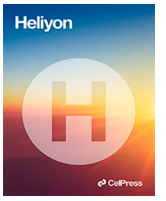
Heliyon 11 (2025) e43536

Contents lists available at ScienceDirect

Heliyon

journal homepage: www.cell.com/heliyon



Research article

Effects of biochar, ligneous soil amendments, and a microbial stimulant on soil biological activity, and carbon content and stability after two-years of their application in a boreal cropland

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ARTICLE INFO

Keywords:
Organic soil amendments
Microbial stimulant
Biochar
Pulp mill sludge
Soil organic carbon
Soil microbiology

ABSTRACT

The concerns for soil health and climate change have initiated actions to slow down soil carbon loss. The activities, collectively known as carbon farming, encompass various practices that seek to mitigate climate change and improve soil health. Among these practices, biochar alongside crop cultivation has been recognized as having potential for mitigating climate change. However, in heavily forested countries there is a wide variety of other carbon-rich side streams from the forest industry that could be utilized as organic soil amendments, or as microbial stimulants but their climate change mitigation potential, as well as the mechanisms underlying their observed effects on soil health, are not yet fully understood.

The aim of this study was to assess and compare the use of various wood-based, i.e. ligneous soil amendments and a stimulant that could be used alongside conventional farming practices in a field experiment to boost soil biological activity and carbon sequestration. The studied treatments included two biochar (*Salix* sp. and *Picea abies*), two pulp mill sludge, and one microbial stimulant treatment. The economically relevant application rates of the amendments ranged from 9000 to 21900 kg/ha (on dry weight basis) and they were applied once, whereas the microbial seed stimulant was used yearly. We investigated their impacts on soil organic carbon content and its stability, as well as on soil microbial abundance, activities, and community structures. Based on the extensive data we collected, mainly biochars increased soil organic carbon content enough to

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https://doi.org/10.1016/j.heliyon.2025.e43536
Received 9 June 2025; Received in revised form 13 June 2025; Accepted 18 June 2025
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be detectable after two years. In contrast, non-biochar amendments did not have the same effect on soil carbon, likely due to smaller application rates and higher decomposition rates. On the other hand, both the sludge and biochar treatments led to an increase in soil pH, but the observed changes in soil chemical properties had little impact on soil microbiology. Microbial stimulant was shown to be ineffective and did not alter soil biology as expected. Overall, the studied amendments had no detectable negative effects on measured soil physico-chemical parameters and had a marginal positive impact on soil biology. This suggests that these amendments could be promising options for recycling ligneous side streams from the forest industry to support primary production in arable fields.

1. Introduction

The global depletion of soil organic carbon (SOC) pools has contributed approximately 20 % to all released CO_2 emissions since the industrial revolution [1]. Furthermore, in northern boreal regions, such as in Finland, where soils naturally have relatively high SOC content, approximately 4 permille of SOC is lost from agricultural soils annually [2,3]. However, the concerns for climate change and soil health have initiated actions to slow down SOC loss, and whenever possible, to even turn cultivated soils into carbon (C) sinks following the so called '4 per 1000' initiative [4,5]. In the latest report of Intergovernmental Panel for Climate Change, it was estimated that there is an almost 3.5 Gt CO_2-eq/yr potential in C sequestration into agricultural soils [6]. This shift towards carbon farming has also been suggested to be one of the most cost-efficient tools to mitigate climate change by removing CO_2 from the atmosphere and consolidating it into agricultural soils [7,8]. This has the additional potential to enhance soil health through improved soil moisture and nutrient retention, as well as soil aeration [9–11]. Despite the overall importance of soil C sequestration has been acknowledged, there are still a plethora of uncertainties and research gaps on how in practice C can be sequestered into soils and how long-lasting the obtained effects are.

A recent assessment evaluated the potential of various agricultural practices to mitigate global climate change [12]. Among the assessed practices, biochar use and planting trees in croplands (agroforestry) were identified as having the greatest potential for mitigating climate change [12]. However, in densely forested countries like Finland, there is a wide variety of other C rich side streams from forest industry that could be used as organic soils amendments, in addition to biochars [13–15]. The overall motivation is that in addition to direct C addition, organic amendments and microbial stimulants improve soil biological activity that leads to e.g. improved aggregation, microbial residue formation and therefore to increased soil C sequestration. For example, soil application of pulp mill sludge has been studied in the past years and it has been shown to improve soil properties such as soil C content and soil aggregation as well as reducing soil bulk density [16–20]. Regular application of C rich biomass has also been shown to increase biological activity through increased microbial biomass C, crop yields, and plant nitrogen use efficiency [19–22]. The application rates in the previously mentioned studies ranged from 8000 to 225000 kg/ha (dry-weight basis), the rate playing a big role in the soil and ecosystem responses. However, the climate change mitigation potential of pulp mill sludges and other C rich biomasses as well as the mechanisms behind their observed benefits are far from sufficiently understood, and their impacts on soil health are not well quantified. Additionally, the type of organic C, whether it is active or stable [23], whether it can be classified as particulate organic matter (POM) or whether it stabilizes as mineral-associated organic matter (MAOM), together with soil aggregation and other soil interactions that protect C from decomposers, all influence the ability of C inputs to stimulate biological activity [24]. For these reasons, it is essential to understand how different types of organic materials behave after they have been incorporated into soil. Furthermore, the use of organic soil amendments incurs additional costs for farmers, and thus, it is vital to assess their usefulness for both farmers and society as a tool for improving soil health and mitigating climate change.

Soil organic matter and soil health are known to be closely related while diverse soil biology has been commonly associated with high SOC content and robust soil structure. For example, total organic carbon content (TOC) has been shown to correlate positively with microbial biomass and microbial activity [25] while microbial, especially fungal [26,27], activity and organic C inputs have been shown to support soil aggregation through glomalin-related soil proteins [28–30]. Furthermore, in a recent review, Hannula and Morriën [24] suggested that soil fungi play a crucial role in stabilizing C in the soil and affecting the structure of the soil food web all the way to ecosystem level. Based on their review they recommended soil amendments as one of the targets for future agriculture and policy development. However, the interactions between organic substrates and their effects on microbial diversity, fungal-to-bacterial abundance and ratios, as well as the corresponding consequences on SOC stability, and soil functions [31,32] are not well understood. While wood-derived i.e., ligneous amendments could potentially favor fungal growth over bacteria and lead to increased amounts of fungal necromass and to the formation of more stable SOC [24], the connection between ligneous soil amendments, fungi, SOC, and soil aggregations have not been actively studied, especially in boreal field conditions. Recently, it has been shown that fungi play an important part in the formation of soil macroaggregates [26] and that deciduous wood sawdust and paper pulp-derived organic amendment stimulated the growth of saprotrophic ascomycete fungi [33]. Humic acid treatment has also been shown to increase arbuscular mycorrhizal fungal colonization in roots, and mycelial length [34], potentially also affecting soil biological activity and fungal-to-bacteria ratio in soil. Even though there is evidence for the potential of organic soil amendments influencing soil biology, it is still uncertain how long do the effects of organic soil amendments persist in cultivated soils. Recent studies have reported that soil biological responses can persist even 8 years after application [35], an effect credited to the portion of C that persists in the soil for the long-term [36,37]. Therefore, in addition to knowledge gaps in the effects of organic amendments on soil biology and SOC, too little is

known how these effects last in time.

We targeted the abovementioned knowledge gaps by investigating soil microbial abundances, activities, and community structures, which were then compared to soil physico-chemical properties. Soil greenhouse gas (GHG) production (CO_2, N_2O and CH_4) were analyzed as indicators for soil microbial activities. Our first hypothesis (Hypothesis 1) was that biochar would persist in soil as stable SOC and increase soil water retention capacity as well as providing means of soil to retain mineral nitrogen (NH_4^+-N and NO_3^--N) forms and mediating soil N_2O fluxes. However, due to the recalcitrance of biochar-derived C, it would have little impact on other microbial properties. Our second hypothesis (Hypothesis 2) was that because the pulp mill sludges consist of more labile C, they would decompose more rapidly, impact soil microbial activity and microbial community structure, and promote fungal over bacterial abundance leading to increased soil aggregation but have limited effect on soil nitrogen (N) after two years. Our third hypothesis (Hypothesis 3) was that the microbial stimulant (humic water treatment on seeds) used for stimulating mycorrhiza formation would lead to increased fungal/bacteria-ratio and soil aggregation, but due to the relatively large size of the existing C stock, we expected no detectable impacts on the SOC stability. Finally, based on the collected data covering plants, microbes, and soil properties our aim was to compare biological parameters and soil physico-chemical properties with the intention of uncovering persisting interactions caused by our treatments.

