HoliSoils
Working together for forest soils

D1.2 **Methodologies for characterization of soil organic matter properties & identification of those responding to forest management.**

Holistic management practices, modelling and monitoring for European forest soils, HoliSoils

Project duration: 1.5.2021–31.10.2025

H2020 Grant Agreement No 101000289

Coordinator: Natural Resources Institute Finland (Luke)

Deliverable D2.1: Methodologies for characterization of soil organic matter properties & identification of those responding to forest management		
D2.1. describes methodology of SOM characteristics used in HoliSoils.		
Due date	30.04.2024	
Authors	Bartosz Adamczyk (lead author) et al.,	
Date of publication	30.4.2024	
Dissemination level		
PU	Public, fully open e.g. web	X
CO	Confidential, only for members of the consortium (including the Commission Services)	
CI	Classified, information as referred to in Commission Decision 2001/844/EC	
Nature of the Deliverable		
R	Document, report	X
DEM	Demonstration, pilot, prototype, plan design	
DEC	Websites, patents filing, market studies, press & media actions, videos etc.	
OTHER	Software, technical diagram etc.	
Ethics	Ethics deliverables	

This project has received funding from the European Union's H2020 research and innovation programme under grant agreement No 101000289.

Contents

1. Introduction

Soil organic matter (SOM) consists of plant and animal litter and microbial residues at various stages of decomposition. SOM is of crucial importance to soil structure stabilization, retention and release of nutrients, and maintenance of water-holding capacity. SOM contains roughly 50% soil organic C (SOC) by mass. SOM content ranges from around 1% in deserts, to ≥90% for peatlands. The three common functional SOM fractions are POM (particulate organic matter) consisting of organic matter fragments with low degree of decomposition, organic matter associated with minerals (MAOM, mineral-associated organic matter) and a mobile fraction that can be described as dissolved organic matter (DOM). In mineral soils POM and MAOM forms soil aggregates of different shape and size. On the other hand, peat soils consist of partially decomposed plant organic matter.

During the last few decades, we have been witnessing a rapid evolution of thinking about SOM genesis (Adamczyk et al. 2021). The focus of studies moved from aboveground plant litter inputs to belowground ones, in congruence with studies showing that a significant part of the stored soil C is derived from roots and root-associated microorganisms (Clemmensen et al. 2013). We shifted from thinking that chemically resistant C compounds form the most stable SOM (Melillo et al. 1982) to stable SOM genesis from both more labile and chemically resistant compounds (Cotrufo et al. 2015). In line with this change, the paradigm of "humification" as a mechanism for stable SOM formation has finally been rejected, and we have started to see SOM as "a continuum of progressively decomposing organic compounds" (Lehmann and Kleber 2015). Turnover of SOM is driven by fungi and bacteria in interaction with plant roots (Adamczyk et al., 2019; Fanin et al., 2022). Fungi are efficient decomposers of organic matter via secreted enzymes and also via non-enzymatic routes (Lindahl & Tunlid, 2015). Not only free-living fungi are powerful decomposers but also some of the fungi living in symbioses with trees (ectomycorrhizal fungi) and with shrubs (ericoid mycorrhizal fungi) may participate (e.g. Lindahl et al., 2021). Recent advances in soil science underline the role of microorganisms in channeling fresh C from inputs to more persistent forms (Camenzind et al., 2023): while living

microbial biomass consists below 5% of soil organic C, microbial necromass may account for even more than half of it (Liang et al., 2019). Amino sugars have been recognised as components of microbial necromass previously (Liang et al., 2017) with muramic acid as an indicator of bacterial necromass and glucosamine (monomer of chitin) as an indicator of fungal necromass (Amelung et al., 1999). Fungal necromass may be further stabilized in soil via interaction with minerals, moreover, also interaction of necromass with root-derived compounds, tannins may increase SOM persistence (Adamczyk et al. 2019).

The stability of the SOM is dependent on climatic conditions, soil properties (minerals), activities of soil fauna, microorganisms and their interaction with plant roots. In the HoliSoils project we aimed to characterize SOM properties and find those which respond to forest management. We surveyed for potential methods and have selected those which are potentially explanatory to SOM dynamics and C stabilization in the soil. We tested these methods across HoliSoils experimental sites (of WP4 and WP5). Methods and protocols were adopted when necessary, so that their applicability can cover the width of forest soil diversity across Europe.

