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Reduction of forest soil biota impacts tree performance but not greenhouse gas fluxes

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ABSTRACT

Soil communities are essential to ecosystem functioning, yet the impact of reducing soil biota on root-associated communities, tree performance, and greenhouse gas (GHG) fluxes remains unclear. This study examines how different size fractions of soil biota from young and mature forests influence *Alnus glutinosa* performance, root-associated community composition, and GHG fluxes. We conducted a mesocosm experiment using soil community fractions (wet sieving through 250, 20, 11, and 3 μm) from young and mature forest developmental stages as inocula. The results indicate that the root-associated community composition was shaped by forest developmental stage but not by the size of the community fractions. Inoculation with the largest size fraction from mature forests negatively affected tree growth, likely due to increased competition between the plants and soil biota. In addition, GHG fluxes were not significantly impacted by either size fraction or forest developmental stage despite the different community composition supplied. Overall, our research indicates that *A. glutinosa* strongly selects the composition of the root-associated community, despite differences in the initial inoculum, and this composition varies depending on the stage of ecosystem development, impacting the performance of the trees but not GHG fluxes.

1. Introduction

Soil biodiversity plays a crucial role in driving ecosystem functions, and especially affects plant performance (Tilman et al., 1996; Balvanera et al., 2006; Zavaleta et al., 2010). Soil serves as a hub for biological interactions and is regarded as one of the most biologically diverse environments on our planet, exhibiting much higher biodiversity per unit area than what is typically observed aboveground (Brussaard, 1997; Bardgett and Van der Putten, 2014). Consequently, the removal of groups of soil biota can hinder various ecosystem functions, such as plant growth and the exchange of greenhouse gases (GHG; such as Carbon dioxide (CO_2), methane (CH_4), and nitrous oxide (N_2O)) between the soil and the atmosphere (Helgason et al., 1998; Van der Heijden et al., 2008; Wall et al., 2010; De Graaff et al., 2015). Therefore, understanding the role of different compartments of the soil community is essential for predicting soil functions.

Due to their differences in size, soil communities can be successfully manipulated via means of wet sieving methods (e.g. Wagg et al., 2014; Wang et al., 2019; Li et al., 2020). Size fractions of the soil community can be utilized to assess the effects of reductions in soil biodiversity and to partly disentangle the effects of specific groups of organisms (e.g. soil fauna, bacteria and fungi) on the performance of trees and GHG fluxes. The effects of larger soil organisms such as meso- and microfauna can be excluded by wet-sieving at small mesh sizes (e.g. 20 μm), while sieving at even lower levels (e.g. 11 and 5 μm) filters out larger sized fungi, allowing to disentangle the effects of different sized fungi on plant performance (Wang et al., 2019). Wet sieving at mesh sizes such as 3 μm, where only bacteria are expected to pass through, allows for the comparison of bacterial effects versus those of bacteria and fungi from the larger filter sizes (20 and 11 μm). Manipulating the soil community

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composition and abundance via wet sieving and inoculating with different size fractions has been shown to affect plant-soil feedback (Van de Voorde et al., 2012), litter decomposition (Li et al., 2020), plant performance, and nutrient cycling (Wagg et al., 2014; Wang et al., 2019). The extent to which size-based removal of soil biota groups affects the root-associated communities of trees, and consequently their performance and GHG fluxes, remains largely unexplored.

Organisms in the soil can differentially affect plant growth either through beneficial mutualisms, pathogenic activity or competition for nutrients (Philippot et al., 2013). For instance, soil fauna can influence tree performance by decomposing organic matter, which makes soil nutrients more available, but also leads to the release of CH_4 and N_2O, showing they can also influence GHG fluxes (Petersen and Luxton, 1982; Bradford et al., 2002). Some bacteria can form beneficial symbiotic relationships with plants, allowing them to fix atmospheric N (Cocking, 2003; Menge et al., 2023), while fungi can form mycorrhizal associations with plants, which can extend their root systems and assist in nutrient acquisition (Brundrett and Tedersoo, 2018; Mortier et al., 2020). For example, *Alnus glutinosa*, a tree species that is the focus in the current study, can form symbiotic relationships with the N-fixing bacteria *Frankia alni* as well as with both ectomycorrhiza (EcM) and arbuscular mycorrhiza fungi (AMF) in order to meet its nutrient demands (Orfanoudakis et al., 2010). With regards to GHG fluxes, bacteria can mediate the processes of methane oxidation, nitrification and denitrification (Nielsen et al., 2015; Chen et al., 2018). Oxidation of atmospheric methane by methanotrophic bacteria in soils is a significant sink globally, and across various soil types is considered to be strongest in forest soils (Hiltbrunner et al., 2012; Rowlings et al., 2012; Bárcena et al., 2014). It has also been demonstrated for a wide range of soils that loss of N_2O-reducing bacteria can explain increased N_2O emissions (Domeignoz-Horta et al., 2018). Additionally, the loss of soil biodiversity can significantly reduce carbon sequestration (Wagg et al., 2014), highlighting the importance of biodiversity for regulating the magnitude of emissions. To date, research on ecosystem functions (including plant performance and GHG fluxes), has largely focused either on specific groups of organisms, such as soil fauna (e.g. Bradford et al., 2002) and mycorrhizal fungi, (e.g. Maherali and Klironomos, 2007) or on comparisons of these effects on entire soil communities and on ecosystem performance (De Vries et al., 2013). There is a significant research gap on how plant growth and GHG fluxes depend on the presence of different size fractions of the soil biotic community and how this differs between ecosystem developmental stages.

Different developmental stages of an ecosystem can exhibit entirely different soil communities (Mäder et al., 2002; Nielsen et al., 2015). This can influence the impact of community fractionation on tree performance and, in turn, alters the available communities that a tree can associate with (Wang et al., 2017). For instance, as tree plantations develop into mature forests, it is expected that soil communities will shift from a predominance of bacteria to fungi (Kang et al., 2018). This shift is partially explained by the tighter association of fungi with tree roots than bacteria whose composition is mediated partly by soil abiotic characteristics (Urbanová et al., 2015). It is therefore expected that fungal communities play a larger role in plant development in soils mature forest soils than in young forest soil. Consequently, the removal of fungi, such as EcM and AMFs, through community fractionation is likely to negatively impact plant performance most at the later stage of development. Although past studies have shown that a general reduction in soil biodiversity negatively impacts plant and ecosystem performance (Wagg et al., 2014), research on how this is further affected by the ecosystem developmental stage is lacking.

In this study we test the effects of decreasing community size fractions from two forest developmental stages on the root associated community composition of *A. glutinosa*. We also examine how size fraction reduction impacts tree performance and soil community GHG response expressed as net CO_2, CH_4 and N_2O fluxes (regarded as proxies for total heterotrophic respiration, bacterial CH_4 oxidation and total denitrification). A mesocosm experiment was established where trees were grown in soils that were inoculated with different size fractions of the soil community (250, 20, 11 and 3 μm). The soils originated from young (afforested ~10 years previously) and mature forests (>100 years). We hypothesize that inoculating trees with fractions extracted from soils from young and mature forest developmental stages will result in different root associated communities (Li et al., 2020), characteristic of the affiliated forest developmental stage (Ruiz Palomino et al., 2005). In particular, because we anticipate that young soils are dominated by bacteria while fungi are more adapted to soils of mature forests (Kang et al., 2018), we hypothesize that there will be higher variation in fungal communities among fractions in young forest treatments. For each of the two developmental stages, we further hypothesize that a decrease in community size fraction will result in a decrease in richness of root associated communities (Li et al., 2020) and that this, in turn, will negatively impact tree performance (Wagg et al., 2014). We also hypothesize that gas fluxes (particularly CO_2 and N_2O emissions) will decrease as size fractions decrease due to reduced community diversity and activity (Wagg et al., 2014).

