays are easier to carry out than fatty acid extraction (Baldrian et al., 2013; Wallander et al., 2013). Ergosterol is a sterol found ubiquitously in cell membranes of filamentous fungi (Olsson et al., 2003; Lau et al., 2006; Beni et al., 2014; Beni et al., 2017). Chitin is a long polymer of N-acetylglucosamine and a derivative of glucose, and is the second most abundant polysaccharide on the planet (Gooday, 1990; Jacquiod et al., 2013; Sharp, 2013). Along with chitin, chitosan is found in the cell walls of fungi, and is associated with increased integrity and protection against high temperatures. The difficulty with using these latter compounds is that they are not only found in fungi, but are also found in the spines of diatoms, many arthropods, and many cephalopods, nematodes, and exoskeletons of molluscs (Bhuiyan et al., 2013; Yeul and Rayalu, 2013; Debode et al., 2016). However, the contribution from these non-fungal sources is probably minimal as their biomass is typically below 0.5% of the fungal biomass in soil (Beare et al., 1997; Simpson et al., 2004; Wallander et al., 2013), furthermore macroinvertebrates and larger individuals of mesoinvertebrates are removed from soil samples prior to testing. Chitin and chitosan are not found in the cell walls of all fungal species, but are found in the most important groups in the soil as Basidiomycetes, Ascomycetes, Zygomycetes, and Deuteromycetes (Abo Elsoud and El Kady, 2019).

We earlier explored the use of ergosterol to determine live fungal biomass (Beni et al., 2014; Beni et al., 2017). However, methods based on ergosterol can estimate the biomass of live fungi but do not provide information about fungal dead cells or necromass (Frey et al., 1994; Stahl and Parkin, 1996; Adamczyk et al., 2020). Studies involving both dead and living fungal biomass are not common, due to methodological drawbacks. However, chitin, as a relatively persistent compound in soil, may be used as a marker representing the total fungal mass (living and dead fungal mass) (Nilsson and Bjurman, 1998; Eklad et al., 2013). Although chitin is not commonly used as a fungal marker, it was chosen in this study as a marker compound for fungi as it is a highly stable biopolymer and thus the sample will not degrade during storage and it can be analyzed by field flow fractionation. Thus, fungal death rate can be estimated by using the chitin and previously developed ergosterol (Beni et al., 2017) methods together.

Field-Flow Fractionation (FFF) is a group of separation techniques which use different types of external fields, such as centrifugation, temperature, electric fields, or cross-flow, to separate samples components. Within FFF, Asymmetrical Field-Flow Fractionation (AF4) separates particles that are dissolved or dispersed in a solvent inside an open flow channel without a packing or a stationary phase. Perpendicular flow pushes molecules towards the bottom of the channel. These molecules also undergo a counter acting diffusion back into the channel against the carrier flow, as determined by their natural Brownian motion based on size. The channel has a laminar flow (a parabolic pattern) and the speed of the flow increases towards the center of the channel and decreases towards the sides. Therefore, smaller particles located in the center of the channel will be transported with a greater velocity than larger ones. This results in a gentle separation of particles based on mass (Fuente et al., 2019). The AF4 method can separate molecules over a wide size (1 nm – 1 μm) range while maintaining high resolution (Malik and Pasch, 2016; Guo et al., 2019).

In this study, chitin was extracted from soil, deacylated to chitosan, and the chitosan content was determined by AF4. Our proposed method needs fewer sample preparation steps than traditional chitin determination methods, because it avoids conversion of chitosan to glucosamine by acidic hydrolysis and other derivitazation steps. Our goal was to optimize sample preparation and AF4 parameters for chitosan as determined by sample linearity and repeatability. Our additional goal was a comparison between the AF4 and Gel Permeation Chromatograph (GPC) methods as the AF4 method is a newer technique not yet widely used in soil microbiology. The AF4 system has a better limit of detection and separation, and thus we hypothesized that lower soil fungal mass levels in soil can be more accurately determined using the A4 method.