2. Materials and methods

2.1. Experimental setup

The field experiment was established in autumn 2016 at Qvidja farm, South-West of Finland (60° 17′ 44″ N 22° 23′ 35″ E). The field soil was classified as Vertic Endogleyic Stagnic Cambisol (clayic) [38], with texture consisting of 54 % clay, 34 % silt and 12 % sand [39], and with average SOC content of 2.4 %. Soil properties were analyzed when the experimental field was established in 2016, before application of organic amendments (Supplementary Table 1). For more information about the experimental site and the application rates see Kulmala et al. [40].

Three replicates of the seven different treatments were applied into randomly chosen plots (gross 9 m × 20 m, net 5 m × 16 m) in each replicate block (20 m × 135 m). The treatments were non-fertilized control (C0N), control fertilized annually with 80 kg N/ha (C80N), and four organic soil amendments, including fibre sludge (FibreS), lime-stabilized mixed pulp mill sludge (LimeS), as well as spruce biochar (SprB), and willow biochar (WilB), both created through slow pyrolysis at 450 °C. For more information on the pulp mill sludges, see Rasa et al. [41]. A humic water (HW) treatment was also included, where seeds were pre-treated with humic water extracted from peat soil before sowing them in 2016 (wheat), 2017 (wheat) and 2018 (oat). The peat water is known to contain humic substances, which have been shown to stimulate arbuscular mycorrhiza formation [34,42] and plant growth [43]. All treatments, except for the non-fertilized control, received chemical fertilization annually equal to 80 kg N/ha at sowing. The total quantities of the amendments, their pH and electric conductivity (EC) and corresponding element inputs are presented in Table 1, excluding the HW treatment, which was assumed to have negligible contribution to soil inputs.

The ligneous soil amendments were applied in autumn 2016 and harrowed down to a maximum depth of 10 cm with a cultivator (Horsch Terrano 3.5 FX). The application rates were chosen based on agricultural recommendations. The next autumn the soil was again harrowed down to 5–10 cm depth with a disc cultivator to control the volume of the soil mixed with the soil amendments. In spring 2018 the soil was prepared for sowing by doing a single pass with a tine harrow (5 cm). Before the experiment in 2012–2014, the cultivated species had been cumin (*Cuminum cyminum* L.) and in 2015–2017 wheat (*Triticum aestivum* L., cultivars Skagen and

Table 1
Chemical and physical characteristics of the studied organic soil amendments (fibre sludge (FibreS), Lime-stabilized mixed pulp mill sludge (LimeS), Spruce biochar (SprB), and Willow biochar (WilB)) and their application rates. Carbon and nitrogen were analyzed according to ISO 16948:2015, and other elements after HNO_3-HCl digestion (ISO 11885:09).

Treatment	FibreS	LimeS	SprB	WilB
Application rate (kg/ha fw)	47100	24200	20600	33400
Dry matter content (%)	29.7	37.2	94.4	65.7
Application rate (kg/ha dw)	14000	9000	19400	21900
C (%)	41	37	90	75
N (%)	0.043	1.0	0.44	1.7
C/N-ratio	950	37	203	44
pH (H_2O)	9.2	8.9	8.3	9.8
EC (mS/cm)	54	170	94	300
Carbon input (C) (kg/ha)	5707	3349	17 424	16 524
Nitrogen input (N) (kg/ha)	6.0	90	86	373
Calcium input (Ca) (kg/ha)	685	706	188	418
Magnesium input (Mg) (kg/ha)	18	8.0	15	53
Potassium input (K) (kg/ha)	[a]	4.0	55	235
Phosphorus input (P) (kg/ha)	1.0	15	5.8	82
Manganese input (Mn) (kg/ha)	0.1	0.7	4.5	7.2
Sodium input (Na) (kg/ha)	7.8	13	1.0	15

[a] below the detection limit. fw = fresh weight, dw = dry weight.

Anniina). During the sampling year (2018), the field was sown with oat (*Avena sativa* L., cultivar Matty; [40]).

In 2018, the mean annual temperature and precipitation were 6.6 °C and 486 mm while the long-term (1981–2010; [44]) means measured at a nearby weather station (Yltöinen, 13 km from Qvidja) were 5.4 °C and 679 mm, respectively. In short, in the summer 2018 the weather was exceptionally dry and hot compared to the long-term average (for more details see Kulmala et al. [40]). To alleviate water stress during the extended dry period extending from May to July, the field was irrigated once with ~40–50 mm of water during a period of 18 h at the end of June.

2.2. Sample processing and analyses

2.2.1. Soil sampling in 2016, before the application of organic amendments

The field was sampled in 2016 by collecting seven individual samples around a circle (Ø = 50 cm) to a depth of 20 cm using a soil probe (Ø = 2.5 cm). These were taken from three fixed locations in each plot and combined to form one composite sample.

2.2.2. Sampling in 2018

Soil chemical and biological properties were analyzed monthly (May to September) during the growing season in 2018. Each month, soil samples were collected from three random locations inside each treatment plot to a depth of 10 cm using a soil probe (Ø = 2.5 cm). For determining soil bulk density and aggregate size distribution, three 5 cm deep soil cores (250 cm^3) were taken from the surface of each plot once, in August. All samples were taken during one day and stored in cold boxes for transportation. Afterwards, the samples were stored at +4 °C until the next day when they were first sieved (Ø = 10 mm mesh) and then divided into subsamples, which were kept at +4 °C (fresh samples), air-dried, or stored at −20 °C for later analyses. During this process, approx. 10 g of soil was oven dried at 105 °C overnight to determine soil water content (w/w) at each sampling time, hereafter referred to as soil moisture.

Soil biological activity measurements were done from fresh soil samples the next day after sieving. Soil physico-chemical analyses were performed separately from each individual soil sample, but the data was averaged to obtain one value per plot, leading to a total number of three samples per treatment (N = 3). During soil sampling, plant (oat) samples were collected from three locations within each plot. Roots were separated from a 15 × 15 cm wide and 10 cm deep volume of soil. Because roots were stored for further DNA-based analysis, they could not be dried, and only fresh weight (fw) was recorded. An overview of the conducted analyses is summarized in Table 2 while a detailed description of each method is given below.

2.2.3. Physico-chemical analysis

Analysis using Rock-Eval 6 (Vinci Technologies, France) was performed for dried and ground soil samples as described in Disnar et al. [45] and Barré et al. [46]. Shortly, Rock-Eval 6 consists of two-steps, with ramped heating pyrolysis followed by ramped heating oxidation. The hydrocarbons, CO_2 and CO produced during the ramped heating pyrolysis and oxidation enables an estimation of SOC characteristics and stability [46,47]. The measured parameters in Rock-Eval 6 analysis include hydrogen and oxygen index, temperatures at which 50 % of CO_2 is evolved during the pyrolysis step (t50_CO_2_pyr) or oxidation step (t50_CO_2_ox) and proportion of pyrolysable C. Based on these parameters and TOC content, the active and centennially stable SOC proportions, hereafter referred to as "stable SOC", and their quantities can be estimated using PARTYsoc-model [48].

Soil aggregates were fractionated into four aggregate size fractions i) large macroaggregates (>2 mm), ii) small macroaggregates (250–2000 μm), iii) microaggregates (53–250 μm) and iv) silt + clay fraction (<53 μm). The fractionation was achieved using manual wet-sieving method adopted from Six et al. [49] with minor modifications. In short, field-moist soil was gently pushed through a 20 mm sieve and then air-dried. Then 50 g (dw) of air-dry soil was placed evenly on top of a 2 mm sieve and slaked in deionized water for 2 h. After slaking, the sieve was moved up and down in water 50 times during a period of 2 min and the fraction that remained on top of the sieve was collected as large macroaggregates (>2 mm). Procedure was repeated by pouring the fraction that passed through the 2 mm sieve to a 250 μm sieve and subsequently to a 53 μm sieve. Each sieve was backwashed to transfer the aggregates to a vessel for

Table 2
Summary of conducted analyses and their timing.