2. Materials and methods

2.1. Forest soil sampling and tree species

In this study, the focus was on investigating the impact of community size fractions from two forest developmental stages on tree performance. To study the effect of a young and mature forest soil community, forests spanning two distinct developmental stages were selected. The forests were categorized based on their time of planting as either "young" (2010–2015) or "mature" (1880–1927). Within each developmental stage, six individual forests were sampled (6 young and 6 mature), each with their own soil community, characteristic of each developmental stage. Each individual forest acted as a replicate for its corresponding development stage. All sampled forests were located in the province of Drenthe in the Netherlands (SI, Table S1), had sandy soil and were initially planted with oak (*Quercus robur* and *Quercus petraea*). In each forest stand, soil was collected 15 cm deep from multiple points around the stand (February 2022). Each point was at least 10 m apart from the previous one and the soil from all points was placed in one bag to make one homogenate per forest stand. Additionally, three soil cores (150 cm^3, 12 cm deep) were collected from each stand to measure soil properties (SI, Table S2). In total, we collected 12 bags of soil (one from each forest) and 36 soil cores. The soil from each bag was initially sieved through a 2 cm mesh to further homogenize the soil and remove large debris and stones. After homogenizing the soil of each forest, a subsample was stored at −20 °C for DNA analysis. The bags were then stored at 4 °C for one day. The soil from the cores (150 cm^3, 129.5 ± 0.76 g) was oven dried at 40 °C for 96 h to measure soil moisture, pH (1:2 soil:water ratio) and the bulk density of the soil (Schofield and Taylor, 1955). The loss on ignition (LOI) method was used to measure the soil organic matter of the soil by first drying the soil at 105 °C for 48 h and then heating at 550 °C inside a muffle furnace for 4 h (Heiri et al., 2001). The soil heated at 550 °C was then used to determine the sand/silt fractions using a HAVER EML 200 Premium automated shaker (Oelde, Germany). Soil texture was classified into gravel (>2 mm), Sand (2–0.045 mm) and Silt and Clay (<0.045 mm). Black alder (*Alnus glutinosa*, (L.) Gaertnr) was selected as the species to grow in the inoculated sterile soils of our experiments. *A. glutinosa* trees were present in all of the sampled sites despite a clear dominance of oak trees (*Quercus robur* and *Quercus petraea*). Seeds of native alder were acquired from the Nature Agency Staatsbosbeheer located in the Netherlands. The seeds were germinated in a nursery using commercial potting soil where they were grown for 3 weeks and subjected to a watering regime of three times a week.

2.2. Community size fractions

In order to assess the impact of soil community size on *A. glutinosa* tree performance, we prepared four community size fractions (250, 20, 11 and 3 μm) from each of the forest soil samples, under sterile conditions, following methods from studies that have reliably proven that with reducing size fraction, the soil community simplifies (e.g. Wagg et al., 2014; Wang et al., 2019; Li et al., 2020). We prepared phosphate buffer (1 g KH_2PO_4 in 1 L DI H_2O, pH 6.5) and mixed it with soil (1:2, w/w) in four batches, each followed by sieving through 1 mm and 250 μm meshes. One-quarter of the filtrate was stored at 4 °C (the 250 μm fraction), and the rest was further processed. To prevent clogging of the filter papers, the filtrate was passed through 63 and 45 μm sieves to remove more soil particles, and then through 20, 11 and 3 μm Whatman filter papers to generate the remaining fractions. These steps were done using a Buchner funnel and a LABOPORT N816.3 KT.18 vacuum pump (KNF, Utrecht, the Netherlands). After each step, portions of the recovered filtrate (1/3rd at 20 μm, 1/2 at 11 μm and the rest at 3 μm) were stored at 4 °C, representing the respective size fractions. All of the equipment and the workstation were thoroughly cleaned with 70% ethanol prior to the filtering process and lab coats and gloves were worn at all times during the filtering process (see detailed description of the filtering process below). In between each forest soil sieving and the filtering of each size fraction, all materials were thoroughly rinsed with water and surface sterilized with ethanol to minimize the possibility of any contamination. We used this configuration under the assumption that solutions that were filtered through 3 μm sieves would primarily consist of small size microbes (e.g. bacteria and viruses; Wang et al., 2019). However, it is important to acknowledge the possibility that some fungal spores and yeasts can also be present in this 3 μm size fraction (Duarte et al., 2008). Furthermore, we predicted a reduction in the diversity of soil fauna and fungi with decreasing pore sizes, while bacteria were generally expected to be present in all the size fractions. We expected that in the 250 μm fraction, soil fauna, fungi and bacteria would be present. Filtering through 20 μm was expected to entirely remove the soil fauna (Van de Voorde et al., 2012). Larger fungi (e.g. mycorrhiza) were anticipated to be filtered out in the 11 μm fraction which was expected to primarily consist of smaller fungi and bacteria (Wagg et al., 2014). Despite the expected exclusion of certain taxa from each size fraction, all three groups of organisms were sequenced from the roots of trees regardless of which size fraction they received. This was done to account for the possibility of small eggs, fungal spores and very thin soil fauna such as some nematodes (<5 μm; Andriuzzi and Wall, 2018) passing through the filters.

Due to the number of samples and duration of the protocols, generation of the fractions had to be split in two days. Thus, the sieved fractions were stored for 1 day at 4 °C before centrifuging and pelleting until all fractions were obtained. Following the sieving, each fraction of each forest was then centrifuged at 4700 rpm for 10 min (10 °C) to obtain a pellet. The supernatant was decanted, and the pellets were resuspended using 125 and 5 ml LB medium for the 250 and 20 μm fractions respectively, and 4 ml for the 11 and 3 μm fractions to account for the differences in pellet size between fractions. Each resuspension was stored in either falcon or Eppendorf tubes in 3:2 extract to glycerol (80%) ratio and stored at −20 °C (Riis et al., 1998). An additional 2 ml from each resuspension was stored at −20 °C for DNA analysis.

To prepare the inocula, each fraction stock was thawed and centrifuged to decant the supernatant (LB medium + glycerol). For each of the four fractions of each forest, the pellets were then resuspended in KH_2PO_4 buffer and transferred to a flask that was filled up to 200 ml with KH_2PO_4 buffer. The same procedure was followed for each of the four fractions of all twelve forests, resulting in a total of 48 liquid inocula with each individual forest acting as a replicate in its forest development stage (six replicates for young and six replicates for mature). Each of these inocula was randomly applied to 3.15 L (15 × 15 × 20cm) pots that were filled with gamma-sterilized grassland soil (Salonius et al., 1967). Finally, ten pots with gamma-sterilized grassland soil received just the KH_2PO_4 buffer without any community fraction inocula and another ten pots with gamma-sterilized grassland soil were left completely untreated and received neither buffer nor inocula bringing the total number of pots to 68. In this research, we aimed to compare the effects of different soil community fractions from young plantations and mature forests. Hence, a background soil was used that is close in properties to the plantations (e.g. agricultural soil or grassland). This soil was poor in available ammonia (NH_4: 37.82 ± 1.61 mg/kg soil), nitrate (NO_3: 15.41 ± 0.57 mg/kg soil) and orthophosphate (PO_4: 3.86 ± 0.41 mg/mg soil). Soil subsamples from the sterilized soil were oven-dried for 48 h at 40 °C for nutrient analysis, specifically targeting NH_4, NO_3, and PO_4. NH_4 and NO_3 were extracted using a 1 M potassium chloride (KCl) method (Kachurina et al., 2000), while PO_4 was extracted with a 0.01 M calcium chloride (CaCl) method (Houba et al., 2000). Final nutrient concentrations were expressed in mg of each available nutrient per kg of soil. Two additional experiments were performed using the same setup but supplemented with either bactericide or fungicide with the aim of disentangling the effects of fungi and bacteria from each fraction (SI, Methods S1).

2.3. Plant growth

After receiving the inocula, the pots were placed in a climate room with relative humidity 70%, light regime of 16 h:8 h (light:dark), air temperature of 20 °C (light) and 18 °C (dark). The pots were then left to settle for four days while the soil was kept moist. On the fifth day after receiving the inocula, one four-week-old *A. glutinosa* seedling, germinated in autoclaved potting soil, of similar stem height (~2 cm) and number of leaves (at least two) was planted in each pot. The seeds were initially surface-sterilized with bleach solution (14%) to minimize surface contaminants (vertically shaking at 200 rpm for 10 min) before being thoroughly rinsed with autoclaved MQ H_2O to remove residual bleach. Seedlings were watered three times per week. Each pot was fully saturated with water during each watering event. During the first week, four seedlings died and these were immediately replaced. After 18 weeks, stem height (cm) and diameter (mm) were measured. Hereafter, CO_2, N_2O and CH_4 gas fluxes were measured for each tree, see below. When measuring GHG fluxes, the 11 μm size fraction was excluded from the measurements. Since access to the necessary equipment was limited, this size fraction was excluded as it was deemed to be the most redundant based on stem height measurements from the last week before the harvest.

