SOIL SCIENCE ANNUAL
2021, 72(3), 143901

https://doi.org/10.37501/soilsa/143901

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

Áron Béni[1]*, Kate Lajtha[2], Daniel Osorio[3], István Fekete[4]

[1] University of Debrecen, Institute of Agricultural Chemistry and Soil Science, Böszörményi út 138, H4032, Debrecen, Hungary
[2] Oregon State University, Department of Crop and Soil Science, 3017 Agricultural and Life Sciences Building, OR 97331, Corvallis, USA
[3] Lund University, Engineering and Nutrition, Faculty of Engineering, LTH, Department of Food Technology, John Ericssons väg 3, SE-221 00 Lund, Sweden
[4] University of Nyiregyhaza, Institute of Environmental Sciences, Sóstóiút 31/B, H-4400 Nyiregyhaza, Hungary

* PhD A. Béni, beniaron@agr.unideb.hu, ORCID iD: https://orcid.org/0000-0001-5436-6911

Received: 27.07.2021
Accepted: 12.11.2021
Associated editor: A. Gałązka

Keywords

AF4
Chitin
Fungal biomass and necromass
Gel permeation

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 potentially effective way to monitor changes in total fungal mass (biomass and necromass) under different environmental conditions, because chitinis 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.

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; Kotroczó 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-chain 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; Ekblad 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 centre 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 (Fuentes 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 Versapor 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 Aglient 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 x 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 sample through the separation chamber. Cross-flow (V_x) is applied perpendicular to the channel, against the sample flow. In

Table 1
The optimized AF4 method for chitosan. Parameter settings: outflow (Detector flow) 1 mL min^{-1}, Focus Flow 2 mL min^{-1}, Inject flow 0.2 mL min^{-1}

Step	Aim	Start, min	End, min	Duration, min	Mode*	V_x, start**, mL min^{-1}	V_x, end**, mL min^{-1}
I.	Cleaning	0	1	1	E	2.0	2.0
II	Cleaning	1	2	1	F		
III	Injecting	2	4	2	F + I		
IV	Focusing	4	6	2	F		
V	Eluting	6	16	10	E	1.5	0
VI	Eluting	16	26	10	E	0	0
VII	Cleaning	26	30	4	E + I		

*E: elution F: sample focus and I: sample inject programme phases, **V_x: 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.

3.2.3. 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 2
Comparison of AF4 detectors and GPC techniques, measured calibration curves with R^2 values, and limits of detection

Detector	standard curve	R^2	Limit of detection chitosan, μg mL^{-1}
AF4/MALS	A = 13.396c + 0.09933	0.992	0.24
AF4/UV	A = 0.4489c + 0.000529	0.989	0.32
AF4/RI	A = 0.0008555c – 0.000001138	0.982	1.18
GPC/UV	A = 0.3208c + 0.0922	0.983	0.45

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.

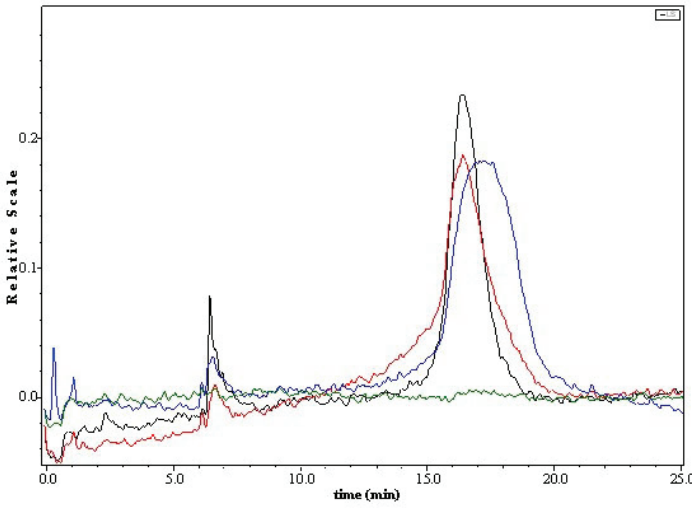

Figure 1. Chromatograms of the chitosan standard (black) the Luvisol (red), Chernozem (blue) and Arenosol (green). A AF4- light scattering LS-9 detector was used

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)

Chitin spike (μg g^{-1})	Measured (μg g^{-1})	Recovery (%)
50	48.3 ± 7.1[a]	97
100	101.7 ± 5.3[b]	98
200	203.2 ± 4.8[c]	98

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 fugal 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 is showed the best results for chernozem.