Target properties	Methods	Monthly sampling (2018)	Yearly sampling (autumn)
Physico-chemical analysis	SOC stability (Rock Eval6 thermal analysis)	–	2016, 2018
	Soil aggregates (manual wet-sieving)	–	2018
	Total C and N (Variomax)	Yes	2016, 2018
	Bulk density (dried soil cores)	–	2018
	Soil pH (H_2O)	Yes	2016, 2018
	Soil moisture (oven drying 105 °C)	Yes	–
	Mineral N: NH_4^+-N, NO_3^--N (1 M KCl extraction)	Yes	–
Biological analysis	Microbial biomass (chloroform fumigation extraction)	Yes	–
	CO_2, N_2O, CH_4 fluxes (72 h incubation)	Yes	–
	Enzyme activities (fluorescent substrates)	Yes	–
	Community-Level Physiological Profiling (Biolog assay)	Yes	–
	Fungal/bacterial-ratio (qPCR)	–	2016, 2018
	Decomposition (Teabag index)	–	2018
	Soil microbial community analysis (MiSeq sequencing, ITS, 16S)		2016, 2018
	Plant analysis (biomass, yield, total protein)	Yes	2018

drying in 40 °C for one week before weighing.

In 2016 (before amendment application), TOC and N contents were analyzed using the standard SFS-EN ISO 16948. In 2018, TOC and N contents were determined from dried and ground soil samples by a Vario MAX CN elemental analyzer (ELEMENTAR Analysensysteme, Hanau, Germany).

In 2016 (before amendment application), soil pH was measured once from water-soil suspension using air-dried and ground soil. In 2018, soil pH was measured every month from mixed water-soil suspension (14 g fw soil in 35 mL distilled water) using a pH meter (Orion research SA720 pH/ISE, pH electrode Orion 8102BN). The same fresh soil was also used for enzyme and Biolog assays (see below).

Soil mineral N (NH_4^+-N and NO_3^-N) was measured monthly using 5 g of fresh sieved soil that was extracted with 25 mL 1 M KCl. The soil suspension was shaken for 30 min in an orbital shaker (200 rounds per minute) and filtered through SartoriusTM Grade 3-HW folded filters (Ø = 150 mm) and stored frozen (−20 °C) before being analyzed with an automated flow analyzer Lachat QuikChem 8000 (Zellweger Analytics, Milwaukee, Wisconsin, United States).

2.2.4. Biological analyses

Microbial biomass C (MBC) and N (MBN) were determined from one pooled soil sample per plot each month in 2018 using the chloroform fumigation extraction (CFE) method by Vance et al. [50] with the following modifications. About 8 g of fresh sieved soil was fumigated with chloroform inside a desiccator for 24 h in the dark, and then extracted with 40 mL of 0.05 M K_2SO_4. A control for the same sample without fumigation was extracted in the same way. The extracts were filtered through Whatman No. 42 filter paper and frozen (−20 °C). Thawed extracts were then filtered with 0.45 μm syringe filters (Minisart highflow (PES)) before being analyzed for dissolved organic carbon (DOC) and total dissolved nitrogen (TN) using a Schimadzu TOC-V cph/cpn analyzer (Kyoto, Japan). The MBC and MBN were calculated as the difference in DOC and TN contents in chloroform fumigated and control samples, respectively. No correction factor was used [51,52].

Soil CO_2, N_2O and CH_4 production potential was estimated from 30 g (fw) soil (10 g from each of the three locations within a plot), which was inserted into a 500 mL air-tight incubation bottle with 1 mL of sterile distilled water. The bottles were immediately closed with rubber caps and the first 15 mL gas sample was drawn from the headspace using a syringe and a needle. The gas sample was then injected into a pre-evacuated 12 mL vial and the negative pressure released from the bottle before re-closing it. The samples were then incubated in a dark chamber at +20 °C and re-sampled after 24 and 72 h. The gas samples were analyzed for their CO_2, CH_4 and N_2O content with a gas chromatograph (7890A, Agilent Technologies, California, USA) equipped with a flame ionization detector (FID) and a methanizer for CO_2 and CH_4, and an electron capture detector (ECD) for N_2O [53]. Analyses were carried out in accordance with the quality requirements of Integrated Carbon Observation System ICOS (www.icos-cp.eu). Finally, the fluxes were calculated according to Peltokangas et al. [37] and given as μg CO_2 g^{-1} soil h^{-1}.

DNA was extracted from 0.5 g (dw) of freshly frozen soil samples using NucleoSpin Soil kit (Macherey-Nagel). The chemical lysis was carried out using SL1 lysis buffer and 100 μL of enhancer, and the mechanical lysis was performed with FastPrep 5 m/s for 30 s. Whenever the soils were dry and absorbed the lysis buffer, an additional amount of SL1 buffer was added up to the 1.5 mL mark according to the manufacturer's instructions. Rest of the steps followed the protocol provided by the manufacturer. Each extraction was performed using two technical replicates and after the DNA elution step, the elutes from these two replicate extractions were combined. The purity and concentration of extracted DNA was measured with NanoDrop One (Thermo Scientific). DNA-based estimate of fungal/bacteria (F/B)-ratio was determined from each sample with quantitative PCR (qPCR) using FF390 and FR1 primers [54] for fungi while Eub338F and Eub518R primers [55] were used for bacteria. The qPCR reactions were carried out following the protocol described in Helin et al. [56] with SsoAdvanced SYBR Green supermix (Bio-Rad) and using 4–8 ng of DNA as template. The fungal Internal Transcribed Spacer (ITS2) and bacterial V4 region of 16S rDNA from the extracted DNAs were sequenced using Illumina® MiSeq v3 2 × 300 bp flowcell at the Institute of Genomics, University of Tartu. Prior to sequencing a two-step PCR was conducted at Institute of Genomics using the fITS7 and ITS4 primers for fungi [57] while 515F and 806R primers were used for bacteria [58] during the first PCR. During the second PCR indexes and sequencing adapters were attached to the PCR products in the indexing PCR with 7 cycles, using Illumina Nextera XT dual index primers (Illumina Inc., San Diego, CA).

The adapter sequences were removed from the raw ITS and 16S rDNA reads at the Institute of Genomics, and the general read quality was checked with FastQC software (http://www.bioinformatics.babraham.ac.uk/projects/fastqc/). Adapter and barcode sequences were cut away using Cutadapt software [59]. The nucleotide sequence data are available in the NCBI database under Bioproject number PRJNA1099140 (SUB14374545 for bacteria and SUB14379306 for fungi). Fungal sequences were further filtered, de-noised and clustered to operational taxonomic units (OTUs) using mothur (version 1.42.0) [60]. For filtering, de-noising and clustering fungal sequences to OTUs, the pipeline by Sietiö et al. [61] was followed. For identification, the representative fungal sequences were aligned against the UNITE-database (UNITE + INSD version 8 [62], in mothur with classify.seqs. Bacterial sequences were filtered, de-noised and clustered to OTUs using QIIME2 and the workflow recommend to it (https://docs.qiime2.org/2019.4/tutorials/moving-pictures/, accessed 19.6.2019). For identification, the representative bacterial sequences were aligned against SILVA database (version 132, [63,64]). The phylum names were updated according to Oren et al. [65]. In case of fungi, all global doubletons were removed from the downstream analyses after calculation of alpha diversity indices with estimate richness-function from vegan package [66] using R version 4.2.1 [67]. In case of bacteria, the data was collapsed at genus-level for further downstream analyses, except the alpha diversity indices were calculated from the non-collapsed data in QIIME2.

