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Precise method to measure fungal and bacterial necromass using high pressure liquid chromatography with fluorescence detector adjusted to inorganic, organic and peat soils

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ABSTRACT

Soil organic matter is the dominant pool of carbon (C) in terrestrial ecosystems. Recent advances in understanding of the mechanisms of C stabilization in the soil emphasize microbes as the main drivers. Special attention is placed on the accumulation of bacterial and fungal necromasses. This calls for development of fast and reliable methods to estimate microbial necromass in a various type of soils, including peat soils. Here we provide precise method to measure fungal and bacterial necromasses with high-pressure liquid chromatography-fluorescence detector (HPLC-FLD) and its comparison with gas chromatography method. Purity of the chromatographic peaks was confirmed with mass spectrometry. The HPLC-FLD method provides reliable results for mineral, organic and highly organic peat soils.

Soil organic matter (SOM) is the largest terrestrial carbon (C) stock (Bradford et al., 2019). To sustain and/or increase this soil C stock to mitigate climate change through proper management, we need to understand the mechanisms that control C persistence in the soil (Adamczyk, 2021; Liang et al., 2019; Mäkipää et al., 2023). Recent advances in soil science underline the role of microorganisms in channeling fresh C from inputs to more persistent soil fractions (Camenzind et al., 2023; Joergensen, 2018). Though living microbial biomass consists below 5 % of soil organic C, microbial necromass may account for even more than half of it (Joergensen, 2018; Liang et al., 2019). The concept of amino sugars as a proxy for microbial necromass has been proposed 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). Remaining amino sugars found in the soil, i.e. mannosamine and galactosamine cannot be easily ascribed to one group of microorganisms (Amelung et al., 1999; Joergensen, 2018). Available protocols to measure soil amino sugars (Indorf et al., 2011; Liang et al., 2012; Salas et al., 2023) are not tested yet for peat soil, which store even more C than forests (Beaulne et al., 2021; Lehtonen et al., 2023). Analyses of organic peat soils with methods developed for forest soils (mineral and organic layers), may be difficult since organic matter of peat may interfere with analyses due to far higher organic matter content and lack of mineral admixture often found in humus layer of forest soils (Adamczyk et al., 2020; Laiho, 2006). Due to known importance of microbial necromass for accounting C stocks, we need fast and reliable methods to determine amino sugars from a wide set of soil types.

Here we report development of our earlier method to study glucosamine with high-pressure liquid chromatography with fluorescence detector (HPLC-FLD) (Adamczyk et al., 2020) into a method with HPLC-FLD aiming to account for muramic acid and glucosamine within the same runs. We confirmed our method with mass spectrometry (MS) to obtain full confidence about chromatographic peak purity. Like in our previous method for glucosamine estimation (Adamczyk et al., 2020), we propose purification steps with 0.2 M NaOH to remove free amino sugars and amino acids, followed by acid hydrolysis releasing amino sugars from microbial residues followed with derivatization with FMOC-Cl (9-fluorenyl methoxycarbonyl chloride). To adjust the method to mass spectrometry (MS), we replaced acetic acid-sodium buffer with ammonium acetate buffer, as sodium buffers cannot be used with MS. Moreover, we changed the buffer pH from 4.2 to 5.7 which made possible to separate chromatographic peak of muramic acid. Moreover, we estimated the conditions suitable for mass detection and effective ionization in electrospray ionization (precise description of the proposed protocol in Supplementary Data, Table S1).