2. Materials and methods

2.1. Reagents

All aqueous solutions were prepared using water deionized through a Millipore system. The chitin and chitosan compounds (Sigma-Aldrich) were >95% pure. Pro-analysis grade potassium-hydroxide was used to convert chitin to chitosan. Buffer solution for mobile phase was prepared from sodium acetate trihydrate (pro analysis, 99.5%) and glacial acetic acid (Ph Eur, 99.8%). All prepared solutions solvents were filtered through a 1.2 μm Vesper membrane.

2.2. Apparatus

Laboratory equipment used were a Pierce 18971 Reacti-Therm Heating/Stirring module, a rocker shaker made by Linson instrument (AB Lars Ljungberg & Co) and a Beckman Coulter Allegra X-15R Centrifuge. The chitosan content of the prepared samples was determined with an Agilent 1200 UPLC pump and autosampler coupled with Wyatt Eclipse 3+ AF4 separation system. A Millipore RC 10 kDa membrane and a spacer (350 μm height and 21.5 mm wide) were installed in the AF4 cell and the channel was 152 mm. This system contained three detectors: a Wyatt DAWN Helios-II MALS (multi angle light scattering), a Wyatt T-rEX RI (refractive index) and a Jasco UV-975 with 8 μL flowcell. UV detection was done at 214 nm. The whole system was controlled by Agilent Chemstation and all data were collected by Wyatt Astra software.

System setup tests (focus and channel test) were performed on the assembled AF4 channel by injection of blue dextran,
a compound that easily shows flow problems in the cell. The measurements were done at room temperature and were replicated in triplicate. Living soil fungal biomass was assessed using ergosterol content measured by HPLC (Béni et al. 2017).

2.3. Site description

We took soil samples from 3 different sites in order to test our newly developed method. One soil was a Luvisol in an oak forest at the Síkfőkút Project (Fekete et al., 2017). This area is located in the southern part of the Bükk Mountains (N 47.933° E 20.4167°). The second soil was a Chernozem from the Kerecsend lowland oak forest in northeastern Hungary (Fekete et al., 2020a) (N 47.79252° E 20.32493°). The third soil was an Arenosol (poor in nutrients and organic matter) at a clearing area of the Sóstói Forest near to the University of Nyíregyháza (N4 7.97644° E 21.71159°).

We established three randomly located 4 × 4 m plots in every site. Soil cores were collected from the 0–5 cm layer from the mineral soil with an Oakfield soil corer (Oakfield Apparatus Company, USA) at five randomly selected locations in each plot at May 2015. Soil samples were sieved to < 2 mm and hand-picked to remove roots, stones and invertebrates of visible size. Samples were air dried at room temperature and then stored in airtight plastic bag until analysis.

2.4. Statistical analyses

Total soil fungi mass of the sites was compared by one-way ANOVA followed by Tukey's HSD post-hoc test. These statistical analyses were conducted using Statistica 8.0. The limit of detection (LoD) was calculated from the calibration graph and was calculated as 3σ/S, where σ is the residual of the standard deviation of regression and S is the slope of the calibration. Each sample was analyzed three times and the standard deviation (SE) was calculated.

2.5. Method development

The first steps of the new method are digestion of fungi and extraction and deacetylation of chitin from dry soil by concentrated potassium hydroxide (60.0% (w/w)) to convert chitin to chitosan. The sample: reagent ratio and temperature and reaction duration needed to be optimized. The final step is the determination of chitosan in the extracts by AF4. The crucial parameters of the AF4 separations that we needed to optimize were membrane and mobile phase type, timing, and flow rates.