Enzyme activities were measured from mixed soil-water suspension (14 g fw soil in 35 mL distilled water) that was made in 50 mL Falcon tube, vortexed for 15 s and left to settle at +4 °C for 36–40 h. After new vortexing, the slurry was centrifuged (5 min 3000 rcf) and 4 mL of liquid was collected from the upper part of the tube. A 1 mL portion of the solution was filtered using Costar® Spin-X®

CLS8162 (Corning Inc., NY, USA) containing a cellulose acetate membrane (pore size 0.45 mm) by centrifuging at +4 °C and 15 700 g for 30 min and the filtrate was used for the enzyme assays. The measurement time was 72 h after sampling. In 96-well plates, activities of acid phosphatase (EC 3.1.3.2, Pho), chitinase (EC 3.2.1.14, Nag), b-glucosidase (EC 3.2.1.21, Gls), b-glucuronidase (EC 3.2.1.31, Glr), b-xylosidase (EC 3.2.1.37, Xyl), cellobiohydrolase (EC 3.2.1.91, Cel), and leucine aminopeptidase (EC 3.4.11.1, Leu) were measured using a fluorometric assay based on Pritsch et al. [68]. The reactions were carried out at room temperature (close to +22 °C) in buffered pH 4.5, except for leucine aminopeptidase where pH 6.5 buffer was used [69]. After stopping the reaction, the fluorescence was measured with a Victor3 plate reader (PerkinElmer, Inc., USA) using excitation at 355 nm and emission at 460 nm. The obtained fluorescence counts were compared to standard curves prepared from 4-methylumbelliferone, or aminomethylcoumarin in case of leucine aminopeptidase. For determining the background-fluorescence of the samples, 1 mL of each sample was heat inactivated (+85 °C, 2 h) to eliminate enzyme activity, cooled to +22 °C and analyzed as the actual samples [70]. The enzyme activities were given as pmol/min/g.

The analysis of community-level physiological profiling using Biolog EcoPlates was performed using the same soil slurry (14 g (fw) soil and 35 mL sterile-MilliQ water) as for enzyme assays, and it was let stand for 12 h in +4 °C before diluting it with Ringer solution and pipetting onto BioLog EcoPlates resulting in 1:100 end dilution on the plate. The color reaction was read every 24 h until 96 h/144 h with Infinite plate reader (Tecan) or SparkControl plate reader (Tecan) using wavelength of 590 nm. The data was given as (OD590nm*h)/g (dw).

Organic matter decomposition was studied using standardized TeaBag Index method ([71], http://www.teatime4science.org/). In short, three green tea and three rooibos tea bags were buried in 8 cm depth into each plot in June 2018 and harvested exactly after three months in September. Based on the mass loss of green tea (labile organic matter) and rooibos tea (recalcitrant organic matter), decomposition rate (k) and litter stabilization factor (S) were calculated as described in Keuskamp et al. [71].

Root and shoot biomass were determined during monthly samplings in June, July and August after the shoot biomass was dried for 48 h at 60 °C. Roots were separated from a volume 15 × 15 × 10 cm^3 (15 cm width, 10 cm depth) under each sampled plant, and weighed for fresh weight (fw). Yield estimate was obtained when a 1.5 m × 13 m wide sector from each plot was harvested with field plot combine harvester (Wintersteiger, Ried, Austria) at the end of August. The grain samples were dried, cleaned and weighed. Crude protein and starch as well as hectolitre weight were analyzed with NIR grain analyzer at Natural Resources Institute Finland.

2.2.5. Statistical analyses

Before other analyses the normality of the data was confirmed using Shapiro-Wilk's test and visual evaluation of Q-Q plots. If the test value was below $p < 0.05$ and Q-Q plot indicated skewed distribution, Log_{10} and square-root transformations were performed for scale data, arcsine square root transformation for percentage data. If the data was not normally distributed, non-parametric Kruskal-Wallis with pairwise comparisons was used, and organic amendment treatments were compared to fertilized control C80N.

In all analyses, the focus was to compare the amended treatments to the fertilized unamended control (C80N). However, whenever appropriate, the amendment treatments were compared against each other and the non-fertilized control (C0N). Treatment-related differences were then analyzed using general linear model with investigated parameter as Dependent, Treatment as Fixed, and Month (if the parameter was measured monthly) as Random factor. The model included treatment, month, and their interaction terms. In case the interaction was non-significant, it was removed from the final model. If the interaction between treatment and month was significant, the treatment-related differences within each month was also analyzed as described but using split file function (grouped based on month). Tukey's HSD post hoc test with p-value <0.05 was regarded statistically significant different unless otherwise stated.

Pearson or Spearman's correlation coefficient for parametric and non-parametric data, respectively, was used to describe general patterns in the whole data within each month. TOC, active and stable C were analyzed for correlations with yield, grain protein content and plant biomass compartments and greenhouse gas production potential. Additionally, the correlations between soil C and N content (%) and all biological parameters were studied.

For the chemistry data, partitioned canonical correspondence analysis (pCCA) was performed to observe general trends of the treatment effects on soil chemical and biological properties using data normalized to 0 … 1 with decostand-function′s range-method [66]. Prior to pCCA, data was checked for collinearity with corvif package [72] using threshold value 3. The pCCA was performed with cca-function from the vegan package [66] using R version 4.2.1 [67], data from June–August 2018 was used, treatments were introduced to explanatory variables as dummy variables (0 or 1) and the effect of sampling month was partitioned out. Statistical significance of the model, terms, and axes were analyzed with anova.cca. The ordination was visualized by using scaling "symmetric" and the species-scores of individual variables were plotted to the ordination.

The statistical significances of fungal and bacterial alpha diversities in 2016 and 2018 were tested with non-parametric Kruskal-Wallis test with kruskal.test-function from stats package [67] by comparing the values of each treatment to those of C80N control treatment. The effects of soil amendment treatments on change observed in fungal and bacterial communities were assessed with CCA, where treatments were used explanatory variables as dummy variables. To minimize the within field variation in the original soil microbial community, the sequences were calculated to percentage data to normalize the variation on library sizes, and these values were further calculated to fold change values by comparing the original microbial community distribution in 2016 in each plot to situation in 2018 prior to CCA. Statistical significances of the models, terms and axes were analyzed with anova.cca.

All other statistical tests were performed with IBM SPSS Statistics version 28.0.0.0 but for multivariate analysis, R version 4.2.1 [67] was used.

3. Results

3.1. Soil properties

Soil organic C content was measured in 2016, before the organic soil amendments were applied, and again two years later in September 2018. As we assumed, no significant differences were observed in 2016, confirming that the field was homogenous to start with (Fig. 1). However, in 2018 the soil TOC content was significantly higher in SprB than in WilB and both were higher compared to all other amendment treatments and to C80N control ($p < 0.001$), although WilB did not differ significantly from LimeS (Fig. 1). In 2018, we also measured SOC stability and found that the stable SOC content was significantly higher in SprB and WilB compared to all other treatments and to C80N control ($p < 0.001$), but the active SOC was not affected by the treatments (Fig. 1).

The soil bulk density was not affected by the soil amendment treatments compared to the controls. The average (±SD) bulk density (g cm^{-3}) was 1.11 (±0.11) in C80N and 1.09 (±0.12) in FibreS, 1.09 (±0.07) in LimeS, 1.15 (±0.02) in WilB, 1.08 (±0.07) in SprB and 1.16 (±0.09) in HW, respectively (Supplementary Table 2). Overall, the bulk densities were significantly lower than on average with well-structured clay soils (1.3 g/cm^3).

The proportions of mass-based size fractions of soil aggregates in 2018 (>2 mm, 2 mm–250 μm, 250–53 μm, and <53 μm) were not affected by the soil amendment treatments (Supplementary Table 3**). However, the C content in 2 mm–250 μm fractions was significantly higher in SprB treatment than in controls C0N and C80N ($p = 0.031$ and $p < 0.001$, respectively) (**Supplementary Table 3**). No treatment-related significant differences were found in the N content or C/N-ratio of different size aggregates (**Supplementary Table 3).

No differences were observed in soil moisture between any of the treatments compared to C80N control during the monthly sampling campaign (data not shown). In contrast, soil pH was observed to be significantly higher in FibreS and WilB treatments compared to both controls, and there was a tendency of higher pH also in LimeS (Fig. 2) even after two years had passed since the application of the organic soil amendments (in 2018).

Soil mineral N (NH_4^+-N and NO_3^-N) contents did not show any systematic treatment-related differences between C80N control and soil amendment treatments when the whole growing season is considered (Supplementary Table 4**). Overall, the non-fertilized control (C0N) had the lowest mineral N content compared to other treatments, and all the fertilized treatments exhibited higher NH_4^+-N contents after fertilization in May and in June. However, the soil NH_4^+-N -contents decreased rapidly after fertilization while the soil NO_3^-N -contents initially increased in June, but then began to rapidly decrease in July. In monthly comparisons, HW showed significantly higher soil NH_4^+-N -contents in July, while SprB showed a significantly higher NO_3^-N -contents in September (**Supplementary Table 4).