2. Methodologies for characterization of soil organic matter

Characterisation of SOM can be done using different methods. Overall chemical quality of the SOM can be estimated for example with Fourier-transformed Infrared Spectroscopy (FTIR, Adamczyk et al. 2016), nuclear magnetic resonance spectroscopy (NMR) (Kögel-Knabner et 2008), or analytical pyrolysis (Saiz-Jimenez, 1994). However, these methods do not provide information about potential stability and dynamics of different soil fractions. Thus, based on mechanistic understanding of SOM processes, we pin-point following markers of SOM characteristics:

- physical characterization (MAOM vs POM),
- markers of microorganisms, ergosterol (fungal biomass) and amino sugars (bacterial and fungal necromass) and PLFA (phospholipid fatty acids, markers of living bacteria and fungi),
- plant compounds, tannins and lignins,
- chemically stable vs chemically labile organic C pools
- pyrogenic C

2.1 Physical characterization/fractionation of SOM: MAOM and POM

The fractionation of SOM between POM (particulate organic matter) and MAOM (mineral-associated organic matter is done by wet sieving combined with ultrasonic dispersion. Figure 1 shows the main steps. The sample undergoes a first dispersion by agitation in water, followed by sieving through a set of three sieves (200, 50 and 20 µm). The material retained in the 50 and 20 µm sieves is recovered, pooled, made up with water to about 125 mL, and submitted to a second dispersion by ultrasonics (100 W nominal output). The so dispersed material is sieved again through 50 and 20 µm sieves. By this method, we isolate three POM fractions (coarse: 2000—200 µm; medium: 200—50 µm; and fine: 50—20 µm). The materials < 20 µm are taken as the organo-mineral complex (OMC), and therefore its organic matter is the

MAOM; however note that two different MAOM fractions are generated: one, released without the need of ultrasonic dispersion (MAOM I), and another, after obtained ultrasonic dispersion (MAOM II). From our observations, the second organo-mineral complex (released after ultrasonics) is always richer in organic matter than the first one (released just after agitation).