For testing the protocol, we have chosen two forest sites and forested

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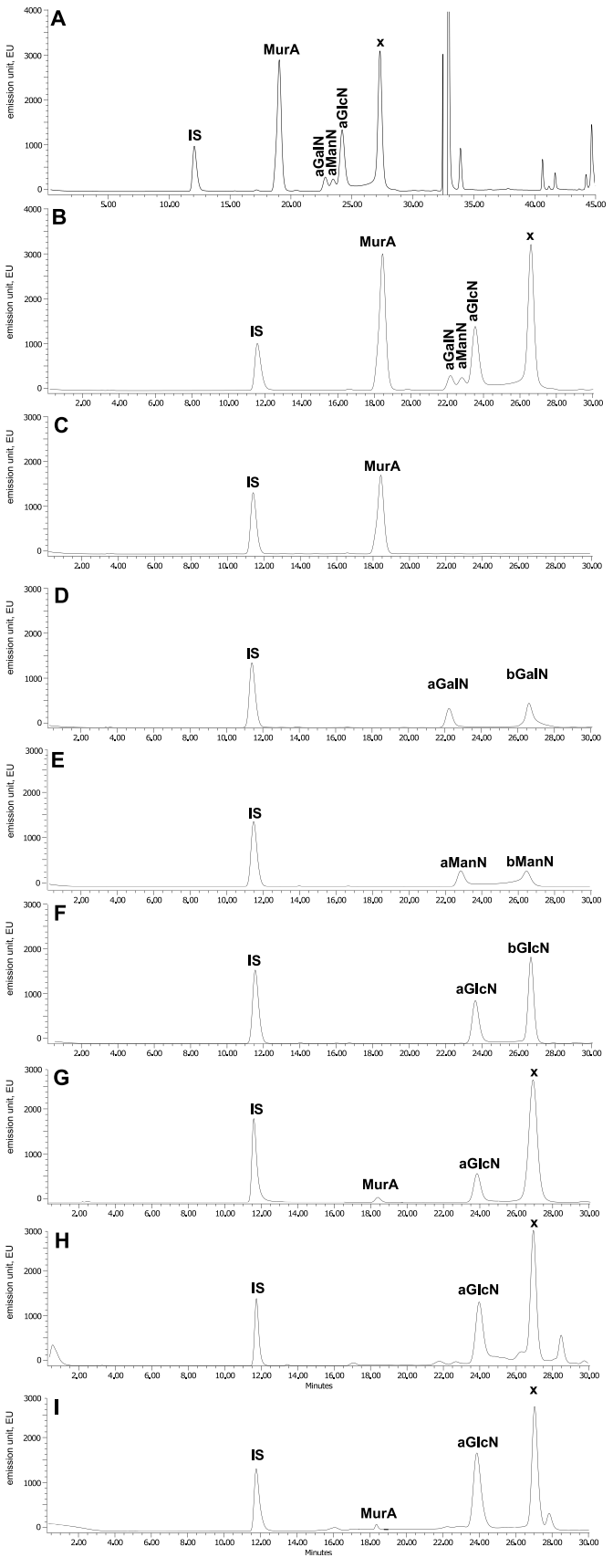

(caption on next column)

Fig. 1. Chromatograms of amino sugar standards and samples. A) full chromatogram of the proposed method, B) 0–30 min part of chromatogram with all peaks of amino sugars. C) chromatogram of muramic acid standard, D) chromatogram of galactosamine standard, E) chromatogram of mannosamine standard, F) chromatogram of glucosamine standard, G) chromatogram of peat soil sample, H) chromatogram of forest soil from Karstula, I) chromatogram of forest soil from Hyytiälä. IS – internal standard, FMOC-homocysteic acid, MurA – FMOC-muramic acid, GlcN – FMOC-glucosamine, ManN – FMOC-mannosamine, GalN – FMOC-galactosamine. X – peak of mixed beta-anomers of FMOC-amino sugars. a and b – alfa and beta-anomers of GalN, GlcN, ManN. Standards of amino sugars (glucosamine, muramic acid, mannosamine and galactosamine) were purchased from Sigma-Aldrich. Samples were run on Arc HPLC Waters equipped with fluorescence detector (FLD) and mass spectrometer (Acquity qDa, Waters) with software EMPOWER 3.6.

peatland. Forest site soil is Podzol on glacial till and forested peatland is well drained nutrient rich peat (for precise site description see Supplementary Data). Chromatograms of standards of FMOC-amino sugars detected with fluorescence detector are shown at Fig. 1 (^{1}A–^{1}B). Though modifications of buffer pH in eluent let us to obtain one peak for muramic acid (MurA), glucosamine is represented by two peaks, like in our earlier work (Adamczyk et al., 2020). Amino sugars may provide two peaks because they form anomers (alfa and beta) with different retention time (Jahnel and Frimmel, 1996), which is visible for galactosamine (GalN), mannosamine (ManN) and glucosamine (GlcN) (Fig. 1 C-F). Chromatogram of a mixture of all amino sugars showed well separated peak of MurA, and alfa anomers of ManN, GalN and GlcN (Fig. 1A-B) and all beta anomers of ManN, GalN and GlcN are overlapping under peak "x" (Fig. 1A-B). Thus, for detection and quantification we propose to use peak of alfa GlcN, providing high linearity with no need to consider also beta anomer of GlcN (see Fig. S1). As galactosamine and mannosamine do not represent well microbial necromass of specific origin, detection of these amino sugars was not the aim of this protocol. Examples of chromatograms from soil samples (see Supplementary data for site descriptions) are on Fig. 1 G-I. Peak purity is provided as Fig. S2 with explanation of fragmentation patterns.

The recovery percent of glucosamine was studied with addition of chitin (0–10 mg) to soil and recovery percent of muramic acid (0–5 mg) was studied with addition of peptidoglycan to soil (soil from all studied sites). The average recovery percent was 75 (±2)% for glucosamine and 85 (±3)% for muramic acid. The precision of the method estimated as relative to standard deviation from analyses of five laboratory replicates was ± 1.5 % (average for all studied sites). Increasing additions of chitin or peptidoglycan to soil resulted in a linear response of measured amino sugars (Fig. S3). The limit of detection and limit of quantitation was 2 and 1 μg g DW for glucosamine and 4 and 2 μg g DW for muramic acid, respectively.