2.4. Gas fluxes measurements

Two days prior to the harvest of the pots, CO_2, N_2O and CH_4 gas fluxes were measured for each of the trees (pot + tree system). The measurements were conducted using an UGGA GLA-915-0011 (Los Gatos Research, Inc., Mountain View, CA, USA) gas analyzer instrument for CO_2 and CH_4 fluxes and a LICOR LI-7820 (LI-COR Environmental., Lincoln, Nebraska USA) instrument for N_2O fluxes. Each pot was placed in a 30 cm diameter PVC base on top of which we fitted a 100 × 30 cm (70 L) plexiglass chamber covered with double-layer black plastic bag to prevent photosynthesis and the fluxes were measured over a 5-min enclosure time. A fan was placed at the top of the chamber, to ensure mixing during flux measurements. Between each flux measurement, the chamber was removed from the base and placed on its side so that it was flushed with atmospheric air. After each flux measurement soil moisture and temperature were measured in each pot using a Delta T probe HH2 moisture meter (Delta-T Devices Ltd, Cambridge, United Kingdom) and a HANNA checktemp1 (HANNA instruments, Nieuwegein, Netherlands) instrument, respectively. These two instruments were thoroughly washed with ethanol in between samples to avoid cross contamination.

Two days later, all plants were harvested. After removing the soil, roots were thoroughly rinsed with running tap water, and the root

nodule density was recorded. Root subsamples were then collected for both morphological characterization (preserved in water at 4 °C) and DNA analysis (preserved at −20 °C). Root scanning was conducted using an Epson Perfection V850 Pro scanner at 1200 dpi, and the WinRHIZO software (Regent Instruments, Quebec, QC, Canada) was employed to determine root length, specific root length, and the percentage of fine roots (defined as those with a diameter <0.3 mm). The subsample was then oven-dried for 96 h (40 °C). After removing the leaves, the stems were separated from the roots and all of them were oven-dried, similar to the root subsamples. The dry weight of the stems, leaves, roots and root subsamples was then recorded and this was used to calculate the above and belowground dry biomass of the trees.

2.5. Soil respiration

After the plants were unpotted, the soil from each pot was thoroughly mixed to homogenize the top and bottom layer and subsamples of ~100 g of fresh soil were kept from each pot and stored at 4 °C for a week. The subsamples were sent to the university of Copenhagen to measure soil respiration. Roughly 50 g of each sample was placed in an incubator and all samples were normalized to 10% soil moisture. The temperature sensitivity of soil heterotrophic respiration (Q10) rate was measured for moisture-normalized samples (10% gravimetric water content) using a respirometer (Nordgren, 1988). Shortly, the soil sample was placed in a sealed cup and the respired CO_2 produced from the soil dissolved in a 0.1 M KOH-solution placed inside the cup. The change in electric conductivity of the KOH-solution is directly proportional to the CO_2 production. Data was recorded automatically on a computer. Soil CO_2 respiration was measured at 9 temperature intervals from 5 °C to 25 °C with increments of +2.5 °C.

2.6. Soil and root-associated communities

Forest soil DNA was isolated utilizing the DNeasy PowerSoil pro kit (Qiagen Inc., Hilden, Germany). Extraction of DNA from root samples was done by using the DNeasy plant Pro kit (Qiagen Inc., Hilden, Germany) following the manufacturer's protocols. For bacteria, the indexed primers 515 F (GTG YCA GCM GCC GCG GTA A) and 926 R (GGC CGY CAA TTY MTT TRA GTT T) (Quince et al., 2011; Parada et al., 2016) were used, targeting the V4 region of the 16Sr RNA gene. For fungi, the rRNA ITS2 region was targeted using the primers gITS7ngs (GTG ART CAT CRA RTY TTT G) and ITS4ngsUni (CCT SCS CTT ANT DAT ATG C) (Tedersoo and Lindahl, 2016). For soil fauna, the 313 bp mitochondrial cytochrome oxidase 1 (COI) gene was targeted using the primers mlCOIintF (5′ GGW ACW GGW TGA ACW GTW TAY CCY CC) (Leray et al., 2013) and jgHCO2198 (3′ TAI ACY TCI GGR TGI CCR AAR AAY CA) (Geller et al., 2013). PCR procedures were performed in duplicate for all reactions and then pooled for further analysis. To validate the success of the PCR, 5 μl of the resulting products were used for 1% agarose gel electrophoresis. For library preparation, 1–20 μl of PCR product was taken, depending on the size of the amplicon band on the gel. Libraries were prepared using the NEBNext Ultra II FS DNA library prep kit (SPT labtech, Melbourne, UK) and were sequenced with 2 × 250 paired-end chemistry in Illumina NovaSeq 6000 device at NovoGene UK. To verify that the fractionation led to community simplification with each filtration step, we quantified soil animals, bacteria and fungi using quantitative real-time PCR (qPCR). This step aimed to ascertain that the absolute abundance of soil animals, fungi, and to a lesser extent bacteria (as we expect them in each size fraction), would reduce with reducing size fraction. DNA was extracted from 2 ml resuspended pellets in LB medium. The pellets were collected from all inocula from all fractions and were kept at −20 °C (see community size fractions session). DNA was extracted using the DNeasy PowerSoil pro kit (Qiagen Inc., Hilden, Germany). DNA concentration was measured using a NanoDrop 1000 spectrophotometer (Thermo Scientific, Wilmington, DE). qPCR was performed using a CFX96 Touch Real-Time PCR Detection System (BioRad, Hercules, CA). We selected the same primers as for high throughput sequencing, as mentioned above for each of the three groups (qPCR details can be found in SI, Methods S2). The final number of copies for soil fauna, fungi and bacteria in each fraction was corrected based on the DNA concentration of each size fraction measured from the nanodrop and then further extrapolated to the concentration of inoculum added to the pots. As expected, the abundance of soil animals decreased significantly to almost zero after filtering past the 20 μm size fraction (Fig. 1A–D; Table S3). The absolute abundance of fungi reduced with decreasing size fraction for both young and mature forest inocula (Fig. 1B–E; Table S3) and despite not being completely absent in the 3 μm, only $9.29 \times 10^{11} \pm 8.73 \times 10^{11}$ and $1.21 \times 10^{12} \pm 1.15 \times 10^{12}$ number of copies were detected in inocula originating from young and mature forests, respectively. The abundance of bacteria remained high in all fractions with a significant reduction only in the 3 μm fraction inocula originating from young forests (Fig. 1C; Table S3).

2.7. Bioinformatics

High-throughput sequencing of one COI library and seven 16 S and ITS2 libraries provided 9,972,478 raw paired-end sequences for the COI (soil fauna), 74,170,482 for the 16 S (bacteria) and 69,332,423 for the ITS (fungi). Sequence data were processed using PipeCraft2 v1.0.0 (Anslan et al., 2017). Raw data was demultiplexed to sample-wise fastq files with" demultiplexing" module in PipeCraft2, which utilizes Cutadapt v4.4 (Martin, 2011). Demultiplexing resulted in an average of 149, 181, 107,482, and 69,633 reads per sample for the COI, 16 S, and ITS2 libraries, respectively (SI, Fig. S1). Demultiplexed sequences underwent quality filtering, denoising, and assembly using the default settings of DADA2 v1.28 (Callahan et al., 2016) within PipeCraft2. Putative chimeric sequences were removed with the "consensus" method in DADA2. From the resulting ASV tables, tag-jumps were corrected based on UNCROSS2 score ("filter tag-jumps" module) (Edgar, 2018). For the ITS2 library, the generated ASVs were passed through ITSx v1.1.3 (Bengtsson-Palme et al., 2013) to extract ITS2 region without conservative gene fragments. For all the libraries, ASVs were clustered to OTUs using vsearch (–iddeff = 2, similarity threshold of 97%; "ASV to OTU" module) (Rognes et al., 2016) and post-clustered using LULU (Frøslev et al., 2017). All statistical analysis of the sequencing data was performed on the OTUs. The match list for LULU was generated using BLASTn (Camacho et al., 2009). For the COI OTUs, NCBI's ORFfinder (Sayers et al., 2022) with the invertebrate mitochondrial code (genetic code 5) was used to remove off-target OTUs and pseudogenes (by translating sequences to open reading frames (ORFs) and retaining the longest ORF per sequence if the length of the ORF was 309–317 bp). For the COI library, taxonomy was assigned to filtered OTUs using BLASTn search against CO1Classifier v5.0.0 database (Porter and Hajibabaei, 2018) while for the 16 S and ITS2 libraries, the BLASTn search was used against SILVA v138.1 (Quast et al., 2012) and UNITE v9 (Nilsson et al., 2019) databases, respectively. Following the outcomes of taxonomy assignment, all soil fauna OTUs not classified within the kingdom Metazoa were removed. An OTU was classified at the phylum level when its top BLASTn match, with ≥80% identity against a reference sequence (annotated at the phylum level), was identified. Certain OTUs were most closely aligned with Hydrozoa and Porifera at <89% sequence similarity. However, as these aquatic organisms are uncommon in terrestrial environments, these OTUs were designated as unclassified Metazoa. Additionally, all bacterial OTUs not classified within the kingdom Bacteria, along with those identified as chloroplast and mitochondria, and all fungal OTUs not attributed to the kingdom Fungi were excluded prior to statistical analyses. Bacterial and fungal OTUs sequenced from the roots were considered root associated OTUs while soil animal OTUs were considered eDNA remnants from soil animals that potentially associate with the plants.