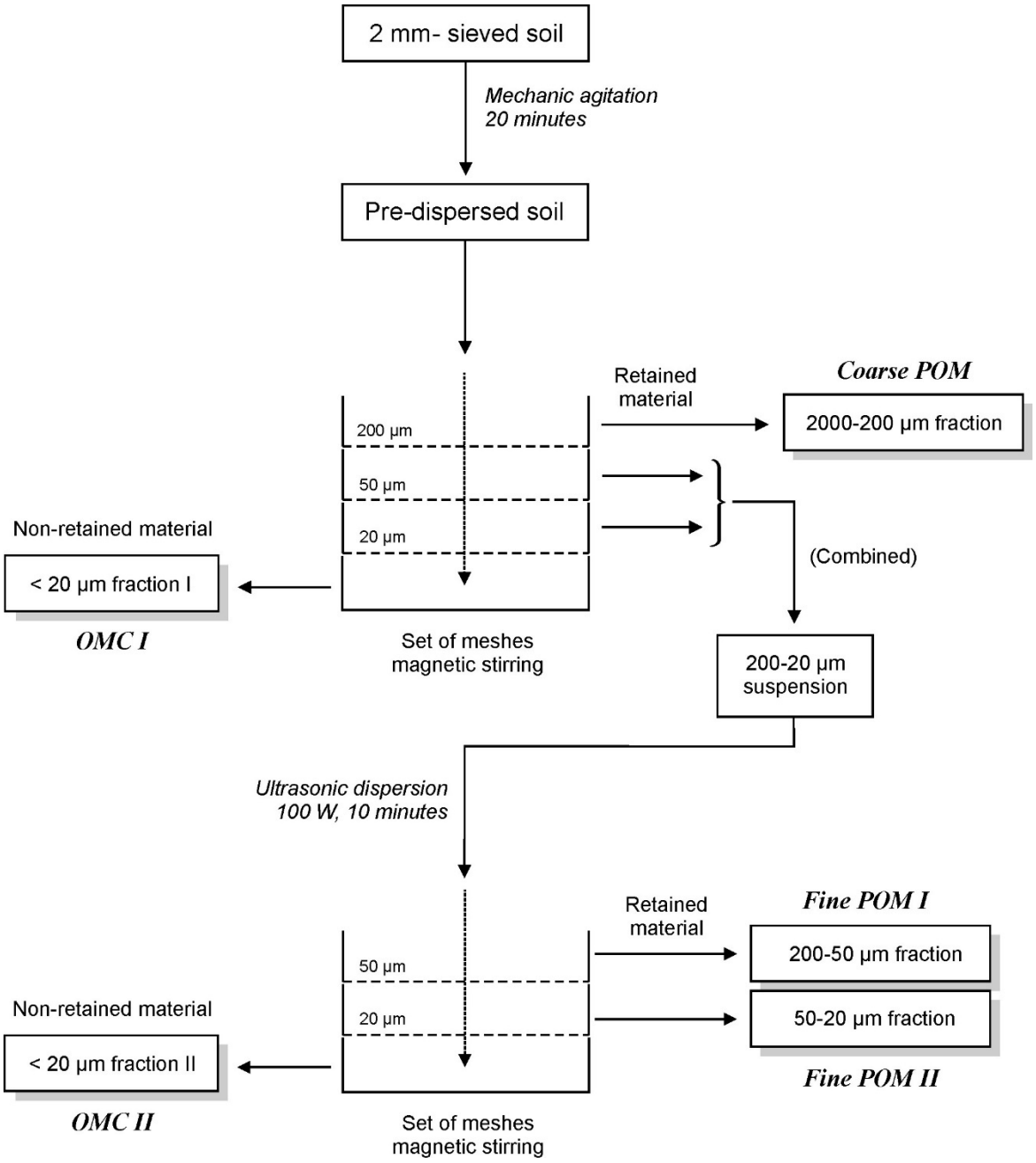

Figure 1. Flow diagram of the method for size-fractionation of soil samples.

A paper is in preparation, describing exhaustively the method, in combination with acid hydrolysis, for an exhaustive characterization of soil organic matter, from both the viewpoint of physical protection (free versus protected fractions) and biochemical quality (labile versus recalcitrant fractions).

2.2 Biochemical methods for characterization SOM

Soil sampling

Precise description of the protocol for soil sampling for further characterization of SOM is described elsewhere, in deliverable 4.1 “Protocol of site, soil property and GHG measurements at management test sites”.

Concentration of amino sugars

Amino sugars are measured with high-performance liquid chromatography (HPLC) with fluorescence detector as in Adamczyk et al. (2020) and modified in Adamczyk et al (2024, manuscript submitted). This method is adjusted to wide range of soils, including highly organic peat soils.

Concentration of ergosterol

Concentration of ergosterol is measured with classic HPLC method with UV detector. Our modification during HoliSoils project will let to assure peak purity with mass spectrometry and increased precision of detection of small quantities of ergosterol (Adamczyk et al. 2024). Also, we compared extraction protocols and chosen the most effective for a wide set of HoliSoils sites, which is 10% KOH in methanol followed with liquid-liquid extraction with cyclohexane.

Chemically stable and labile C pools

Chemically stable and labile C pools are determined following the protocol by Kallenbach et al. (2016) which uses acid hydrolysis to fractionate SOM into chemically stable and chemically labile pools. The chemically stable C is estimated based on the difference in C remaining in the non-acid hydrolysable pool and the initial C content of the sample. C content of the stable C pool and the total C pool is measured with elemental analyzer (LECO).

In addition to acid hydrolysis procedures, the overall recalcitrance of organic matter pools is being quantified by means of *Differential Scanning Calorimetry* (DSC), using a TA Q100 Scanning Calorimeter (WATERS). DSC gives a panoramic view of the release of energy (by combustion) along a growing gradient of heat (from 100 to 600°C), allowing the calculation of the total energy content of organic matter and T50 (the temperature at which 50% of the total energy content is released) as a measure of the recalcitrance of a given organic matter pool (either total OM, or a fraction) (Rovira et al. 2008). The combustion temperature, (at which combustion starts) is also a measure of organic matter recalcitrance.