3.3. Method validation

The equation of the calibration curve was A = 13.396c + 0.09933 (R^2 = 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 (R^2 = 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)

Sample	method/detector				
	AF4/ MALS	AF4/ UV	AF4/RI	GPC / UV	C18 / UV
	Chitosan				Ergosterol
	fungal biomass, mg g^{-1} dry soil Chitosan or ergosterol, (μg · g^{-1} dry soil)				
Chernozem	**1.77 ± 0.17[c]** (88 ± 9[c])	**2.01 ± 0.26[c]** (101 ± 13[c])	**1.58 ± 0.34[b]** (79 ± 17[b])	**2.00 ± 0.21[c]** (100 ± 10.5[c])	**1.01 ± 0.017[c]** (4.04± 0.07[c])
Luvisol	**1.01 ± 0.18[b]** (51 ± 9[b])	**1.27 ± 0.28[b]** (63 ± 14[b])	**0.78 ± 0.31[b]** (39 ± 15[b])	**1.34 ± 0.24[b]** (67 ± 12[b])	**0.64 ± 0.01[b]** (2.56 ± 0.04[b])
Arenosol	**0.12 ± 0.03[a]** (6 ± 1[a])	**<0.16*** (<8*)	**<0.59*** (<30*)	**<0.22*** (<11*)	**<0.02*** (<0.08*)

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

Table 5
Comparison of sample preparation steps for chitin determination

Steps	Adamczyk *et al.*, 2020 method	Our method
I.	0.05 g peat sample + 5 mL 0.2 mol L^{-1} NaOH	1.00 g soil sample + 10 mL 60% (w/w) KOH
II.	1st-Incubation: 6 h at 20°C on a planar shaker (120 rpm) and every 2 h, the samples centrifuged (2500 g, 5 min) and the supernatant removed and fresh 0.2 mol/L NaOH added	24 h stirring and heating at 130°C
III.	2nd-Incubation: fresh 0.2 mol L^{-1} NaOH at 100°C overnight (12 h)	10 min centrifugation at 1500 g
IV.	Sample washing four times with water	filtration
V.	5 min centrifugation at 2500 g supernatant removal	4 h dissolution in 10 mL 4.7 buffer
VI.	add 5 ml of H_2O and 2.5 mL 6 mol L^{-1} HCl	fill up to 25 mL with distilled water
VII.	3rd-Incubattion: 7 h at 100°C cooling down and add 0.4 mL of water evaporate 0.4 mL of supernatant under a stream of N_2 at 40°C	
***VIII.**	mix 400 µL of hydrolysatedsample with internal standard	
***IX.**	mix 200 µL of mixture + 50 µL of borate buffer pH 6.3 + 250 µL of FMOC-Cl	
***X.**	4th-Incubation 10 min at room temperature	
***XI.**	add 1 mL of heptane	
***XII.**	5 min centrifugation at 2500 g	
***XIII.**	filtration of lower phase	

**Derivatization steps with 9-Fluorenylmethoxycarbonyl chloride (FMOC-Cl)*

Table 6
Comparison of chitin determination methods for soils

	Adamczyk et al., 2020 method	Our methods	
Separation principle:	Reversed-phase	Asymmetrical flow field-flow fractionation	Gel Permeation
Column:*	C18 (5µm, 250 × 4 mm)	10 kDa membrane	GPC (250x4.6 mm; 6 µm)
Eluent:	methanol-4.2 pH buffer gradient elution	4.2 pH phosphate buffer	4.2 pH phosphate buffer, isocratic elution
Flow rates:	1 mL min^{-1}	(Table 1)	3 mL min^{-1}
Detection:	Fluoresces (excitation at 260 nm, emission at 330 nm)	absorbance at 214 nm or MALS at 90°	absorbance at 220 nm
Runtime:	40 min	25 min	20 min

* for AF4 is separation membrane

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). 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 deacylated 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

Financial support was provided by the National Research, Development and Innovation Fund of Hungary, under the KH_17 funding scheme, Projects 126478 and 125688. Support was also provided by the European Union and the European Social Fund under EFOP-3.6.1-16-2016-00022 project as well as the Campus Hungary project of the Balassi Institute.