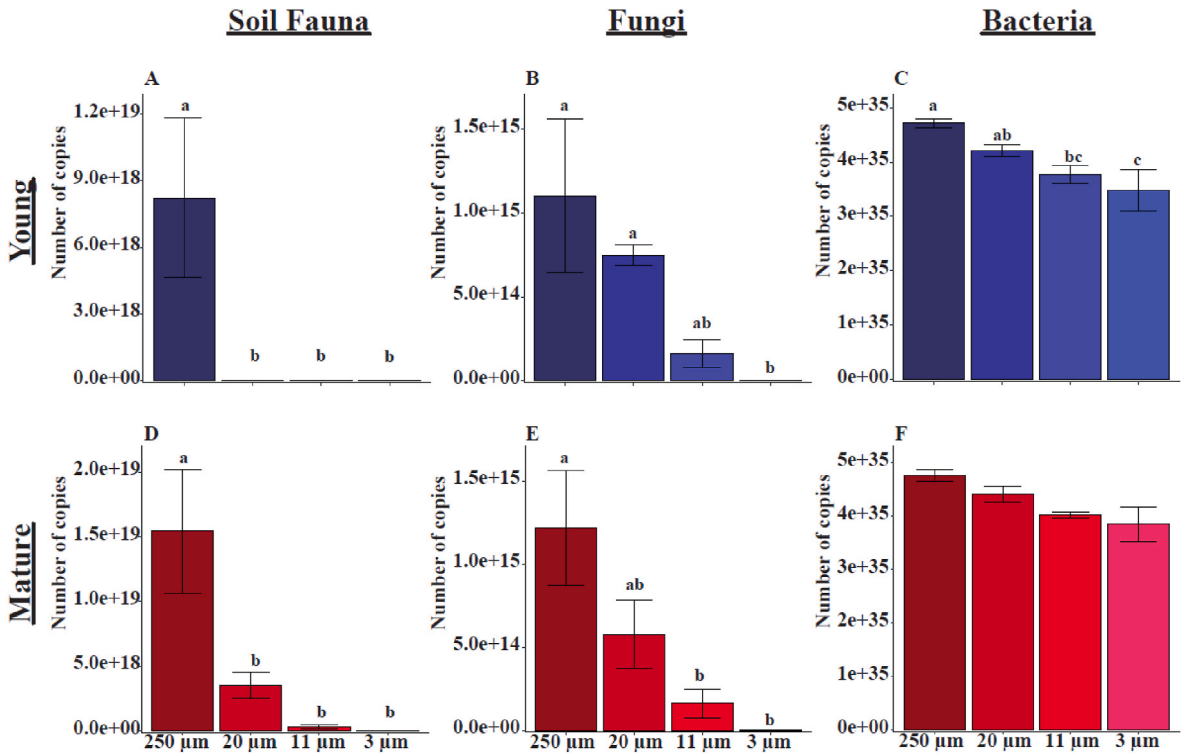


Fig. 1. Absolute abundance of soil fauna (A, D), Fungi (B, E) and Bacteria (C, F) in inocula from the liquid size fraction from both forest development stages (young in blue, and mature in red) that were used to inoculate the pots in the experiment. The absolute abundance of each community is expressed in copy numbers (in 200 ml of liquid inoculum) that were obtained from a qPCR. The reducing colour vibrancy represents the reducing community size fractions. These are calculated from n = 6 replicates per forest development stage. Details of the statistical output of the full linear mixed effects model can be found in Table S3. Different letters above the boxplots refer to significantly different means based on a Tukey post-hoc test. The values shown are mean ± se.

2.8. Statistical analysis

2.8.1. Community composition and microbiomes

Following the bioinformatics, the soil and root sample datasets were treated independently as soil and root DNA was extracted using different kits. Low abundant OTUs (represented by less than 0.01% of total reads) were removed prior to rarefying each dataset to increase the reliability of community composition (Nikodemova et al., 2023). Soil fauna, bacterial and fungal OTU tables were rarefied to 269, 9683 and 2020 for roots and 541, 55514 and 10146 for soils, respectively, in order to normalize the sequencing depth for each dataset (SI, Fig. S2). Soil fauna rarefaction curves did not reach a perfect asymptote but were rarefied to the lowest possible depth that allowed for the presence of at least five samples from each treatment to be able to conduct reliable statistical analysis (one sample from each fraction was lost from roots and one from each developmental stage from soils). To examine variations in community structure between the two forest development stages and community size fractions, a principal coordinate analysis (PCoA) was conducted based on Bray-Curtis distances, utilizing the Hellinger transformed counts calculated with the vegdist and pcoa functions of *vegan* R package v2.6–4 (Oksanen, 2015). Differences in soil fauna, bacterial and fungal communities between the two forest development stages and the four different size fractions were evaluated through a nested Permanova analysis using the same package. To ensure that the results were not influenced by different ingroup variations, data overdispersion was assessed using the betadisper function of the *pairwiseAdonis* R package v0.4.1 (Martinez Arbizu, 2020). Pairwise comparison tests were conducted with the same package when significant differences were observed in the Permanova results. In addition to correlations with above- and belowground biomass production, soil fauna, fungal and bacterial OTUs were correlated with the CO_2, N_2O and CH_4 flux measurements to explore the potential effect of individual taxa on GHG fluxes. Correlations between all OTUs and above- and belowground biomass and GHG fluxes (CO_2, N_2O and CH_4) were analysed using Spearman correlations with the *Hmisc* R package (Harrell Jr and Harrell Jr, 2024). The p values were adjusted using false discovery rate (FDR) with the Benjamini-Hochberg procedure in R. Correlations were considered significant only when the adjusted p values were equal or lower than 0.01 and then the correlation coefficient (R) was higher than 0.5 (or lower than −0.5 in the case of a negative correlation) to only account for strong correlations (Akoglu, 2018). A linear mixed effects model (LMM) was used to test the effect of community size fraction (soil, 250 µm, 20 µm, 11 µm, 3 µm) on OTU numbers found in each size fraction within each developmental stage (SI, Table S4) using the *lme4* R package v1.1–35.1 (Bates et al., 2015). Pairwise comparisons were carried out using a Tukey post-hoc test. Similarly, a LMM was used to test the effect on the absolute abundance (number of copies) of soil fauna, fungi and bacteria in each of the inocula of the different fractions.

2.8.2. Tree performance

In the mesocosm experiment, firstly a LMM, excluding the control, was used to test the interaction between forest development stage (young vs mature) and community size fraction (250 µm, 20 µm, 11 µm, 3 µm) on each of the tree performance parameters (see SI, Table S5) which were individually measured using the *lme4* R package v1.1–35.1 (Bates et al., 2015). Forest identity was used as a random factor due to sampling from multiple different subplots within each forest. Pairwise comparisons were carried out using a Tukey post-hoc test. To test the effect of inoculating with the different size fractions, the LMM was repeated, including the control as part of the forest development stage (Control, young, mature). When the output was significant, differences in relation to the control treatment were tested with a Dunett's comparison test using the *DescTools* R package v0.99.51 (Signorell et al., 2023). For each model, we examined the normality of the residuals and

their distribution through a Shapiro-Wilk normality test and a qqplot, respectively. Moreover, a histogram was employed to visually inspect the skewness of the data. We also assessed the homogeneity of variance between samples using a Levene's test. The data for belowground biomass, root diameter, root volume, the percentage of fine roots and root nodule density were square-root-transformed prior to analyses. Correlations between aboveground and belowground biomass, stem width, number of leaves, chlorophyll content and root nodule density were analysed using Pearson correlations through the *Perform-anceanalytics* R package v2.0.4 (Peterson et al., 2020).

2.8.3. Gas flux measurements

The gas flux data from the mesocosm experiment was expressed in μmol h^{-1} kg-1 where kg is the dry soil weight in kilograms. This was done to account for potential differences in soil moisture levels at the time of measuring gas fluxes.

Analysis for the gas fluxes experiment was performed similarly to the tree performance analysis with either the CO_2, N_2O, CH_4 flux or the Q10 used as the response variables. The data was normally distributed and no further transformation was necessary.

The graphical representations were generated using the ggplot 2 R package v3.4.4 (Wickham, 2011).

2.9. Accession numbers

The raw Illumina reads are deposited in European Nucleotide Archive (ENA) at EMBL-EBI under accession number PRJEB72733 (BioSample accessions ERS20251472-ERS20251533).