2.5.1. Optimization of sample preparation

We assessed the optimal parameters for chitin extraction and analysis using artificial sand to which 2.5 mg chitin was added. Similar to the former HPLC method (Ekblad and Näsholm, 1996), we found an optimal mix of 1 g sample and 10 mL 60% KOH to extract chitin and convert chitin to chitosan. The extraction and conversion were performed in 20 × 150 mm test tubes which were mixed vigorously by vortex mixer after which the mixtures were placed in a block heater and stirrer at 130°C for 24 h with continuous stirring, a duration selected to ensure the highest yield of chitosan. Shorter times were determined to be less efficient; the deacetylation yield of chitosan has been reported as 70% at 120°C over 1–3 h (Ravi Kumar, 2000). Samples were centrifuged at 1500 g for 10 min. at 2°C, filtered, and the solid particles introduced into 10 mL 4.7 pH buffer with the pH adjusted by glacial acetic acid. A pH of 4.7 was ideal for the dissolution of the chitosan. The solution was homogenized by 2 min vortex mixing followed by 4 h rocker shaking. The solution was brought to 25 mL with distilled water and filtered through a 1.2 μm membrane filter prior to AF4 analysis.

2.5.2. Optimization of the AF4 separation

The optimization of separation parameters was established using a 0.02 mg mL−1 chitosan standard solution in 4.7 pH acetic buffer. The 10 kD a cut-off membrane and spacer was used as recommended for chitosan. The 0.01 mol L−1 pH 4.2 acetic buffer solution was the eluent at the analysis.

We needed to optimize injection, focusing, and elution parameters, and thus different timing, duration, and rate of flows (cross, outflow, focus and injection flow) in the focus and elution steps were tested. The aim was to reach the narrowest and highest peak for the 0.02 mg/mL chitosan standard solution.

Our proposed AF4 method is shown in Table 1. The first and second steps of this proposed method are the cleaning procedures of the AF4 device. Step III is sample injection mode. Fractionation after sample injection leads to line broadening and insufficient separation, because the sample particles are spread out randomly from the injection site and the beginning velocity and place of the all particles are not same. Thus, focusing of the injected sample (Step IV, focusing mode) is needed. Step V is the elution mode (fractionation), the laminar flow that carries the injected sample (Step IV, focusing mode) is needed. Step V is the elution mode (fractionation), the laminar flow that carries the sample through the separation chamber. Cross-flow (Vf) is applied perpendicular to the channel, against the sample flow. In

<table>
<thead>
<tr>
<th>Table 1</th>
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<tbody>
<tr>
<td>The optimized AF4 method for chitosan. Parameter settings: outflow (Detector flow) 1 mL min−1, Focus Flow 2 mL min−1, Injext flow 0.2 mL min−1</td>
</tr>
<tr>
<td>Step</td>
</tr>
<tr>
<td>---</td>
</tr>
<tr>
<td>I</td>
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<tr>
<td>II</td>
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<td>III</td>
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<td>IV</td>
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<td>V</td>
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<tr>
<td>VI</td>
</tr>
<tr>
<td>VII</td>
</tr>
</tbody>
</table>

*E: elution F: sample focus and I: sample inject programme phases, **Vf: cross flow (this flow is perpendicular to the eluent flow)
this step the rate of crossflow is reduced every minute by 0.15 to reach 0 mL min⁻¹. The elution continues without crossflow at Step VI. Finally, the last step is cleaning out the AF4 system from residual molecules (step VII).

2.6. Gel permeation optimization and measurements

To check this new method based on the AF4 analysis, chitin contents of these soil samples were measured by gel permeation chromatography. The soil sample extraction method was the same as for AF4 measurements, using 1 g samples and 25 mL as a final volume.

The chitosan contents of the soils were determined with a Gilson 506C HPLC system with autosampler and UV detection. The measurements were done at 220 nm. The 0.01 mol L⁻¹ pH 4.2 acetic buffer solution was the mobile phase. The chromatography column was ZORBAX GF-450 (250 x 4.6 mm; 6 μm) and the flow rate was 3 mL/minute and the temperature of the column was room temperature. The chromatograms were processed with Gilson 712 software.