The greenhouse gas (GHG) flux data was analyzed using a general linear univariate model with month as a fixed factor. Based on the analysis, only FibreS ($p = 0.041$, Supplementary Table 5**) had a significant impact on soil CO_2 production potential by increasing it 1.19 to 1.52 -fold compared to the fertilized control C80N. The other greenhouse gases, N_2O and CH_4 fluxes, did not show any treatment-related significant differences compared to the control ($p = 0.208$ and $p = 0.189$, respectively;** Supplementary Table 5).

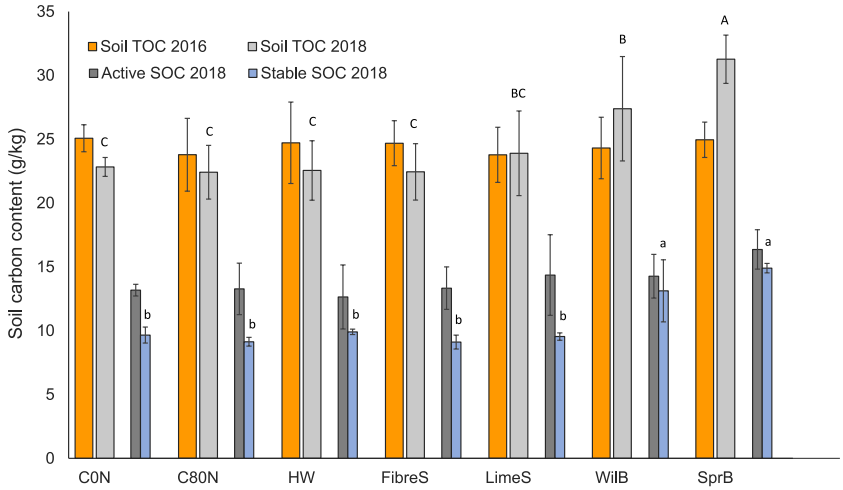

Fig. 1. The content of total organic C (TOC) in soil samples in 2016 and 2018 as well as the active and stable SOC fractions in 2018. Error bars indicate standard deviation (N = 3), and different letters indicate statistical differences between treatments (Tukey's HSD $p < 0.05$). No letters indicate a lack of statistical differences in that parameter. 'C0N' indicates non-fertilized control, 'C80N' fertilized control, 'HW' humic water pre-treatment, 'FibreS' fibre sludge, 'LimeS' lime-stabilized pulp mill sludge, 'SprB' Picea and 'WilB' Salix biochar.

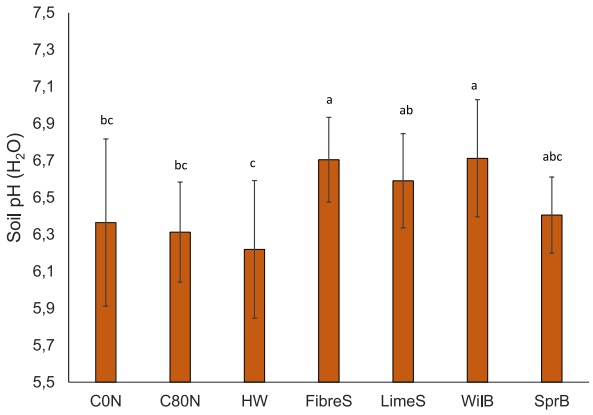

Fig. 2. Average soil pH of the soil samples collected in 2018 (from May to September). The overall difference (visualized as average) was analyzed using the general linear univariate model with the month as a random variable. Both treatment and month were significant ($p < 0.001$) and their interaction non-significant ($p = 0.992$). Error bars indicate standard deviation ($N = 3$) and different letters statistically significant difference between the treatments (Tukey's HSD $p < 0.05$). The abbreviations for the treatments are presented in Fig. 1.

3.2. Biological parameters

Neither the organic soil amendments or the humic water treatment had any significant treatment effects on the following measured parameters compared to C80N: microbial biomass (C, N and C/N-ratio) (Supplementary Table 6), fungal/bacteria-ratio (Supplementary Table 2**), enzyme activities (**Supplementary Table 7**), community-level physiological profiling using Biolog EcoPlates (**Supplementary Table 8**), or decomposition rate and stability index (TeaBag k and S index,** Supplementary Table 2**). Also, root or shoot biomass or grain yield (g fw) and its protein content (%) were not significantly different between C80N, C0N, or any of the amendment treatments (**Supplementary Table 2).

Regarding the microbial communities, we obtained 6046 good quality fungal OTUs without singletons and doubletons, and 1512 good quality bacterial OTUs which were collapsed at genus-level. In FibreS treatments, both fungal and bacterial Shannon diversities differed in 2018 from those in C80N control treatments ($p \leq 0.05$, with non-parametric Kruskal-Wallis test, Supplementary Table 9**). The number of observed OTUs were similar across all treatments (**Supplementary Table 9). The soil fungal community was dominated by fungi from phylum Ascomycota, followed with Basidiomycota and Mortierellomycota, while other phyla were in minority (Supplementary Fig. 1A and B). The willow biochar (WilB) treatments differed in soil fungal community structures in 2018 when compared to the soil original fungal community structure in each experimental plot before starting the experiment in 2016 (Fig. 3).

In case of bacterial community, the most abundant phyla were Pseudomonadota, Acidobacteriota and Actinomycetota

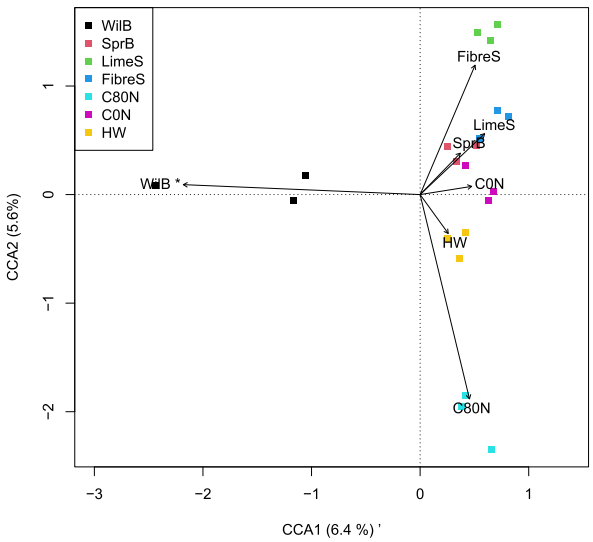

Fig. 3. Canonical correspondence analysis of the change in soil fungal community structures between 2016 and 2018 in the different soil amendment treatments. The abbreviations for the treatments are presented in Fig. 1. The statistical significances of the explanatory variables and axes are presented; * $p \leq 0.05$. The CCA model was statistically significant $p \leq 0.01$.

(Supplementary Fig. 2A and B). The FibreS treatment caused significant change in soil bacterial community structures in 2018 when compared to the soil original soil bacterial community structures in 2016 ($p \leq 0.05$, Fig. 4). The bacterial community structures in LimeS treatment changed as a trend when compared to the soil original bacterial community structures ($p \leq 0.1$). Similarly, C80N control caused a significant change and C0N tended to change the bacterial community structures from 2016 to 2018 ($p \leq 0.1$).

3.3. Comparisons between soil properties and biological parameters

The overall impact of the treatments on all soil chemical and biological parameters was studied using multivariate partitioned canonical correspondence analysis. *Picea* biochar (SprB) treatment separated significantly from other treatments in CCA ($p \leq 0.001$, Fig. 5). This separation was mostly due to higher soil SOC content and C/N-ratios in plots treated with SprB compared to other plots. Similarly, FibreS treatments separated significantly from other treatments ($p \leq 0.01$) mainly due to higher soil CO_2 and CH_4 respiration potential as well as lower bacterial polymer utilization capability compared to other treatments. Separation of LimeS treatment from others ($p \leq 0.05$) was likely driven by the combination of higher-than-average soil MBC and N and P acquisition related enzyme activities detected from these soils.

The whole data, irrespective of the treatments, was investigated for overall correlations. TOC, active and stable SOC, N%, moisture, and pH were correlated to yield, grain protein content as well as to shoot and root biomass. Yield correlated positively with TOC, active and stable SOC, and moisture in the September sampling (at harvest), grain protein content only to moisture whereas shoot biomass had in general positive correlations with TOC, N%, and moisture towards the end of the growing season. Root biomass correlated positively with soil N% over the whole summer except in May, soon after sowing (Supplementary Table 10).