3. Results

3.1. Soil community composition and richness

In the soils collected from the twelve fields, soil fauna (Permanova: pseudoF = 4.49, R^2 = 0.36, p = 0.010), bacterial (Permanova: pseudoF = 13.70, R^2 = 0.58, p = 0.006) and fungal (Permanova: pseudoF = 5.70, R^2 = 0.36, p = 0.004) community composition significantly differed between the two forest development stages (SI, Figs. S3A, D, G). The soil fauna and bacterial richness were significantly higher in young forest soils than in mature forest soils (SI, Figs. S3B and E). No significant differences were observed in the richness of soil fungi between the two forest development stages (SI, Fig. S3H).

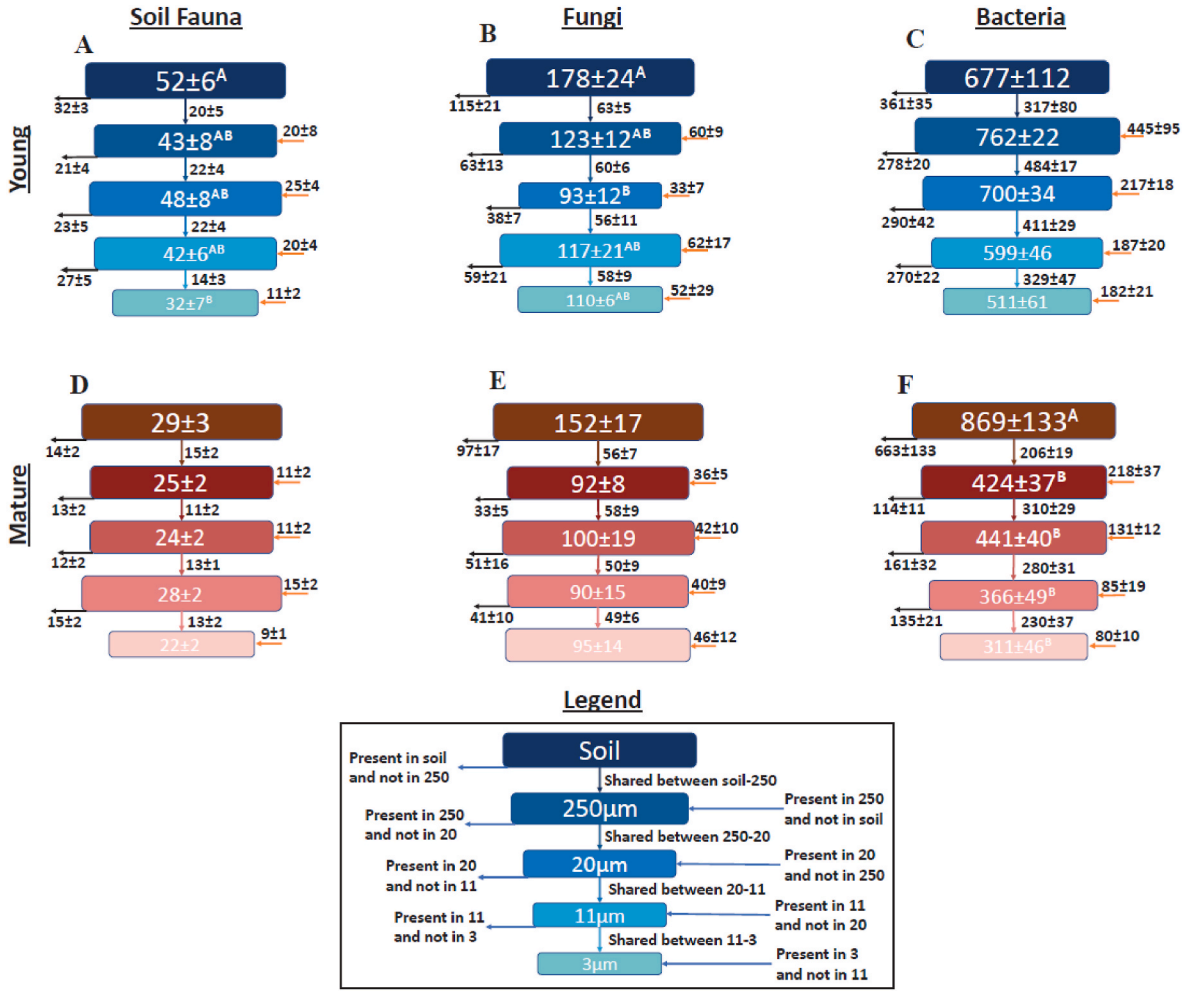


Fig. 2. Number of OTUs for soil fauna (A, D), fungi (B, E) and bacteria (C, F) present in all stages of the experiment in the fractions. Mean ± se of number of OTUs is presented for the initial soils where inocula were originated, and for the subsequent four community size fractions (250, 20, 11 and 3 μm) in the two forest developmental stages (young and mature). Size of the boxes reflects the richness. The vertical arrows and numbers linking two boxes denote the number of OTUs shared between them. The horizontal arrows to the left of each box denote the number of OTUs that are filtered out when comparing one size fraction with the next while the arrows to the right represent the number of OTUs that were not present in the previous size fraction. Means and standard errors were calculated from averaging 6 replicates per forest development stage. Significant differences from a post-hoc test between size fractions within each developmental stage are indicated with capital letters in superscript. No letters are presented in the case that there are no significant differences from the post-hoc test.

3.2. Size fractions experiment

Altogether, in the roots of trees growing in sterile soils that were inoculated with the size fractions 393 soil fauna, 2612 bacterial and 988 fungal OTUs were present.

Generally, the number of root-associated OTUs in the largest fraction (250 μm) decreased compared to the number of OTUs found in the soils where they originated from. An exception to this were the root-associated bacteria of trees that were inoculated with communities from young forests where the largest fraction had more OTUs than what was found in the soils (Fig. 2C). On average, the number of soil fauna and root-associated fungal OTUs did not change with decreasing size fraction for either of the two forest development stages (Fig. 2A, B, D, E). However, the number of soil fauna OTUs decreased in the smallest size fraction (3 μm) compared to the larger fractions (Fig. 2A–D). On average, two adjacent size fractions had half of the soil fauna and root associated fungal OTUs in common. The remaining half were filtered out with a roughly similar number of new OTUs appearing into the next fraction (Fig. 2A, B, D, E). On the other hand, the number of root-associated bacterial OTUs decreased with decreasing size fraction in both forest development stages (Fig. 2C–F). Roughly 66% of the root associated bacterial OTUs were common from one size fraction to the next and the remaining 34% were filtered out as size fractions decrease (Fig. 2C–F). In the case of bacteria, the new OTUs that were coming into each decreasing size fraction were half as many as those that were filtered out with the exception of the 250 μm fraction where they were double the amount of what was filtered out (Fig. 2C–F).

3.3. Root-associated community composition and richness

The soil fauna (Fig. 3A; Permanova: pseudoF = 15.26, $R^2 = 0.27$, p = 0.001), bacterial (Fig. 3D; Permanova: pseudoF = 32.08, $R^2 = 0.41$, p = 0.001) and root associated fungal (Fig. 3G; Permanova: pseudoF = 8.27, $R^2 = 0.16$, p = 0.001) community composition significantly differed between the roots of trees grown in soils of the two different forest development stages. However, neither of the communities differed between the four different size fractions and no interaction effect was observed between forest development stage and size fraction on either the soil fauna or root associated bacterial and fungal community composition. Bacterial richness decreased with decreasing size fraction in the roots of trees grown in young forest soils and was on average 66.8% higher than in the roots of trees grown in mature forest soils (Fig. 3E). There were no differences in either the soil fauna or fungal richness of the root associated communities between either the two forest development stages or the four different size fractions (Fig. 3B–H).