2.7. The optimized AF4 method for total fungal mass estimation

2.7.1. Chitin conversion to chitosan

1 g dry sieved soil and 10 mL of a 60% w/w potassium hydroxide solution was added to 20 x 150 mm test tube or 25 mL glass beaker and subjected to 24 h stirring and heating at 130°C. After that the sample was centrifuged at 1500 g for 10 min. at 2°C and the soil extract was filtered (2–4 μm pore size). The residue was introduced to a 10 mL 4.7 pH acetate buffer with the pH adjusted by glacial acetic acid. The solution was homogenized with a vortex mixer for 2 min and shaken for 4 h. Finally, the solution was brought up to 25 mL with distilled water.

2.7.2. AF4 analysis

Samples were passed through a 1.2 μm membrane filter prior to injection. A 100 μL sample was injected into the AF4 system and eluted with 0.01 mol/L sodium acetate/acetic acid at a pH of 4.2. Our recommended parameters are shown in Table 1. With these parameters the elution time was close to 16.5 minutes.

2.8. Method validation

A 0.20 mg/mL chitosan stock solution was prepared with 50 mg chitosan dissolved in 250 mL water at pH 4.7. Three standards were made by dilution with distilled water. Linearity and repeatability of the final AF4 method were determined.

2.8.1. Recovery and precision

The reliability of the technique was studied by determining the recovery of chitin from spiked soil. Solid chitin was added to 1 g of the sandy soil (Arenosol), and spiked standards were processed along with soil samples. Each of the spiked samples were replicated three times.

2.9. Total fungal mass estimation

When analyzing soil samples collected at the same time and place, these methods are well suited for detecting the effects of differing soil treatments and changing ecological conditions on fungal mass, as long as the fungal community composition remains relatively constant. In addition, because 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 Ascomycetes, Basidiomycetes and Deuteromycetes Zygomycetes (Appuhn and Joergensen, 2006), a chitin:total fungal mass: ratio of 1:20 can be used to convert chitin concentration to total fungal mass in more ecosystems of forest soils.

3. Results

3.1. Comparison of detection techniques

The AF4 system has three different detectors, which are multi angle light scattering (MALS), refractive index (RI) and ultra violet (UV). The Wyatt MALS has 16 detectors which detect the light scattering at different angles. We found that the 90° detector (LS-9) had the highest sensitivity. The UV detection was performed at 214 nm. All detectors were calibrated by chitosan standard solutions in a 0.008–0.200 mg mL⁻¹ concentration range. The injected volume was 100 μL and the peak area was used for evaluation.

The MALS had highest sensitivity while the RI detector had the lowest sensitivity, and RI had the highest, or least sensitive, limit of detection value of the three techniques due to the low sensitivity of the RI detector (Table 2). The MALS and UV detectors has similar limit of detection values. These detectors have highly linear standard curves for a wide range of chitosan values and thus are all useful for these analyses. However, the estimation of soil fungal mass could be problematic with the RI detector below 0.15 mg g⁻¹ as was found for the Arenosol.

3.2. Analysis of soil samples

The retention times of the standard and samples were nearly the same (Figure 1). The total fungal mass calculated for the

<table>
<thead>
<tr>
<th>Detector</th>
<th>standard curve</th>
<th>R²</th>
<th>Limit of detection chitosan, μg mL⁻¹</th>
</tr>
</thead>
<tbody>
<tr>
<td>AF4/MALS</td>
<td>A = 13.396c + 0.09933</td>
<td>0.992</td>
<td>0.24</td>
</tr>
<tr>
<td>AF4/UV</td>
<td>A = 0.4489c + 0.000529</td>
<td>0.989</td>
<td>0.32</td>
</tr>
<tr>
<td>AF4/RI</td>
<td>A = 0.00008555c – 0.0000001138</td>
<td>0.982</td>
<td>1.18</td>
</tr>
<tr>
<td>GPC/UV</td>
<td>A = 0.3208c + 0.0922</td>
<td>0.983</td>
<td>0.45</td>
</tr>
</tbody>
</table>

Table 2: Comparison of AF4 detectors and GPC techniques, measured calibration curves with R² values, and limits of detection

Multi angle light scattering (MALS), ultra violet (UV) and refractive index (RI) detectors, “A” is the peak-area of chitosan on the chromatogram and “c” is the concentration of chitosan.
Figure 1. Chromatograms of the chitosan standard (black) the Luvisol (red), Chernozem (blue) and Arenosol (green). A AF4- light scattering LS-9 detector was used.