References

Abo Elsoud, M.M., El Kady, E.M., 2019. Current trends in fungal biosynthesis of chitin and chitosan. Bulletin of the National Research Centre 43(1), 59. https://doi.org/10.1186/s42269-019-0105-y

Adamczyk, S., Larmola, T., Peltoniemi, K., Laiho, R., Näsholm, T., Adamczyk, B., 2020. An optimized method for studying fungal biomass and necromass in peatlands via chitin concentration. Soil Biology and Biochemistry 149, 107932. https://doi.org/10.1016/j.soilbio.2020.107932

Appuhn, A., Joergensen, R.G., 2006. Microbial colonisation of roots as a function of plant species. Soil Biology and Biochemistry 38(5), 1040–1051. https://doi.org/10.1016/j.soilbio.2005.09.002

Averill, C., 2016. Slowed decomposition in ectomycorrhizal ecosystems is independent of plant chemistry. Soil Biology and Biochemistry 102, 52–54. https://doi.org/10.1016/j.soilbio.2016.08.003

Axelsson, B.-O., Saraf, A., Larsson, L., 1995. Determination of ergosterol in organic dust by gas chromatography-mass spectrometry. Journal of Chromatography B: Biomedical Sciences and Applications 666(1), 77–84. https://doi.org/10.1016/0378-4347(94)00553-H

Baldrian, P., Větrovský, T., Cajthaml, T., Dobiášová, P., Petránková, M., Šnajdr, J., Eichlerová, I., 2013. Estimation of fungal biomass in forest litter and soil. Fungal Ecology 6(1), 1–11. https://doi.org/10.1016/j.funeco.2012.10.002

Baldrian, P., Voříšková, J., Dobiášová, P., Merhautová, V., Lisá, L., Valášková, V., 2011. Production of extracellular enzymes and degradation of biopolymers by saprotrophic microfungi from the upper layers of forest soil. Plant and Soil 338(1), 111–125. https://doi.org/10.1007/s11104-010-0324-3

Bayart, C., Jean, E., Paillagot, M., Renoud, A., Raillard, A., Paladino, J., Le Borgne, M., 2019. Comparison of SEC and AF4 analytical tools for size estimation of typhoid Vi polysaccharides. Analytical Methods 11(37), 4851–4858. https://doi.org/10.1039/C9AY00145J

Beare, M.H., Hu, S., Coleman, D.C., Hendrix, P.F., 1997. Influences of mycelial fungi on soil aggregation and organic matter storage in conventional and no-tillage soils. Applied Soil Ecology 5(3), 211–219. https://doi.org/10.1016/S0929-1393(96)00142-4

Beni, Á., Lajtha, K., Kozma, J., Fekete, I., 2017. Application of a Stir Bar Sorptive Extraction sample preparation method with HPLC for soil fungal biomass determination in soils from a detrital manipulation study. Journal of Microbiological Methods 136, 1–5. https://doi.org/10.1016/j.mimet.2017.02.009

Beni, A., Soki, E., Lajtha, K., Fekete, I., 2014. An optimized HPLC method for soil fungal biomass determination and its application to a detritus manipulation study. Journal of Microbiological Methods 103, 124–130. https://doi.org/10.1016/j.mimet.2014.05.022

Bhuiyan, R.M.A., Shaid, A., Bashar, M.M., Haque, P., Hannan, M.A., 2013. A novel approach of dyeing jute fiber with reactive dye after treating with chitosan. Open Journal of Organic Polymer Materials 3, 87–91. https://doi.org/10.4236/ojopm.2013.34014

Blumenthal, H.J., Roseman, S., 1957. Quantitative estimation of chitin in fungi. J Bacteriol 74(2), 222–224.

Boer, W. d., Folman, L.B., Summerbell, R.C., Boddy, L., 2005. Living in a fungal world: impact of fungi on soil bacterial niche development. Federation of European Microbiological Societies – Microbiology Reviews 29(4), 795–811. https://doi.org/10.1016/j.femsre.2004.11.005

Brzostek, E.R., Dragoni, D., Brown, Z.A., Phillips, R.P., 2015. Mycorrhizal type determines the magnitude and direction of root-induced changes in decomposition in a temperate forest. New Phytologist 206(4), 1274–1282. https://doi.org/10.1111/nph.13303

Chen, G.C., Johnson, B.R., 1983. Improved colorimetric determination of cell wall chitin in wood decay fungi. Applied Environmental Microbiology 46(1), 13–16. https://doi.org/10.1128/aem.46.1.13-16.1983