Comparison of protocols: We compared our new HPLC-FLD protocol with well-established GC protocol (Zhang and Amelung, 1996), for which peak purity was also proven with MS (Amelung et al., 1999; Liang et al., 2012). A new method with UPLC-HRMS (ultra-high-performance liquid chromatography and high-resolution mass spectrometry), although able to provide in the same run estimation of amino sugars, neutral sugars and uronic acids, does not provide good separation of galactosamine and glucosamine and has not been tested for peat soils (Salas et al., 2023). There are also other HPLC methods available, but due to lack of MS confirmation of peak purity we did not take them into consideration (Indorf et al., 2011). We followed precisely description of GC protocol as described in (Zhang and Amelung, 1996), with the use of beta-endosulfan instead of methylglucamine as second internal standard. This change was proposed by Roth et al. (2011) to avoid interference of methylglucamine with iron. We compared the protocols using the same sample set. The results for amino sugars are similar, with lower standard deviation for HPLC and somewhat higher detection of MurA for GC (Table 1). However, GC method did not provide clear peaks for MurA in peat soils and GC chromatograms contain significant number of

Table 1
Concentrations of muramic acid and glucosamine in soil studied with GC and HPLC method. Mean values and standard deviation of three replicates. As bacteria also possess glucosamine in their cell walls, estimation of fungal glucosamine considered subtraction of bacterial glucosamine from total glucosamine assuming that muramic acid and glucosamine occur at 1-to-2 molar ratio in bacterial cells (Amelung et al., 1999).

Site	muramic acid, μg g DW		glucosamine, μg g DW	
	HPLC	GC	HPLC	GC
Karstula (forest on mineral soil)				
organic layer	<4 (0.0)	4 (4.0)-	3002 (322)	2901 (502)
mineral layer	-	-	62 (25)	51 (101)
Hyytiälä (forest on mineral soil)				
organic layer	29 (3.2)	35 (20.1)	5109 (250)	4900 (566)
mineral layer	6 (1.0)	8 (3.2)	110 (10.2)	104 (20.1)
Ränskälänkorpi (forested peatland)				
0–10 cm	19 (3.1)	-	2025 (426)	2001 (998)
10–20 cm	11 (2.2)	-	2390 (357)	2281 (909)
20–30 cm	6 (1.0)	-	1929 (68)	1954 (563)
30–40 cm	7 (0.9)	-	1763 (136)	1706 (342)
40–50 cm	7 (0.7)	-	1837 (159)	1842 (256)

unknown peaks for both humus layer of the soil and especially for peat soil making peak detection challenging (Fig. S4). Following difference is time consumption of the protocols: preparation of batch of samples for HPLC method takes three days, comparing to nine days for GC making HPLC method three times faster. Another issue of GC method is availability of second internal standard, beta-endosulfan which is needed in soils rich in iron (Roth et al., 2011). Beta-endosulfan is highly toxic (Sutherland et al., 2004) and thus it is phasing out globally.

Tracing and quantifying microbial residues in numerous soil types of various ecosystems is crucial for soil health monitoring, soil productivity estimation and mitigation of climate change. As microbial necromass is an important part of stable soil C pool (Camenzind et al., 2023; Liang et al., 2019), it could be used in the estimation of loss of stable soil C, one of descriptors of soil monitoring law, a newly launched EU Commission directive aiming to bring back healthy soil to Europe. Thus, microbial necromass estimation could be applied in the future forest management planning and monitoring which aim to sequester C in mineral soil and at forested peatlands.

In conclusion, this short communication provide method to analyze microbial necromass markers (muramic acid for bacteria and glucosamine for fungi) from both, inorganic and peat soils with HPLC-FLD and confirmation of chromatographic peak purity with mass spectrometry. Taking into account similar reliability of our HPLC to GC method for mineral soils and superior of HPLC for peat soils and time consumption of both methods, we recommend the use of our HPLC protocol. We provide also short troubleshooting (Table S2) to make easier to adjust this method to your laboratory and available equipment.

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CRediT authorship contribution statement

Raisa Mäkipää: Writing – review & editing, Supervision, Funding acquisition, Conceptualization. **Bartosz Adamczyk:** Supervision, Methodology, Funding acquisition, Formal analysis, Data curation, Conceptualization. **Sylwia Adamczyk:** Writing – original draft, Methodology, Investigation, Formal analysis, Data curation, Conceptualization. **Aleksi Lehtonen:** Writing – review & editing, Project administration, Funding acquisition.

Declaration of Competing Interest

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests: Bartosz Adamczyk reports financial support was provided by Natural Resources Institute Finland. Raisa Makipaa reports financial support was provided by H2020 Food Security Sustainable Agriculture and Forestry Marine Maritime and Inland Water Research and the Bioeconomy. Bartosz Adamczyk reports financial support was provided by Research Council of Finland. Bartosz Adamczyk reports a relationship with Natural Resources Institute Finland that includes: employment. If there are other authors, they declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data Availability

Data will be made available on request.

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.pedobi.2024.150977.

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