Condensed tannins

Tannins are measured after extraction from soil with a mixture of acetone and water. Concentration of extracted tannins is measured with acid-butanol assay (Smolander et al. 2005). The product of reaction is measured spectrophotometrically.

Lignin concentration

Lignin concentration is measured with acetyl bromide method (Danise et al. 2020). Soil samples after initial purification with acetone are dried and residue is exposed to acetyl bromide in glacial acetic acid and later hydroxylamine is added. The product of reaction is measured spectrophotometrically.

Total carbohydrates

Total carbohydrates may be quantified spectrophotometrically on the obtained hydrolysates by the phenol-sulfuric acid method (Dubois et al. 1956).

Hydrolysable phenolics

An overall estimation of hydrolysable phenolics may be attained by measuring them in the obtained hydrolysates, spectrophotometrically, by the Folin-Denis (or Folin-Ciocalteau) (Alef and Nannipieri 1995).

Pyrogenic carbon

Pyrogenic C (black C) is estimated as in Wiedemeier et al. (2013). Pyrogenic C in soil samples is digested with nitric acid to produce benzene polycarboxylic acids measured with HPLC.

Analytical pyrolysis

Analytical pyrolysis after in situ methylation with tetramethyl ammonium hydroxide, combined with gas chromatographic separation of the methylated pyrolysis products, is a rapid analytical technique to study changes in the inner composition of biopolymers including lignocellulose or soil organic matter. The mass spectrometric detection makes it possible to estimate the size of pyrolysis fragments and to draw conclusions on their identity (Saiz-Jimenez, 1994). In this way, the contribution of various molecules to the formation of soil organic matter can be assessed (Valášková et al., 2007). Organic compounds originating in carbohydrates, fatty acids, guaiacyl lignin, p-hydroxyphenyl lignin, syringyl lignin or nitrogen-containing compounds can be identified and quantified.

Samples of milled substrates (soil or its fraction, such as humic acids, 1 mg, per sample) are submitted to pyrolysis/methylation—gas chromatography/mass spectrometry in triplicate to ensure robustness. The samples are treated by adding an excess of tetramethylammonium hydroxide (25% aqueous solution) and the mixtures are placed on a wolfram spirals and dried under vacuum at room temperature (Saiz-Jimenez, 1994). Pyrolysis is performed with pyrolyzer. Each sample on the wolfram support is inserted within a coil of platinum filament and this probe is placed into the injector port (240 °C) of a gas chromatograph. The samples are pyrolyzed at 550 °C for 10 s. The pyrolysis is thus performed directly in the injector of a GC/MS system. The GC instrument should be equipped with a split injector (split ratio 1/40) and an HP-5 column can be used for separation (30 m, ID 0.25 mm, 0.25 mm film thickness); using helium as the carrier gas (1 ml min^{-1}). The temperature program is started at 45 °C and the oven was heated to 240 °C at a rate of 5 °C min^{-1}. The detector delay time should be set to 2 min. The injector and transfer line temperature is set to 240 °C. Mass spectra of the pyrolysis products are recorded at 1 scan s^{-1} under electron impact at 70 eV, mass range 50–450 amu.

The pyrolysis products are identified by comparing the mass spectra with the data in the NIST 02 library, and independently by interpreting the fragmentation pattern. The relative percentages of pyrolysis products are calculated from the relative areas of the peaks. Individual fragments detected after pyrolysis are grouped according to their source compounds (carbohydrates, fatty acids, guaiacyl lignin, p-hydroxyphenyl lignin, syringyl lignin or nitrogen-containing compounds).

2.3 Identification of SOM properties most responsive to forest management

SOM properties differed in response to forest management. Though C pools (stable vs labile C pools, total C) did not differ much in a short-term after forest management practices (e.g. experiments with continue cover forestry), N fertilization experiment lasting for more than 50 years provided clear differences (see Table 1 for all sites analyzed). Markers of microbial biomass (ergosterol and PLFA) and necromass were more responsive under even short-term effect of forest management. We observed also changes in the pools of plant derived tannins, but not clear differences in the lignin content.

Thus, we propose to use for process-driven SOM characterization following methods:

MAOM and POM fractions: size fractionation with ultrasonics. See our previous description of the method. We expect greater effects of sylvicultural practices on the POM fractions than in the MAOM fractions, assumed to be more stable.