Other parameters related to soil biology (greenhouse gas fluxes, microbial biomass C, enzyme activities and community-level physiological profiling using Biolog as well as decomposition rates) were analyzed for correlations against TOC, N%, moisture and pH. In most cases, there were no significant correlation or strong patterns, but CO_2 flux correlated significantly with N% and moisture over summer (Supplementary Table 10).

The summary of all treatment-related differences to C80N control are presented in Table 3.

4. Discussion

Soil C sequestration requires a positive balance in C inputs compared to outputs, otherwise soil loses C [7]. Regarding the inputs, organic C can enter the soil in three ways: as root exudates, litter (above- and belowground) or organic amendments [24]. In this study, our aim was to examine the impact of various organic soil amendments and one microbial stimulant on SOC contents, SOC stability, and soil biological activities.

Our hypothesis (Hypothesis 1) was that biochar remains in soil as stable SOC and may provide means for soil to retain mineral N and therefore influence its availability. Due to the stability of aromatic C, we presumed that biochar would have little impact on the measured biological parameters. Our results confirmed partly our first hypothesis. Biochar (SprB and WilB) was shown to increase both TOC and stable SOC content compared to control, even after two years (Fig. 1). However, the increase in stable SOC did not have a significant impact on the mass-based proportions of different soil aggregates even though SprB did exhibit higher C content in the 2 mm–250 μm size fraction compared to control. The second part of our first hypothesis proposed that biochar would retain mineral N by

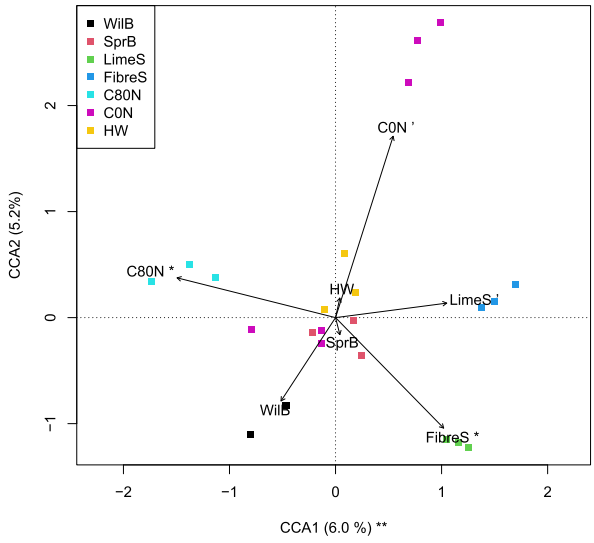

Fig. 4. Canonical correspondence analysis of the change in soil bacterial community structures between 2016 and 2018 in the different soil amendment treatments. The abbreviations for the treatments are presented in Fig. 1. The statistical significances of the explanatory variables and axes are presented; ‘p < 0.1, * p ≤ 0.05. The CCA model was statistically significant p ≤ 0.001.

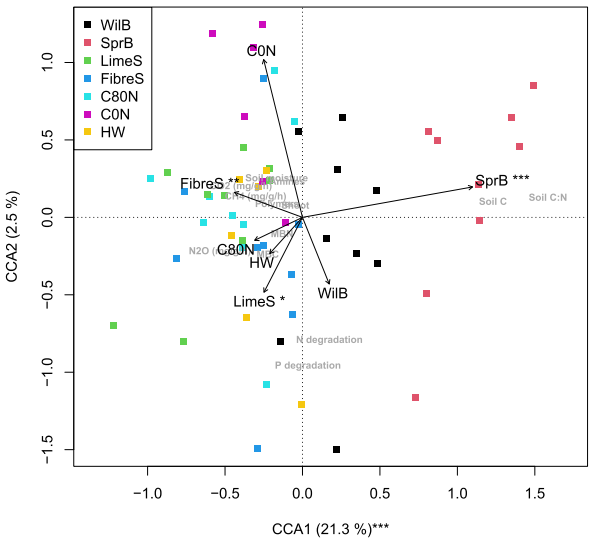

Fig. 5. Partitioned canonical correspondence analysis, where the sampling month has been partitioned out, representing the treatment-related differences caused by the overall soil physico-chemical and biological variables. The abbreviations for the treatments are presented in Fig. 1. The species scores of measured variables are visualized in grey. The statistical significances of the explanatory variables and axes are presented; * $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$. The CCA model was statistically significant $p \leq 0.001$.

absorbing dissolved N into its internal pores due to capillary action and hydrophobic interactions [73,74]. Contrary to our hypothesis, we found no significant biochar-induced differences in soil NH_4^+-N contents. Also, no systematic treatment effects on NO_3^-N contents were found during the growing season. Interestingly, SprB did exhibit significantly higher NO_3^-N content after harvest, which would indicate that SprB can retain NO_3^-N in some circumstances. As NH_4^+ adsorption was not higher, it is improbable that NH_4^+ transformations were causing the observations of higher NO_3^- contents. Keeping this in mind, it is relevant to point out that the plant root, shoot, and yield parameters did not show any significant differences between fertilized and non-fertilized controls. This indicates that N was not the growth limiting factor during the studied year. It is therefore likely that the dry soil conditions in the early growing season limited N availability as well as its mobility, which may have masked any treatment related effects. Furthermore, it is possible that multiple consecutive extractions would have been needed to extract all mineral N retained by biochar during the prolonged dry period. Therefore, it is possible that our results underestimate the retention potential of SprB and similar biochar materials, which have been shown to retain NO_3^-N and prevent its leaching in earlier studies also from the Qvidja site. Karhu et al. [75] observed that during growing season 2017 in Qvidja, the cumulative NO_3^-N leaching in the spruce biochar treatment was reduced by 68 % compared to the fertilized control treatment. Also, in the middle of the 2018 growing season (from 7 June to 5 July), both biochars significantly reduced NO_3^-N leaching [35]. The capacity of biochars to capture NO_3^-N have been observed both during co-composting [76], and field-aging in soil [77], or from surface runoff [78]. The relative importance of the suggested mechanisms for NO_3^- capture, such as physical entrapment inside the biochar pores, electrostatic bonding between NO_3^- and some functional groups on biochar surfaces, or nonconventional H bonding [76,77] remains to be investigated.

The last part of our hypothesis was that biochars would have little impact on biological parameters, and this was partially confirmed: we found no significant differences between biochars and C80N control for microbial biomass I, fungal/bacteria-ratio, enzyme activities, community-level physiological profiling using Biolog EcoPlates, decomposition rate and stability index (TeaBag index), root or shoot biomass, grain yield and its protein content or the number of estimated and observed fungal and bacterial OTUs. Soinne et al. [79] have previously observed that the addition of biochar does not affect soil microbial community structures. In our study, neither of the studied biochars influenced soil bacterial community, but WilB had an impact on soil fungal community, even though we could not see any other indications of altered microbial activities. The difference in the response of willow (WilB) and spruce biochars (SprB) indicate that the origin of biochar has significant role and should be acknowledged more when studying their effect on soil microbial communities. To summarize, biochar had no measurable impact on the more robust microbial parameters but the sensitive DNA-based methods indicated weak changes in the soil fungal community structures.

When all physical, chemical, and biological parameters (except fungal and bacterial community data) were analyzed together in canonical correspondence analysis, SprB separated significantly from the other treatments and control, SOC and C/N-ratio having the largest impact on the separation. This may indicate that a portion of the applied biochar persisted in soil and that the observed effects (Table 3) were related to stable C, rather than active C or other substrate-induced effects. These observations are supported by previous studies [79–81]. Furthermore, it has been shown that a large part of the added biochar C remains in soil for years and even decades [82,83], regardless of the feedstock or production conditions [84] and that biochar itself contains little or no easily available nutrients for microbes, soil fauna, or plants [85]. Therefore, many of the effects of biochars on soil microbiology have been shown to be temporary [86,87]. It is logical that the application of stable C materials does not strongly affect soil microbial activities [86,88], which are primarily affected by relatively active C fractions and other substrates [89]. Nevertheless, it is generally accepted that as highly porous

Table 3
Summary of the main results.