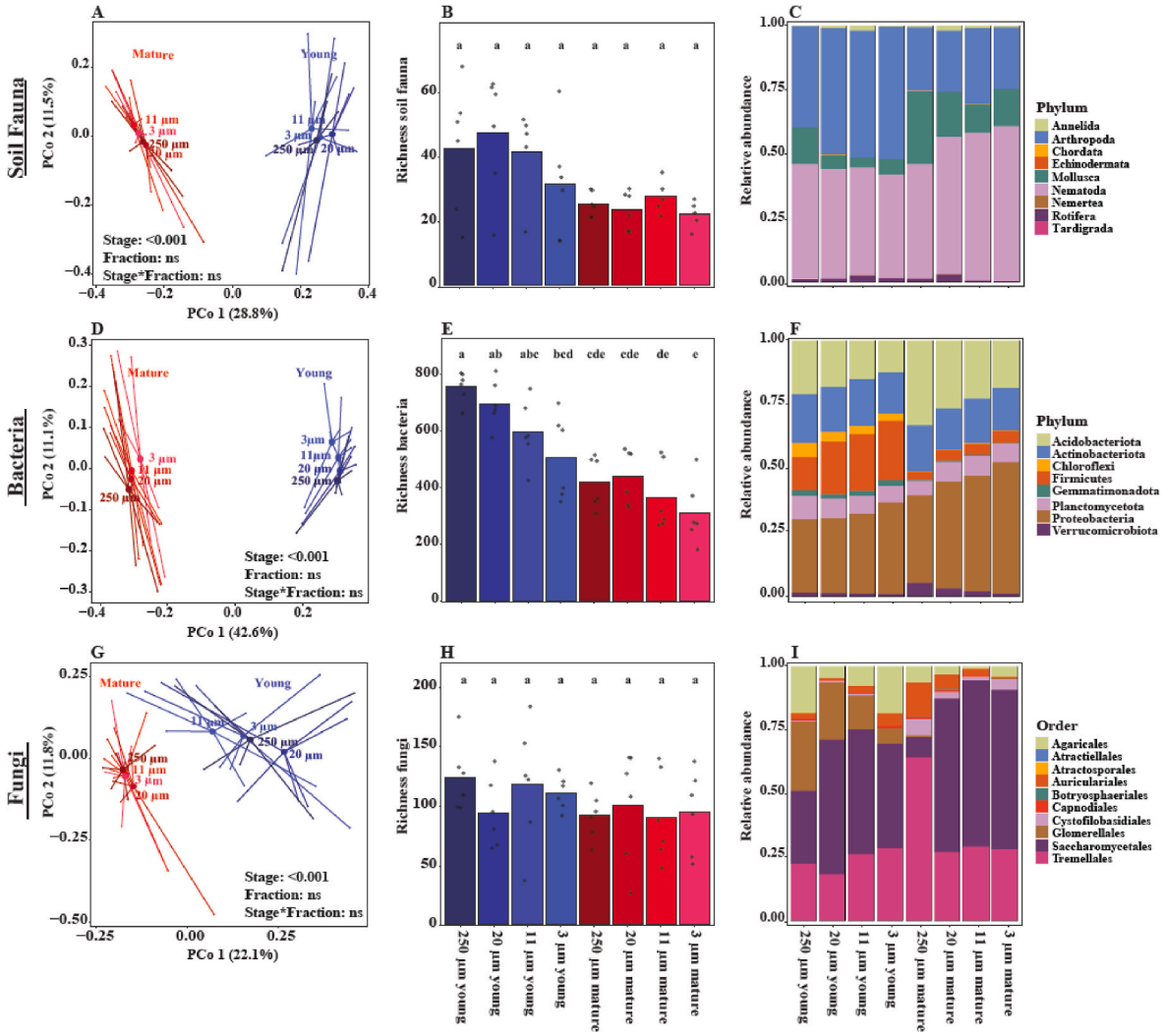


Fig. 3. Soil fauna (A) and root associated bacterial (D) and fungal (G) community composition (based on Bray-Curtis similarity) and alpha diversity (B, E, H); letters indicate significant differences. Taxa bar plots showing the relative abundance of the eight most abundant soil fauna and bacterial phyla (C, F) and the fungal orders (I) present at each forest development stage (young in blue, and mature in red) and community size fraction (250, 20, 11 and 3 μm).

3.4. *Alnus glutinosa* tree performance

Forest development stage and size fraction had a significant effect on aboveground biomass production, stem width and leaf number of *A. glutinosa* trees (Fig. 4A; SI, Table S5). Specifically, trees grown in soils inoculated with size fractions from mature forest soils produced 13.7% less aboveground biomass (averaging over all fractions) than those inoculated with size fractions from young forest soils, with those that received the 250 μm size fraction from the mature forests exhibiting the lowest biomass production (Fig. 4A). Although trees that were inoculated with the 250 μm size fraction from the mature forest sites produced 76.2% less aboveground biomass compared to the control treatment, the difference in biomass production was not significant. The biomass production of the remaining treatments was indistinguishable from the control. As expected, trees that produced more aboveground biomass also produced thicker stems, higher chlorophyll content and a higher number of leaves (SI, Figs. S4A, B, E). However, there was no effect of either forest development stage or size fraction on leaf chlorophyll (Fig. 4B).

Spearman correlations between the relative abundance of soil fauna, root associated bacterial and fungal OTUs and the aboveground biomass production of trees revealed positive correlations with *Gammaproteobacteria* for both forest development stages, namely of the orders *Burkhoderiales* and *Pseudomonadales*. *Bacteroidota* of the family *Chitinophagaceae* positively correlated with aboveground biomass production when trees were inoculated with communities from young forests and negatively correlated when inoculated with communities from mature forest soil communities. *Alphaproteobacteria* of the order *Sphingomonodales* positively correlated with aboveground biomass production in young treatments while *Elsterales* showed both positive and negative correlations. *Rhizobiales, Acidimicrobiia* and *Acidothermus* negatively correlated with aboveground biomass production in mature treatments. Additionally, *Limnochordia* in young treatments negatively correlated with aboveground biomass production. From the fungal OTUs, *Agaricomycetes* and *Mortierella* were positively correlated with aboveground biomass in mature treatments. No fungal OTUs correlated with aboveground biomass in young treatments (SI, Fig. S5). No correlations were found between soil fauna OTUs and aboveground biomass in either young or mature treatments.

Belowground biomass production was not dependent on forest development stage but rather on size fraction (Fig. 4D; SI, Table S5) and was positively correlated with aboveground biomass production ($R^2 = 0.56$, $p < 0.001$; SI, Fig. S4C). Similarly to aboveground biomass, trees grown in soils that received the 250 μm size fraction from the mature forests exhibited the lowest biomass production. Although trees inoculated with the 250 μm size fraction from either the young or the mature forest sites produced 37.2% and 78.6% less biomass, respectively, compared to the control treatment, the difference in biomass production between either of them and the control was not significant. The biomass production of the remaining treatments was indistinguishable from the control. Forest development stage had a significant effect on the specific-root-length of the trees (Fig. 4F; SI, Table S5). The specific-root-length of trees grown in soils inoculated with mature forest soil communities was 38.1% higher than of those inoculated with young forest soil communities. Although differences were not significant between either the forest development stages or the size fractions, trees grown in soils that were inoculated with communities from young forest soils produced on average 66.6% more nodules per gram of dry root biomass than trees grown in soils that were inoculated with communities from mature forest soils, indicating a possible negative effect of mature forest communities on root nodulation (Fig. 4C). As expected, a higher nodule density was positively correlated with aboveground biomass production ($R^2 = 0.15$, $p = 0.010$; SI, Fig. S4D) as well as leaf chlorophyll content ($R^2 = 0.14$, $p = 0.010$; SI, Fig. S4F). There were no significant effects of either forest development stage or size fraction on the percentage of fine roots of *A. glutinosa* (Fig. 4E).

Spearman correlations between the relative abundance of bacterial OTUs and the belowground biomass production of trees revealed positive correlations with *Gammaproteobacteria* (*Legionella* in young and *Pseudomonadales, Burkhoderiales* and *Enterobacterales* in mature) and