Biochemical quality of the overall Soil Organic Matter (SOM) and MAOM and POM fractions: acid hydrolysis and Differential Scanning Calorimetry (DSC). For both POM and MAOM fractions, a quantification of their biochemical quality is achievable by means of acid hydrolysis (to measure the recalcitrance of both C and N), and by differential scanning calorimetry (DSC), to achieve an overall measure of its recalcitrance through its resistance to combustion. Acid hydrolysis may be combined with the characterization of labile (= hydrolysable) fractions for carbohydrates, phenolics, aromaticity, and other indicators.

microbial biomass (ergosterol, PLFA) and necromass (amino sugars)

tannins in tannin-rich forests (when required)

Total carbohydrates: acid hydrolysis + phenol-sulfuric method

Total hydrolysable phenolics: acid hydrolysis + Folin-Denis method

These analyses should be used preferably with analyses of microbial community structure and greenhouse gases to provide explanations to SOM dynamics. Analyses of GHG and microbial community structure with metagenomic tools is provided elsewhere, in deliverables 2.2 and 2.3.

Table 1

The soil from different countries around Europe under different forest management

Soil under different forest managements	Used methods for SOM characterization	Time of measurement
Forest soil under N, P and Ca fertilization, Finland, Karstula, (62°54'43.343"N; 24°34'16.021"E)	*Physical SOM characteristics*: POM and MAOM *Biochemical SOM characteristics:* amino sugars, ergosterol, C and N content, stable C and labile C, NO3, NH4, lignin and tannins	Fall and Winter 2021-22.
Forested peatland, Finland, Ranskanalankorpi (61°10.966'N; 25°15.985'E)	*Biochemical SOM characteristics:* amino sugars, ergosterol, C and N content, stable C and lable C, NO3, NH4, lignin and tannins	Fall 2022 and Fall 2023
Forests. Spain Llobera, Secanella, Madrona (close to Solsona: UTM 31, X 378500, Y 4649775)	*Physical SOM characteristics*: POM and MAOM *Biochemical SOM characteristics*: amino sugars, ergosterol, C and N content, stable C and labile C, NO3, NH4, lignin and tannins. Differential scanning calorimetry (DSC), carbohydrates, phenolics.	Spring 2023
Forests. Germany, 11°39'39.6"E; 48°25'8.4"N Southern Bavaria	*Biochemical SOM characteristics*: amino sugars, ergosterol, C and N content, stable C and labile C, NO3, NH4, lignin and tannins	Fall, 2024

2.4 Methods developed during the project

Table 2

Publications/manuscripts	marker of soil organic matter property:
Adamczyk S, Lehtonen A, Mäkipää R, Adamczyk B (2023) A step forward in fungal biomass estimation – a new protocol for more precise measurements of soil ergosterol with liquid chromatography-mass spectrometry and comparison of extraction methods. New Phytologist DOI: 10.1111/nph.19450.	**ergosterol**
Adamczyk S, Mäkipää R., Lehtonen A., Adamczyk B. Precise method to measure fungal and bacterial necromass using high pressure liquid chromatography with fluorescence detector adjusted to inorganic, organic and peat soil. Manuscript submitted	**amino sugars: glucosamine and muramic acid**
Rovira P, Garcia-Pausas J, Casals P. Evaluating the biochemical quality of soil organic matter by direct Differential Scanning Calorimetry (DSC). In preparation.	**Overall measure of SOM recalcitrance by DSC**
Rovira P, Lopez-Sangil , et al. Isolation of soil organic matter pools differing in turnover: combining size fractionation with acid hydrolysis. In preparation.	**Combined protocol of SOM fractionation**

3. References

Adamczyk B, Sietiö O-M, Straková P, Prommer J, Wild B, Hagner M, Pihlatie M, Fritze H, Richter A, Heinonsalo J (2019) Plant roots increase both decomposition and stable organic matter formation in boreal forest soil. Nature Communications 10: 3982.

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.

Adamczyk S, Lehtonen A, Mäkipää R, Adamczyk B (2024) A step forward in fungal biomass estimation – a new protocol for more precise measurements of soil ergosterol with liquid chromatography-mass spectrometry and comparison of extraction methods. New Phytologist DOI: 10.1111/nph.19450.