	Analysis	Main treatment-related differences	Differences to C80N	Figure/ Table
Physico-chemical analysis	SOC stability	More stable SOC in SprB and WilB	Yes	Fig. 1
	Soil aggregates	No difference in aggregates. In SprB, more organic C in 2 mm–250 μm class compared to controls and FibreS	Yes	Suppl. Table 3
	Total C and N	More SOC in 2018 in SprB, trend in WilB. No change in N	Yes	Fig. 1
	Bulk density	No differences	No	Suppl. Table 2
	Soil pH	FibreS and WilB differ from C80N	Yes	Fig. 2
	Soil moisture	C0N and FibreS differ from HW	No	Data not shown
	Mineral N	NH_4: only in July, HW significantly higher than others NO_3: in May and August no differences, in other months significant differences, most often C0N differed from others	Yes	Suppl. Table 4
	Multivariate analysis: soil chemistry	SprB, LimeS and FibreS differ from other treatments	Yes	Fig. 5
Biological analysis	Microbial biomass C	LimeS differs from C0N	No	Suppl. Table 6
	CO_2, N_2O, CH_4 fluxes	CO_2: FibreS was significantly higher than WilB and C80N N_2O, CH_4: no differences	Yes	Suppl. Table 5
	Enzyme activities	No differences	No	Suppl. Table 7
	Biolog Community-level physiological profiling	LimeS differs from C0N in total, carbohydrate and amino acid activities. HW and FibreS differ from each other in amino acids and amines.	No	Suppl. Table 8
	Fungal/bacterial-ratio	No differences	No	Suppl. Table 2
	Decomposition (Teabag k and S index)	No differences	No	Suppl. Table 2
	Alpha-diversity: Fungal community	Shannon-index differs between FibreS and C80N treatments	Yes	Suppl. Table 9
	Alpha-diversity: Bacterial community	Shannon-index differs between FibreS and C80N treatments	Yes	Suppl. Table 9
	Beta-diversity: Fungal community	WilB differ between 2016 and 2018	Yes	Fig. 3
	Beta-diversity: Bacterial community	C80N and FibreS differ significantly between 2016 and 2018	Yes	Fig. 4
	Plant biomass	No differences	No	Suppl. Table 2
	Yield and total protein	No differences	No	Suppl. Table 2

material, biochars may retain nutrients and water [35,75,76,90,91], as well as providing niches for soil microbes [92]. Therefore, it is possible for biochar to influence soil microbial communities even when the applied C materials are relatively inert. In our study there was no evidence that the biochar treatments affected the water retention or soil structure of the studied boreal clay soil. On the other hand, many of the soil amendments were demonstrated to have significant liming effect (Fig. 2), which likely affected soil microbial activities through soil pH [93,94]. Despite, we found little evidence that the liming effect had an influence on soil biological parameters or potential GHG emissions in field conditions (Table 3), especially during this exceptionally dry summer. It is relevant to note that in a parallel laboratory experiment, the liming effect of the studied soil amendments was shown to correlate with the number of base cations added to soil as well as soil GHG emissions, especially at higher moisture conditions [37]. Therefore, using biochar or other similarly alkaline materials to increase soil pH of otherwise acidic boreal soils may be a significant part of the positive outcomes of using organic soil amendments in the global north. To summarize, as biochar is rather inert material and may affect soil biology through pH effects, increased nutrient and moisture retention as well as through improved soil structure, more studies are needed to better understand the multifactorial impacts of biochar in boreal soils.

In our hypothesis 2, we hypothesized that the pulp mill sludges (LimeS and FibreS), would not have a clear impact on TOC or stable SOC. Our hypothesis was confirmed as TOC and stable SOC were not increased by the pulp mill sludge treatments compared to control (Fig. 1). Our finding means that more than half of the approximately 3000–5000 kg/ha of C added to soil in LimeS and FibreS treatments had been decomposed within the first two years after their application [37], or their quantities were reduced by other means, such as translocation of DOC [41]. As the second part of our hypothesis, we assumed that the decomposition of added C must have accelerated microbial activities soon after application. Indeed, it seemed that the effect persisted even after two years since FibreS exhibited significantly higher CO_2 production potential compared to the other treatments or the control. Beyond these, we found only small microbial community level differences in FibreS, but no changes in fungal abundance over bacteria. Interestingly, we observed that the FibreS treatments shape soil bacterial and fungal Shannon diversity indices when compared to C80N treatment although they did not change the microbial richness estimate, i.e., the number of observed OTUs remained similar in all the treatments. This suggests that the FibreS was preferred as a substrate by certain microbes over others, but it did not induce major differences in the microbial

community composition by introducing new microbial species or excluding old existing species. Rasa et al. [41] discovered that inclusion of pulp mill-based soil amendments affected significantly soil fungal and bacterial communities. Similarly in our study, the soil bacterial community structure changed under the FibreS and LimeS (as trend) treatments. Rasa et al. [41] speculated that the soil amendments affected soil microbial communities through increase in soil pH. Accordingly, in our study LimeS, FibreS and WildB treatments all increased the soil pH, which may have changed the soil microbial community structures. However, there may be other factors than pH affecting the microbial communities thus more research is needed to investigate the effects of organic soil amendments on soil microbiology.

Furthermore, we found no differences in mass-based proportions of different aggregate size classes indicating that potential changes in soil biological activities during the two-year period did not lead to altered or persisting changes in soil aggregation. However, even though we could see only few significant differences in the individual biological parameters, in canonical correspondence analysis with all variables except fungal and bacterial OTUs, FibreS and LimeS separated significantly from the other treatments and control, FibreS mainly due to soil moisture, CO_2 and CH_4 production potential and Biolog assay (polymers), while the separation of LimeS was more affected by N_2O, MBC and N and P degradation enzymes. Therefore, it seems that there are still faint patterns to be seen after two years indicating that LimeS and FibreS had stimulated soil microbial communities and activity.

Although ligneous, non-biochar soil amendments have been rarely studied, our results are supported by the earlier pioneering studies. For example, Calbrix et al. [86] observed that ligneous waste and other organic materials had only transient effects on microbial activity, genetic structure, and quantities of soil microorganisms. Organic materials with higher proportions of active C compared to lignin increased the number of heterotrophic bacteria and the mean metabolic activity of microorganisms. Shortly after, however, no treatment effects were observed indicating that organic amendments have less of an effect on soil microbiology than seasonal variations and other anthropic factors such as soil management [86,95,96]. These results are in line with ours: after longer periods of time, no major changes in soil microbiology were seen. Similarly, Kok et al. [97] demonstrated that the proportion of hot water extractable C functions as a good predictor for priming effect and other microbial responses because of preferential substrate utilization and greater metabolic efficiency. Based on their observations, organic amendments with more easily available C and N induced larger priming effect. However, no differences in SOC content were observed between different soil amendments after 150 days [97]. While we did not determine immediate effects after soil application, the conclusions that can be drawn from our observations after two years are in line with other parallel studies conducted at the experimental field: treatment effects other than on soil pH [37] were shown to be relatively small compared to other factors affecting soil properties like weather and soil management [40]. Therefore, as many observed biological soil impacts seem to be transient or weak, it is recommended that agricultural soils should receive regular inputs of active C if the interest is to maintain soil activity and related soil functions, like aggregation [17].

Our third hypothesis proposed that the microbial stimulant (HW, humic water treatment on seeds) leads to increased fungal/bacteria-ratio and soil aggregation but has no significant impacts on SOC stability. Regarding TOC and stable SOC content, our hypothesis was supported as HW treatment did not show differences compared to control. Regarding fungal/bacterial-ratio and soil aggregation, our hypothesis was rejected as we did not find any significant differences in these parameters, or any other measured biological parameter, although earlier studies have found microbial stimulants to be effective in some cases [43,98,99]. It seems that in our case, the microbial stimulant was not effective, or its effects were not strong enough to be detected in analyses performed from bulk soil samples, two years after the use of the stimulant. Future studies should consider focusing on specific hotspots of soil microbiology, such as the rhizosphere.

In addition to the studied hypothesis, we assumed that the use of several tons of organic amendments per hectare would affect soil bulk density (BD) and soil moisture. However, we did not find any significant changes in the bulk density of the topsoil (5 cm) or soil moisture, likely because much of the applied organic C had been decomposed or translocated away from the measured soil layer [100,101]. In our study we could not determine the rate of potential transport of amendments in the soil profile. Furthermore, it now seems that it is unlikely that biochar will increase soil water retention in clay soils, which share similar pore size characteristics with biochar [79,102]. In addition, Finnish soils are naturally rich in organic matter [2], and thus, the high fertility of the studied soils can limit the potential gains from added organic C, especially from the perspective of crop production [79]. Therefore, it seems that even if soil amendments have a positive effect on soil health in boreal regions, their impact on soil properties is reduced by high clay and organic matter content and impacts on crop production is outweighed by soil management and local climate [37,40].