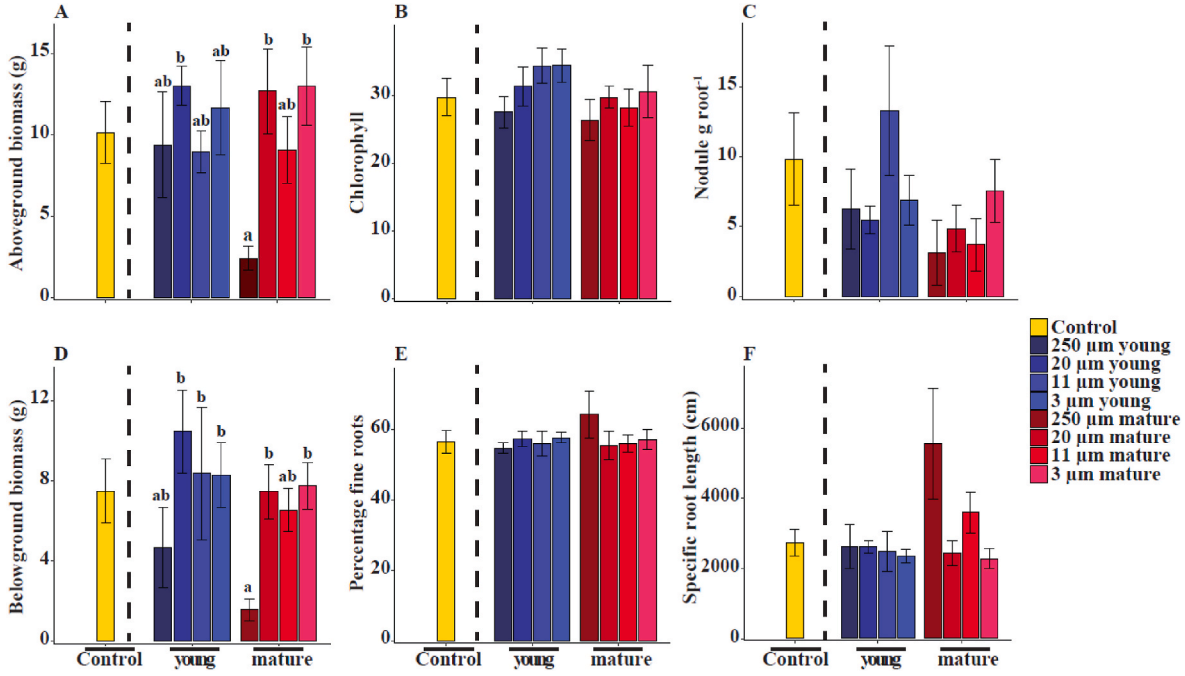


Fig. 4. The effect of forest development stage (young, mature), community size fraction (250, 20, 11 and 3 μm) and their interaction on (A) Aboveground biomass, (B) Chlorophyll content, (C) Nodule density, (D) Belowground biomass, (E) Percentage of fine roots and (F) Specific root length. The blue and red colours represent the two forest development stages (young and mature) while the reducing colour vibrancy represents the reducing community size fractions. These are calculated from n = 6 replicates per forest development stage. Details of the statistical output of the full linear mixed effects model can be found in Table S5. Different letters above the boxplots refer to significantly different means based on a Tukey post-hoc test. The values shown are mean ± se.

Firmicutes (*Clostridia* in mature and *Bacili* in both) for both forest development stages. Actinobacteria both positively and negatively affected belowground biomass (SI, Fig. S6). Specifically, *Nocardioides* in young and Nocardia in mature treatments positively correlated while *Acidimicrobiia* in mature and *Acidothermus* in both treatments negatively correlated with belowground biomass production. *Gemmataceae* positively correlated while *Chloroflexi* (*Ktedonobacteria* and *Anaerolineae*) and negatively correlated with belowground biomass in young treatments. *Acidobacteriae* and *Rhizobiales* also negatively correlated with belowground biomass in mature treatments. From the fungal OTUs, *Thelesphoraceae* negatively correlated with belowground biomass production in young treatments while *Rozellomycota, Mortierellomycota* (*Mortierella*), Ascomycota (*Eurotiomycetes, Oidiodendron, Sordariomycetes, Eurotiomycetes*), *Umbelopsis* and *Agaricomycetes* (*Trechispora* and *Oliveonia*) positively correlated with belowground biomass in mature treatments (SI, Fig. S6). None of the relative abundances of the soil fauna OTUs correlated with belowground biomass production (SI, Fig. S6).

When the control treatment was added as a level in the forest development stage, no significant effects of forest development stage or size fraction were detected for any of the measured variables (SI, Table S6), and thus no significant differences were observed between any of the treatments and the control for any of the measured variables (Fig. 3).

We additionally tested whether forest development stage or size fraction impacted the relative abundance of major symbionts found in the roots of *A. glutinosa*, namely arbuscular mycorrhiza fungi (AMF), ectomycorrhiza fungi (EcM), *Endogonomycetes* and Frankiales and whether the relative abundance of these symbionts affected the above and belowground biomass production of *A. glutinosa.* There was a significant effect of size fraction on the relative abundance of AMF which were relatively more abundant in the 3 μm size fraction (SI, Table S7). However, Tukey post-hoc tests revealed no differences between the treatments (SI, Fig. S7A). Furthermore, a significant interaction was found between forest development stage and fraction side on the relative abundance of Frankiales (SI, Table S7). Specifically, Frankiales were relatively more abundant with increasing size fraction in the roots of trees that were inoculated with the mature forest fractions than those that were inoculated with the young forest fractions. Between the young forest treatments, only the trees that were inoculated with the largest size fraction (250 μm) showed a higher relative abundance of Frankiales than the smaller ones but still lower than the larger fractions of the mature forest soils (250 and 20 μm) (SI, Fig. S7D). In contrast to the nodule density, higher relative abundance of Frankiales negatively correlated with above- and belowground biomass production. However, this was only true for trees inoculated with mature forest size fractions but not for trees inoculated with young forest size fractions (SI, Fig. S8). There were no significant effects of either forest development stage or size fraction on the relative abundance of EcM and *Endogonomycetes* (SI, Table S7).

3.5. Gas fluxes experiment

In the experiment where mesocosm gas fluxes were measured, a significant interaction was detected between forest development stage and size fraction on net CH_4 uptake (Fig. 5C; SI, Table S8). Although the mean net CH_4 uptake did not differ between the two forest development stages, the soils treated with 20 μm size fraction from young forest soils had higher net CH_4 uptake than the mesocosms inoculated with the 20 μm size fraction from mature forest soils (Fig. 5C). Despite a non-significant effect of either forest development stage or size fraction on CO_2 emissions, we observed lower CO_2 emissions in the 250 μm size fraction from both forest development stages (Fig. 5A). Neither forest development stage nor size fraction significantly affected N_2O emissions from the mesocosms (Fig. 5B; SI, Table S8). Furthermore, no effect of either forest development stage or size fraction on the temperature sensitivity of heterotrophic soil respiration rates (Q10) was detected and Q10 values were on average 2.33 ± 0.07 (Fig. 5D; SI, Table S8). When the control treatment was added as a level in the forest development stage, we observed a significant interaction effect on CH_4 fluxes and an effect of size fraction on N_2O fluxes (SI, Table S9). However, no significant differences were observed between any of the treatments and the control.

Spearman correlations between the relative abundance of soil fauna,

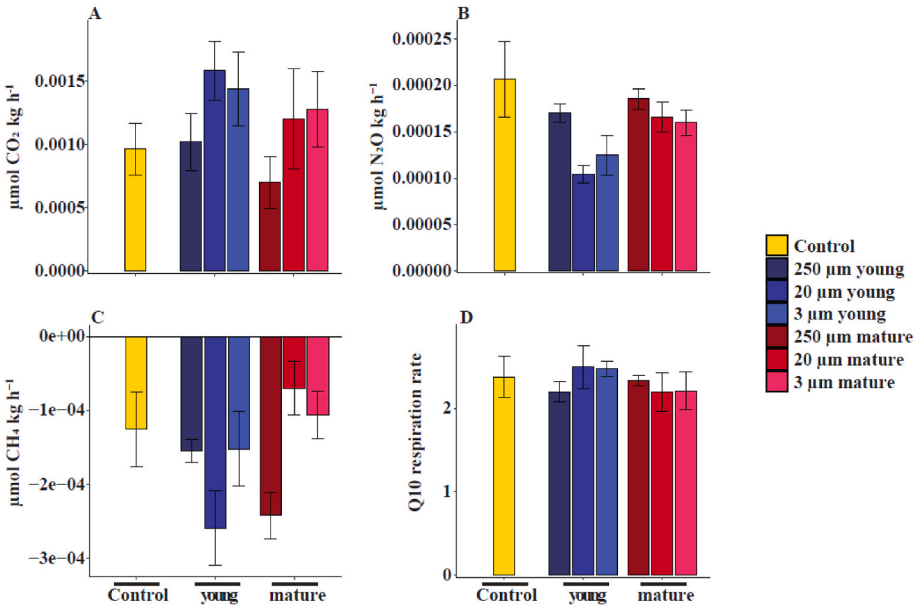


Fig. 5. The effect of forest development stage (young, mature), community size fraction (250, 20, 11 and 3 μm) and their interaction on mesocosm (A) CO_2 flux, (B) N_2O flux, (C) CH_4 flux and (D) Q10 respiration rate. The blue and red colours represent the two forest development stages (young and mature) while the reducing colour vibrancy represents the reducing community size fractions. These are calculated from n = 6 replicates per forest development stage. Details of the statistical output of the full linear mixed effects model can be found in Table S8. Different letters above the boxplots refer to significantly different means based on a Tukey post-hoc test. The values shown are mean ± se.

bacterial and fungal root associated OTUs and CO_2 fluxes from the mesocosms revealed positive correlations between *Acidobacteriota* (*Bryobacteracea* in young and *Vicinamibacterales* in mature), *Planctomycetota* (*Gemmataceae* and *Isosphaeraceae* in young and *Planctomycatales* in mature) and *Verrucomicrobiota* (*Pedospheralles* in young) for both forest developmental stages. Additionally, *Myxococcota* (*Pajaroellobacter*) in young treatments and *Gammaproteobacteria* (*Legionella*) in mature treatments were positively correlated with CO_2 fluxes. Although *Acidobacteriales* was positively correlated with CO_2 fluxes in treatments with size fractions from young forests, it was negatively correlated in mature treatments. *Alphaproteobacteria* (*Elsterales*) were negatively correlated with CO_2 fluxes in young treatments and positively correlated in mature treatments (*Rhizobiales, Elsterales*). There were no correlations between any of the soil fauna and fungal OTUs with mesocosm CO_2 fluxes (SI, Fig. S9).