Debode, J., De Tender, C., Soltaninejad, S., Van Malderghem, C., Haegeman, A., Van der Linden, I., Cottyn, B., Heyndrickx, M., Maes, M., 2016. Chitin Mixed in Potting Soil Alters Lettuce Growth, the Survival of Zoonotic Bacteria on the Leaves and Associated Rhizosphere Microbiology. Frontiers in Microbiology 7(565). https://doi.org/10.3389/fmicb.2016.00565

Ekblad, A., Näsholm, T., 1996. Determination of chitin in fungi and mycorrhizal roots by an improved HPLC analysis of glucosamine. Plant and Soil 178(1), 29–35. https://doi.org/10.1007/BF00011160

Ekblad, A., Wallander, H., Godbold, D.L., Cruz, C., Johnson, D., Baldrian, P., Björk, R.G., Epron, D., Kieliszewska-Rokicka, B., Kjøller, R., Kraigher, H., Matzner, E., Neumann, J., Plassard, C., 2013. The production and turnover of extramatrical mycelium of ectomycorrhizal fungi in forest soils: role in carbon cycling. Plant and Soil 366(1), 1–27. https://doi.org/10.1007/s11104-013-1630-3

Fekete, I., Berki, I., Béni, Á., Balláné, K.A., Madarász, B., Várbíró, G., Makádi, M., Demeter, I., Juhos, K., 2020a. Carbon-sequestration of Hungarian zonal types of deciduous oak forest soils, studied with different annual precipitation values,. Talajvédelem (in Hungarian), 176–190.

Fekete, I., Berki, I., Lajtha, K., Trumbore, S., Francioso, O., Gioacchini, P., Montecchio, D., Várbíró, G., Béni, Á., Makádi, M., Demeter, I., Madarász, B., Juhos, K., Kotroczó, Z., 2020b. How will a drier climate change carbon sequestration in soils of the deciduous forests of Central Europe? Biogeochemistry. https://doi.org/10.1007/s10533-020-00728-w

Fekete, I., Halász, J., Kramoperger, Z., Krausz, E., 2008. Study of litter decomposition intensity in litter manipulative trials in síkfőkút cambisols. Cereal Research Communications 36, 1779–1782.

Fekete, I., Lajtha, K., Kotroczó, Z., Várbíró, G., Varga, C., Tóth, J.A., Demeter, I., Veperdi, G., Berki, I., 2017. Long-term effects of climate change on carbon storage and tree species composition in a dry deciduous forest. Global Change Biology 23(8), 3154–3168. https://doi.org/10.1111/gcb.13669

Fernandez, C.W., See, C.R., Kennedy, P.G., 2020. Decelerated carbon cycling by ectomycorrhizal fungi is controlled by substrate quality and community composition. New Phytologist 226(2), 569–582. https://doi.org/10.1111/nph.16269

Frąc, M., Hannula, S.E., Bełka, M., Jędryczka, M., 2018. Fungal Biodiversity and Their Role in Soil Health. Frontiers in Microbiology 9(707). https://doi.org/10.3389/fmicb.2018.00707

Frey, B., Vilarino, A., Schuepp, H., Arines, J., 1994. Chitin and ergosterol content of extraradical and intraradical mycelium of the vesicular-arbuscular mycorrhizal fungus Glomus intraradices. Soil Biology and Biochemistry 26(6), 711–717. https://doi.org/10.1016/0038-0717(94)90263-1

Fuentes, C., Saari, H., Choi, J., Lee, S., Sjöö, M., Wahlgren, M., Nilsson, L., 2019. Characterization of non-solvent precipitated starch using asymmetrical flow field-flow fractionation coupled with multiple detectors. Carbohydrate Polymers 206, 21–28. https://doi.org/10.1016/j.carbpol.2018.10.100

González-Espinosa, Y., Sabagh, B., Moldenhauer, E., Clarke, P., Goycoolea, F.M., 2019. Characterisation of chitosan molecular weight distribution by multi-detection asymmetric flow-field flow fractionation (AF4) and SEC. International Journal of Biological Macromolecules 136, 911–919. https://doi.org/10.1016/j.ijbiomac.2019.06.122

Gooday, G.W., 1990. The Ecology of Chitin Degradation. In: K.C. Marshall (Eds.), Advances in Microbial Ecology. Springer US, Boston, MA. 387–430.

Goodell, B., Qian, Y., Jellison, J., 2008. Fungal Decay of Wood: Soft Rot-Brown Rot-White Rot. In: T.P. Schultz, H. Militz, M.H. Freeman, B. Goodell and D.D. Nicholas (Eds.), Development of Commercial Wood Preservatives. American Chemical Society, Washingthon, DC. 9–31.