Table 3
Recovery and relative standard deviation (RSD%) of chitin in 1 g spiked sandy soil samples. (n=3.) MALS detection

Different letters denote significant differences within each group (p < 0.05, ANOVA and Tukey’s test)

Luvisol was 1.01 mg g⁻¹ and 1.77 mg g⁻¹ for the Chernozem. The Arenosol had a chitosan content near to the limit of measurement, because the organic matter content was very low in this sandy soil and the estimated total fungal mass was 0.08 mg g⁻¹. The recovery of chitin in spiked samples using the MALS detector ranged from 97% to 98% (Table 3). We found that the developed method showed the best results for chernozem.

3.3. Method validation

The equation of the calibration curve was A = 13.396c + 0.09933 (R² = 0.99), where “A” is the peak-area of chitosan on the chromatogram and “c” is the concentration of chitosan. The limit of detection for this method was 0.24 µg mL⁻¹. The method was validated for linearity with 8.00–20.00 µg mL⁻¹ chitosan and the standard deviation (RSD%) was under 3.

The standard curves of the Gel permeation measurements were A = 0.3208c + 0.0922 (R² = 0.98), where the A is the peak area and the limit of detection was 0.448 µg mL⁻¹ for chitosan.

3.4. Comparison of different AF4 detectors and with GPC methods

Chitin content of the Chernozem and Luvisol soil samples were determined with asymmetrical field flow fractionation (AF4) and gel permeation chromatography (GPC). Both separation techniques showed good agreement with each other (Table 4).

Table 4
Differences in ergosterol and chitin assays and comparison of the AF4 and GPC methods (n = 3) (fungal biomass of soil at the studied sites)

<table>
<thead>
<tr>
<th>Sample</th>
<th>Chitosan</th>
<th>Ergosterol</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>fungal biomass, mg g⁻¹ dry soil</td>
<td>Chitosan or ergosterol, (µg · g⁻¹ dry soil)</td>
</tr>
<tr>
<td></td>
<td>AF4/ MALS</td>
<td>AF4/ UV</td>
</tr>
<tr>
<td>Chernozem</td>
<td>1.77 ± 0.17ᵃ</td>
<td>2.01 ± 0.26ᵃ</td>
</tr>
<tr>
<td> </td>
<td>(88 ± 9)</td>
<td>(101 ± 13)</td>
</tr>
<tr>
<td>Luvisol</td>
<td>1.01 ± 0.18ᵇ</td>
<td>1.27 ± 0.28ᵇ</td>
</tr>
<tr>
<td> </td>
<td>(51 ± 9)</td>
<td>(63 ± 14)</td>
</tr>
<tr>
<td>Arenosol</td>
<td>0.12 ± 0.03ᵃ</td>
<td>&lt;0.16ᵃ</td>
</tr>
<tr>
<td> </td>
<td>(6 ± 1)</td>
<td>(&lt;8)</td>
</tr>
</tbody>
</table>

MALS Multi-Angle Light Scattering detector, RI refractive index detector, UV ultraviolet detector, Different letters denote significant differences within each group (p < 0.05, ANOVA and Tukey’s test)

* below LoD
Total fungal mass (Chitin) contents estimated by AF4 UV at 214 nm were higher than for AF4 MALS or GPC, and contents estimated by AF4 RI were lower than the reference results. The RI detector clearly has a low sensitivity for chitosan and should not be used to estimate chitin in Arenosols or other soils with low carbon, and thus fungal content. There was good agreement between results from the AF4-MALS and the GPC-UV (220 nm) methods (Tables 5–6).