One of the motivations to increase TOC in soils is to increase soil health and consequently to increase plant growth and yields. However, in their meta-analysis, Vendig et al. [103] showed that significant positive SOC responses on yields are usually found with low SOC soils. Still, in our study, when data from all treatments was analyzed together for parameter correlations, we found positive correlations with the yield and TOC, active and stable fractions of SOC, and moisture but not with N% (at harvest date) or pH (Supplementary Table 10). Therefore, our data supports the notion that SOC and grain yields have a positive relationship, as shown previously. Nitrogen and moisture had also a positive correlation with shoot and root biomass during the study period. However, we found no or only occasional positive correlations with TOC, different SOC forms, and pH with grain protein or shoot and root biomass in our monthly measurements. For most soil biological parameters, no correlations, or systematic patterns with TOC, N%, moisture, or pH was seen, except for CO_2 production potential, which showed a positive correlation during several monthly measurements with soil moisture and N%.

The rather weak treatment-related responses in biological measures give reasons for self-criticism: were our analyses appropriate, did the dry weather supersede some of the treatment-effects, or did the experimental field exhibit unknown artefacts? Since our analysis methods are all widely used, state-of-the-art methods, there is no reason to believe that, in our case, we would have had a systematic failure in their use. Regarding the weather conditions, the early summer weather was drier than in average in Finland that could have affected our results. However, similar dry summers have been estimated to become more common in boreal region in the

future [104]. More specifically, in a recent study by Heimsch et al. [105], the weather data of the same location from 2018 (our sampling summer) to 2023 is presented. The average mean annual temperature in 2018–2019 was 7.6 °C, 2019–2020 it was 7.7 °C whereas between 2020 and 2023 the temperature was a bit lower, ranging from 6.6 to 6.8 °C. The precipitation was the lowest in 2018 (473 mm) compared to the other years that ranged from 631 to 855 mm. Even though CO_2 flux, leaf area index (LAI) and soil water content and air temperature of the adjacent grass field were affected by the dry early summer 2018 [105], the patterns were not drastically different, rather indicating normal yearly variation. It is noteworthy that in our experiment the field was irrigated once during the driest period that reduced significantly the impacts of dry weather conditions on plant growth and soil, unlike in Heimsch et al. [105]. Therefore, despite the summer drought, the climatic conditions fall into normal variation in field experiments. What comes to the studied field, our field experiment consisted of three replicated blocks and the treatment plots were randomly distributed in the blocks. Despite careful planning, we found later some uneven patterns in soil chemical parameters, e.g., in micronutrient concentrations (Supplementary Table 1). Also, some parts of the area remained longer water-logged in the spring after snow melt, which would indicate differences in soil compaction or water infiltration and could lead to changes in redox conditions and subsequent changes in soil microbiology. However, our bulk density data did not show any treatment-related differences in the topsoil, but the compaction may have been significant in the deeper subsoil increasing within-treatment variation and masking some potential treatment effects.

Considering the applications rates of the amendments, the native C content of the studied clay soil was approximately 2.5 %, meaning that there was about 27500 kg C per ha. The amount of C added between the ligneous amendments varied from 3300 to 17400 kg C per ha, ranging from approximately 12 %–63 % of added C vs. native C calculated to a depth of 10 cm. The biochars had the highest C addition relative to the native C, and still they did not affect microbial activities, whereas FibreS and LimeS amendments showed some changes in biology despite their originally much smaller relative addition rates and the fact that a larger part of the added biomass must have already been lost. In all our biological measures, we used bulk soil samples which means that there is the unaltered background microbial community affecting microbial analysis and any weak to moderate amendment-related changes in activities may have been masked by the background activities. From this perspective, even the small alterations that were found in the bulk soil microbiology indicates meaningful changes in specific niches in soil, like on the surfaces of added organic amendments or in the rhizosphere and mycorrhizosphere of the plant. The reason for the variable amount of organic amendment applied was agronomic: the volume, transportation and purchase costs have shaped the levels of current commercial application rates of these products, and these application rates were used in this study. However, as our sampling design did not consist of volumetric sampling except for bulk density, and the field application of the amendment was performed with robust agronomic methods regarding spreading and tilling, we were unable to analyze possible C stock changes in sufficient detail. To summarize, despite the unexpected variables every field experiment face, we feel confident that the above-mentioned challenges do not impair our main findings presented in the conclusions, the largest experimental artefact may be the lack of sensitivity and underestimation of the treatment effects.

5. Conclusions

Our study aimed to assess whether different biochars, ligneous soil amendments and a microbial stimulant increase total SOC and its stability, and whether adding C-rich materials would affect soil biological activities in the long-term. From the studied organic soil amendments, biochars significantly increased TOC and stable SOC content to levels still detectable two years after their application while other ligneous non-biochar amendments did not. Overall, TOC including both stable and active SOC fractions correlated positively with grain yield. We showed that SOC stocks can be increased using biochars with positive reflections to yield whereas the amount of C from pulp mill sludge-based amendments did not lead to detectable increases in SOC but did increase soil microbial activity. Both fibre sludge and willow biochar increased soil pH, while mixed pulp mill sludge had an increasing trend and the increase in pH may have contributed to increased soil biological activities. Furthermore, as the studied amendments did not cause any detectable negative effects on the measured parameters and had a low overall impact on soil biology while mostly impacting soil physical and chemical characteristics, they seem to be promising options for recycling ligneous side streams from the forest industry into primary production in arable fields.

CRediT authorship contribution statement

J. Heinonsalo: Writing – review & editing, Writing – original draft, Visualization, Supervision, Investigation, Funding acquisition, Formal analysis, Conceptualization. **K. Peltokangas:** Writing – review & editing, Writing – original draft, Investigation. **P. Barré:** Writing – review & editing, Investigation. **F. Baudin:** Writing – review & editing, Investigation. **L. Cécillon:** Writing – review & editing, Investigation. **S. Kalu:** Writing – review & editing, Investigation. **S. Kanerva:** Writing – review & editing. **K. Karhu:** Writing – review & editing, Supervision, Funding acquisition. **L. Kulmala:** Writing – review & editing. **J. Liski:** Funding acquisition. **A.-R. Salonen:** Writing – review & editing, Investigation. **R. Shrestha:** Writing – review & editing, Investigation. **H. Soinne:** Writing – review & editing. **E. Virtanen:** Writing – review & editing, Conceptualization. **K. Huusko:** Writing – review & editing, Investigation. **O.-M. Sietiö:** Writing – review & editing, Writing – original draft, Investigation, Formal analysis.

Data availability statement

The raw nucleotide sequence data is deposited to the NCBI database under Bioproject number PRJNA1099140 (SUB14374545 for bacteria and SUB14379306 for fungi). We have uploaded the Supplementary data and it is intended as Supporting Information for the

online publication.

Funding source

This work was supported by the Maj and Tor Nessling foundation [grant number 201800136], Centre for Economic Development, Transport and the Environment [project number 58755], the Strategic Research Council at the Research Council of Finland [grant number 327342, 352431] and Flagship funding at the Research Council of Finland [grant no. 337552].

Declaration of competing interest

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests: Karoliina Huusko reports financial support was provided by Maj and Tor Nessling foundation. Outi-Maaria Sietio reports financial support was provided by Centre for Economic Development, Transport and the Environment. Jussi Heinonsalo reports financial support was provided by the Strategic Research Council at the Research Council of Finland. Eetu Virtanen reports a relationship with Soilfood Oy that includes: employment. Eetu Virtanen used to work in a company that sells the product we studied. He was included as a co-author because of his expertise on the products but there are and were no commercial interests regarding this study and its results. Currently he does not work anymore in the abovementioned company. 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.

Acknowledgements

We thank the staff of Soilfood Oy and, as well as the manager and owners of Qvidja Farm for their contribution in establishing and maintaining the experimental site. We acknowledge CSC IT Center for Science for providing computational resources for processing the sequencing data. Special thanks to Tomi Mattila and Niina Ruoho for their commitment and hard work during the measuring campaign.

Appendix. ASupplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.heliyon.2025.e43536.

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