Guo, P., Li, Y., An, J., Shen, S., Dou, H., 2019. Study on structure-function of starch by asymmetrical flow field-flow fractionation coupled with multiple detectors: A review. Carbohydrate Polymers 226, 115330. https://doi.org/10.1016/j.carbpol.2019.115330

Hansson, K., Laclau, J.-P., Saint-André, L., Mareschal, L., van der Heijden, G., Nys, C., Nicolas, M., Ranger, J., Legout, A., 2020. Chemical fertility of forest ecosystems. Part 1: Common soil chemical analyses were poor predictors of stand productivity across a wide range of acidic forest soils. Forest Ecology and Management 461, 117843. https://doi.org/10.1016/j.foreco.2019.117843

Jacquiod, S., Franqueville, L., Cécillon, S.M., Vogel, T., Simonet, P., 2013. Soil Bacterial Community Shifts after Chitin Enrichment: An Integrative Metagenomic Approach. Public Library of Science One 8(11), e79699. https://doi.org/10.1371/journal.pone.0079699

Kotroczó, Z., Koncz, G., Halász, L.J., Fekete, I., Krakomperger, Z., Tóth, D.M., Balázsy, S., Tóth, J.A., 2009. Litter decomposition intensity and soil organic matter accumulation in síkfőkút dirt site. Acta Microbiologica et Immunologica Hungarica 56, 53–54. https://doi.org/10.1556/amicr.56.2009.suppl.1

Kyaschenko, J., Clemmensen, K.E., Karltun, E., Lindahl, B.D., 2017. Below-ground organic matter accumulation along a boreal forest fertility gradient relates to guild interaction within fungal communities. Ecology Letters 20(12), 1546–1555. https://doi.org/10.1111/ele.12862

Lau, A.P.S., Lee, A.K.Y., Chan, C.K., Fang, M., 2006. Ergosterol as a biomarker for the quantification of the fungal biomass in atmospheric aerosols. Atmospheric Environment 40(2), 249–259. https://doi.org/10.1016/j.atmosenv.2005.09.048

Li, N., Xu, Y.-Z., Han, X.-Z., He, H.-B., Zhang, X.-D., Zhang, B., 2015. Fungi contribute more than bacteria to soil organic matter through necromass accumulation under different agricultural practices during the early pedogenesis of a Mollisol. European Journal of Soil Biology 67, 51–58. https://doi.org/10.1016/j.ejsobi.2015.02.002

Looby, C.I., Treseder, K.K., 2018. Shifts in soil fungi and extracellular enzyme activity with simulated climate change in a tropical montane cloud forest. Soil Biology and Biochemistry 117, 87–96. https://doi.org/10.1016/j.soilbio.2017.11.014

Makádi, M., 2010: Microbiological properties of sandy soils in the Nyírség region (Hungary), affected by organic and inorganic additives (Ph.D dissertation). Szent István University, Gödöllő.

Malik, M.I., Pasch, H., 2016. Field-flow fractionation: New and exciting perspectives in polymer analysis. Progress in Polymer Science 63, 42–85. https://doi.org/10.1016/j.progpolymsci.2016.03.004

Mayer, M., Rewald, B., Matthews, B., Sandén, H., Rosinger, C., Katzensteiner, K., Gorfer, M., Berger, H., Tallian, C., Berger, T.W., Godbold, D.L., 2021. Soil fertility relates to fungal-mediated decomposition and organic matter turnover in a temperate mountain forest. New Phytologist 231(2), 777–790. https://doi.org/10.1111/nph.17421

Medina, A., Probanza, A., Gutierrez Mañero, F.J., Azcón, R., 2003. Interactions of arbuscular-mycorrhizal fungi and Bacillus strains and their effects on plant growth, microbial rhizosphere activity (thymidine and leucine incorporation) and fungal biomass (ergosterol and chi-

tin). Applied Soil Ecology 22(1), 15–28. https://doi.org/10.1016/S0929-1393(02)00112-9

Nilsson, K., Bjurman, J., 1998. Chitin as an indicator of the biomass of two wood-decay fungi in relation to temperature, incubation time, and media composition. Canadian Journal of Microbiology 44(6), 575–581. https://doi.org/10.1139/w98-039

Olsson, P.A., Larsson, L., Bago, B., Wallander, H., Van Aarle, I.M., 2003. Ergosterol and fatty acids for biomass estimation of mycorrhizal fungi. New Phytologist 159(1), 7–10. https://doi.org/10.1046/j.1469-8137.2003.00810.x