4. Discussion

The AF4 results have excellent correlation with size exclusion GPC separation (González-Espinosa et al., 2019), and other studies have suggested that the AF4 technique produced more accurate results for polysaccharide determination (Bayart et al., 2019). The total fungal mass estimation results were the same using both techniques for the Chernozem and the Luvisol, suggesting that both methods are useful for chitin analysis. For the Arenosol, we obtained a measurable result with the AF4-MALS system, as this system had the best detection limit, suggesting that the AF4 system might be the preferred technique for low organic matter soils. SOC concentration was 0.52% (Makádi, 2010) in this Arenosol, thus we expect total fungal mass to be low as well. SOC concentration in the Luvisols from the Síkfőkút Forest is significantly higher at 3.68% (Świtoniak et al., 2014), while that of Chernozem soil in Kerecsend Forest is 5.22% (Fekete et al., 2020b). The effect of SOC content on total fungal mass was clear in the soil types we examined (Table 4).

In this study, chitin:ergosterol ratios, that we interpret as the ratio of total (live fungal biomass and necromass) to live fungal biomass, were broadly similar across our soils at about 2:1 (Table 4).
4. Li et al. (2015) reported a significantly higher total: living biomass ratio than we measured. There could be several reasons for this discrepancy. These authors used different methods to determine live biomass and necromass and they conducted their studies using different soil types in different climatic conditions. Climate is important at microbial necromass distribution in topsoil. (Zhang et al., 2020) The lower temperature and soil pH stimulate the accumulation of fungal necromass. (Wang et al., 2021a). It has connection between the concentration of easily accessible soluble nutrients and microbial biomass (Wang et al., 2021b).

They also sampled the top 20 cm of soil, while we sampled only the top 5 cm. Furthermore, we sampled in May, when the soil conditions (moisture, temperature) are optimal for fungal growth, and we assume that there are significant seasonal trends to live:dead fungal ratios.

It should be noted that although chitin can be accurately determined using this technique, translating chitin to total fungal mass is more complicated. Biomass estimates with biomarkers are strongly dependent on the use of conversion factors. It may be difficult to determine fungal biomass based solely on chitin content because the concentration of chitin in fungi may vary not only by species but also by the state of development of the fungus and even by external conditions. The chitin content of non-wood-decaying fungi reportedly ranges from 2.6 to 26.6% (Blumenthal and Roseman, 1957; Chen and Johnson, 1983). Thus, the chitin: dry fungus ratio should always be taken when determining the amount of fungal biomass in the soil.

There are very few published methods for the determination of chitin in soil. Our new method has fewer sample preparation steps than the method of (Adamczyk et al., 2020) because it is not necessary to hydrolyse and derivatize as for fluorescence HPLC detection. The main difference is that our method produces decyacylated chitin (chitosan) and not glucosamine, and therefore our method does not use standard reverse-phase (C-18) separation as in Adamczyk et al. (2020). C-18 columns are widespread and cheaper than GPC columns or the AF4 device, but the fluorescence detector is not commonly with HPLC systems and derivatization is needed; in contrast, the GPC column can be easily fitted into the HPLC device. In summary, this new method is simpler and faster than the glucosamine-based technique.

The estimated cost of determination of soil fungal biomass with our method is 20 EUR/sample. This value is without technician salary.

5. Conclusion

The AF4 method has been used to measure many kinds of macromolecules in various systems, but has not been used to characterize total soil fungal mass through chitin analysis. Chitin is a biomarker for total fungal mass and an important indicator of soil health and of internal biogeochemical processes. We developed a simple, novel and relatively fast AF4 method for the determination of chitin in soils, and demonstrated its usefulness for the estimation of total fungal mass in different types of soils. Although the analysis of ergosterol in soils can also be used as an indicator of live fungal biomass, chitin is more stable than ergosterol and thus there are fewer problems with storing soils prior to analysis. The major disadvantage of this new method is the longer sample preparation time and the likelihood that it also measures fungal necromass. The final method choice will depend on laboratory capacity and equipment, and the need to store soils prior to analysis.

Acknowledgements

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