Adamczyk S, Mäkipää R., Lehtonen A., Adamczyk B. Precise method to measure fungal and bacterial necromass using high pressure liquid chromatography with fluorescence detector adjusted to inorganic, organic and peat soil. Submitted

Adamczyk B (2021) How do boreal forest soils store carbon? BioEssays, 43:e2100010.

Alef K and Nannipieri P (1995) Methods in Applied Soil Microbiology and Biochemistry. Academic Press.

Amelung W, Zhang X, Flach KW and Zech W (1999), Amino Sugars in Native Grassland Soils along a Climosequence in North America. Soil Science Society of America Journal 63: 86-92.

Camenzind T, Mason-Jones K, Mansour I *et al.* (2023) Formation of necromass-derived soil organic carbon determined by microbial death pathways. *Nature Geoscience* 16, 115–122.

Clemmensen KE et al. (2013) Roots and associated fungi drive long-term carbon sequestration in boreal forest. Science 340:1615.

Cotrufo M et al. (2015) Formation of soil organic matter via biochemical and physical pathways of litter mass loss. Nature Geosci. 8:776.

Cotrufo M et al. (2013) The Microbial Efficiency-Matrix Stabilization (MEMS) framework integrates plant litter decomposition with soil organic matter stabilization: do labile plant inputs form stable soil organic matter? Glob. Change Biol. 19:988.

Danise T, Innangi M, Curcio E, Fioretto A, Guggenberger G (2020) Fast spectrophotometric method as alternative for CuO oxidation to assess lignin in soils with different tree cover. Forests 11:1262.

Dubois M, Gilles KA, Hamilton JK, Rebers PA & Smith F (1956) Colorimetric method for determination of sugars and related substances. Analytical Chemistry 28:350-356.

Fanin N et al. (2022), Ericoid shrubs shape fungal communities and suppress organic matter decomposition in boreal forests. New Phytologist 236:684-697.

Kallenbach C, Frey S, Grandy A (2016) Direct evidence for microbial-derived soil organic matter formation and its ecophysiological controls. Nature Communications 7:13630.

Kögel-Knabner I et al. (2008) Organo-mineral associations in temperate soils: Integrating biology, mineralogy, and organic matter chemistry. Z. Pflanzenernähr. Bodenk. 171:61.

Lehmann J and Kleber M (2015) The contentious nature of soil organic matter. Nature 528:60.
Liang C et al. (2017) The importance of anabolism in microbial control over soil carbon storage. Nature Microbiology 25:17105.

Liang C et al. (2019) Quantitative assessment of microbial necromass contribution to soil organic matter. Global Change Biology 25:3578.

Lindahl BD et al. (2021) A group of ectomycorrhizal fungi restricts organic matter accumulation in boreal forest. Ecology Letters 24:1341.

Lindahl BD and Tunlid A (2015) Ectomycorrhizal fungi – potential organic matter decomposers, yet not saprotrophs. New Phytologist 205:1443–1447.

Mäkipää R et al. (2023). How does management affect soil C sequestration and greenhouse gas fluxes in boreal and temperate forests? – A review. Forest Ecology and Management 529: 120637.

Melillo JM et al. (1982) Nitrogen and lignin control of hardwood leaf litter decomposition dynamics. Ecology 63:621.

Rovira P, Kurz-Besson C, Coûteaux MM, Vallejo VR (2008) Changes in litter properties during decomposition: A study by differential thermogravimetry and scanning calorimetry. Soil Biology & Biochemistry 40:172-185.

Saiz-Jimenez C (1994) Analytical pyrolysis of humic substances—pitfalls, limitations, and possible solutions. Environmental Science and Technology 28:1773–1780.

Valášková V et al. (2007) Production of lignocellulose-degrading enzymes and degradation of leaf litter by saprotrophic basidiomycetes isolated from a *Quercus petraea* forest. Soil Biology and Biochemistry 39:2651-2660.

Wallander H et al. (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.

Wiedemeier DB et al. (2013) Improved assessment of pyrogenic carbon quantity and quality in environmental samples by high-performance liquid chromatography. J Chromatogr A 1304:246-50