Ravi Kumar, M.N.V., 2000. A review of chitin and chitosan applications. Reactive and Functional Polymers 46(1), 1–27. https://doi.org/10.1016/S1381-5148(00)00038-9

Sharp, R.G., 2013. A Review of the Applications of Chitin and Its Derivatives in Agriculture to Modify Plant-Microbial Interactions and Improve Crop Yields. Agronomy 3, 757–793. https://doi.org/10.3390/agronomy3040757

Simpson, R.T., Frey, S.D., Six, J., Thiet, R.K., 2004. Preferential Accumulation of Microbial Carbon in Aggregate Structures of No-Tillage Soils. Soil Science Society of America Journal 68(4), 1249–1255. https://doi.org/10.2136/sssaj2004.1249

Stahl, P.D., Parkin, T.B., 1996. Relationship of soil ergosterol concentration and fungal biomass. Soil Biology and Biochemistry 28(7), 847–855. https://doi.org/10.1016/0038-0717(96)00061-2

Steffen, K.T., Cajthaml, T., Šnajdr, J., Baldrian, P., 2007. Differential degradation of oak (Quercus petraea) leaf litter by litter-decomposing basidiomycetes. Research in Microbiology 158(5), 447–455. https://doi.org/10.1016/j.resmic.2007.04.002

Sun, J.-M., Irzykowski, W., Jedryczka, M., Han, F.-X., 2005. Analysis of the Genetic Structure of Sclerotinia sclerotiorum (Lib.) de Bary Populations from Different Regions and Host Plants by Random Amplified Polymorphic DNA Markers. Journal of Integrative Plant Biology 47(4), 385–395. https://doi.org/10.1111/j.1744-7909.2005.00077.x

Świtoniak, M., Novák, T.J., Charzyński, P., Zalewska, K., Bednarek, R., 2014. Forested hilly landscape of Bükkalja Foothill (Hungary). In: M. Świtoniak and P. Charzyński (Eds.), Soil sequences atlas. Nicolaus Copernicus University Press, Torun. 169–181.

Teste, F.P., Laliberté, E., Lambers, H., Auer, Y., Kramer, S., Kandeler, E., 2016. Mycorrhizal fungal biomass and scavenging declines in phosphorus-impoverished soils during ecosystem retrogression. Soil Biology and Biochemistry 92, 119–132. https://doi.org/10.1016/j.soilbio.2015.09.021

Voříšková, J., Brabcová, V., Cajthaml, T., Baldrian, P., 2014. Seasonal dynamics of fungal communities in a temperate oak forest soil. New Phytologist 201(1), 269–278. https://doi.org/10.1111/nph.12481

Wallander, H., Ekblad, A., Godbold, D.L., Johnson, D., Bahr, A., Baldrian, P., Björk, R.G., Kieliszewska-Rokicka, B., Kjøller, R., Kraigher, H., Plassard, C., Rudawska, M., 2013. Evaluation of methods to estimate production, biomass and turnover of ectomycorrhizal mycelium in forests soils – A review. Soil Biology and Biochemistry 57, 1034–1047. https://doi.org/10.1016/j.soilbio.2012.08.027

Wang, B., An, S., Liang, C., Liu, Y., Kuzyakov, Y., 2021a. Microbial necromass as the source of soil organic carbon in global ecosystems. Soil Biology and Biochemistry 162, 108422. https://doi.org/10.1016/j.soilbio.2021.108422

Wang, B., Liang, C., Yao, H., Yang, E., An, S., 2021b. The accumulation of microbial necromass carbon from litter to mineral soil and its contribution to soil organic carbon sequestration. CATENA 207, 105622. https://doi.org/10.1016/j.catena.2021.105622

Weil, R.R., Brady, N.C., 2017. The Nature and Properties of Soils (15th Edition) Pearson Education, London.

Yeul, V.S., Rayalu, S.S., 2013. Unprecedented Chitin and Chitosan: A Chemical Overview. Journal of Polymers and the Environment 21(2), 606–614. https://doi.org/10.1007/s10924-012-0458-x

Zhang, X., Dai, G., Ma, T., Liu, N., Hu, H., Ma, W., Zhang, J.-B., Wang, Z., Peterse, F., Feng, X., 2020. Links between microbial biomass and necromass components in the top- and subsoils of temperate grasslands along an aridity gradient. Geoderma 379, 114623. https://doi.org/10.1016/j.geoderma.2020.114623