There was a negative correlation between N_2O fluxes, and *Arthropoda* (*Diamesa, Neelus*) in young treatments. *Alphaproteobacteria* (*Acidisoma* in young and *Rhizobiales* in mature) positively correlated with N_2O fluxes. *Planctomycetota* was found to both positively (*Gemmataceae*) and negatively (*Gemmataceae* and *Tepidisphaerales*) correlate with N_2O fluxes in young treatments. *Anaeromyxobacter* was negatively correlated with N_2O fluxes in young treatments while *Bacteroidota* (*Chitinophagaceae* and *Flavobacterium*) and *Pedosphaeraceae* were positively correlated in mature treatments. Lastly, for the fungal OTUs, *Mortierella* in young treatments and *Hypocreaceae* in mature treatments were negatively correlated with mesocosm N_2O fluxes (SI, Fig. S10). There were no correlations between the relative abundance of any OTUs and CH_4 fluxes.

4. Discussion

In this study, we investigated the effects of decreasing community size fractions on tree performance and mesocosm GHG fluxes. Our findings underscore the importance of specific microbial groups for the performance of trees but also the absence of a community effect on GHG fluxes. Moreover, we reveal the selective nature of *A. glutinosa* in root-associated communities depending on the initial inoculum available from each forest ecosystem developmental stage, and regardless of decreased diversity from the same inoculum.

Community richness was substantially reduced when comparing soil and the root associated communities from the highest fraction which was expected due to trees selecting specific biota that they associate with (Marilley et al., 1998; Philippot et al., 2013). However, reducing community size fraction did not result in reduced community richness with the exception of bacteria. Although most, if not all the soil fauna and a large amount of fungi should have been removed in the fractions below 250 μm pore size (Wagg et al., 2014; Wang et al., 2019; Li et al., 2020), it is possible that free flowing eDNA that passes through during the filtering process is still detected on the roots, while for fungi, small fungal spores possibly passed even through the smallest filter and may even have transferred via the air and water as spore sizes vary between 2 and 50 μm (Yamamoto et al., 2012; Patel et al., 2018). Although with this method bacterial richness was expected to remain similar between the size fractions, the observed reduction could be due to a non-intended blockage of bacteria on the filter paper pores by larger organisms which prevent them from passing through.

Our findings reveal significant differences in soil community composition between the two forest developmental stages. Remarkably, while each developmental stage was represented by six unique forests in our study, the soil community composition within each stage remained consistent, showing no significant variation among the six forests of each stage. These results suggest that ecosystem developmental stage, rather than each unique forest, exerts a substantial influence on soil community diversity and composition. This is likely due to variations in environmental conditions and resource availability, characteristic of each developmental stage (Xun et al., 2015; Kang et al., 2018). The observation that *A. glutinosa* associated with a stable root-associated community within each forest developmental stage (i.e. showing no significant variation among the six young forests or among the six mature forests) despite the overall differences in soil community composition between the two stages, suggests functional redundancy within these root-associated communities. This implies that the soil communities in forests within each developmental stage are functionally similar, even though the communities differ between the young and mature developmental stages. Despite a clear difference in the root associated communities of *A. glutinosa* between the two forest development stages, the same was surprisingly not observed for the different size fractions. It is well known that trees select their root-associated communities to optimize their survival, and this selection is contingent on the source and presence of the communities (Lameta and Jay, 1987; Ruiz Palomino et al., 2005), which in our study originated from two different forest developmental stages. Thus, as expected, *A. glutinosa* selected different biota when being provided with different starting communities from young and old forest developmental stages. Interestingly, despite the reduction in community diversity through means of inoculation with different size fraction inocula, *A. glutinosa* associates with similar root communities among all of the size fractions indicating its high selectivity. In accordance to our hypothesis, the root-associated fungal community composition in young treatments differed slightly more among fractions compared to mature treatments albeit not significantly. This variation among the fractions in young treatments could be indicative of a fungal community that is less adapted to a forest and hence more variable compared to mature forests where fungi tend to be more adapted to a forest ecosystem (Kang et al., 2018). Although root-associated community composition was similar between size fractions, trees inoculated with the largest size fraction produced less above- and belowground biomass and similar results have been reported in other studies (Wang et al., 2019). Although differences in community composition among size fractions cannot explain this effect, it is possible that the greater abundance of biota from the 250 μm fraction inoculum resulted in more severe competition between plants, microfauna and microbes for nutrients (Kuzyakov and Xu, 2013). Alternatively, the presence of certain soil fauna species, or the higher abundance of soil fauna in the inoculum originating from soils from mature forests (Fig. 1D) had a direct negative effect on the plants. Despite the difficulties in linking these negative effects to a specific group of fauna, the possible negative effect of soil fauna cannot be excluded as live soil fauna should still be present in the 250 μm fraction where biomass production was lowest while the rest of the fractions likely contain merely remnants of their eDNA explaining the lack of differences in the community composition of soil fauna between fractions.

Importantly, our study also highlights the impact of soil communities on tree performance. *A. glutinosa* trees inoculated with soil communities from mature forests produced less aboveground biomass compared to those inoculated with communities from young forests. Our research revealed correlations between microbial taxa and tree performance variables, highlighting their potential roles in forest ecosystem functioning. Among the bacterial taxa, positive correlations were observed between *Gammaproteobacteria* (including orders *Burkhoderiales* and *Pseudomonadales*) and aboveground biomass production in both forest development stages. *Gammaproteobacteria* are known to play key roles in nutrient cycling and plant growth promotion through processes such as nitrogen fixation and phytohormone production and are shown to associate with alder trees (Bahulikar et al., 2014; Thiem et al., 2018). Similarly, *Firmicutes* (*Clostridia* and *Bacili*) which are known to be plant growth promoters, nutrient uptake enhancers and biocontrol agents for plant pathogens (Hashmi et al., 2020) showed positive correlations with belowground biomass production, suggesting their potential involvement in plant and root development and nutrient uptake. Conversely, negative correlations were observed between certain bacterial taxa and tree performance. For instance, *Acidimicrobiia, Rhizobiales* and *Acidothermus* negatively correlated with aboveground biomass production in

mature treatments. Although these symbiotic taxa are commonly associated with improvements in N-cycling, N-fixation and plant growth, it is possible that the biomass investment required from the plants to form this symbiosis hindered their biomass production (Zhang et al., 2019). Additionally, *Alphaproteobacteria* (*Elsterales*) showed both positive and negative correlations with aboveground biomass production, suggesting context-dependent effects on tree performance. In terms of fungal taxa, positive correlations were found between *Agaricomycetes* (including *Trechispora* and *Oliveonia*) and belowground biomass production in mature treatments. Conversely, negative correlations were observed between certain fungal taxa (e.g., *Thelesphoraceae*) and belowground biomass production in young treatments, indicating potential detrimental effects on tree performance. Thus, the presence of potential beneficial and harmful bacterial and fungal taxa in the different treatments may have contributed to the lack of apparent differences in tree performance between consecutive size fractions within each forest developmental stage. This could have been due to compensatory effects between bacteria and fungi (Orfanoudakis et al., 2010), as we also observed in an additional experiment (SI, Methods S1), where the use of bactericide or fungicide led to no differences in tree performance between the size fractions, following the patterns found when trees were grown without antibiotics as presented in this study.

The main *A. glutinosa* symbiont group, Frankiales was found to be relatively more abundant in the roots of trees inoculated with the largest fraction from the mature forest soil. Surprisingly, despite this higher relative abundance, trees inoculated with different size fractions obtained from mature forest soil produced less root nodules than those inoculated with size fractions obtained from young forest soil. Additionally, we observed a negative correlation of Frankiales with aboveground biomass production. Greater *Frankia* nodulation has been shown to positively affect plant biomass production, showcasing the pivotal role of *F. alni* for the growth of *A. glutinosa* (Ballhorn et al., 2017). It is possible that inoculating with the largest community size fraction from the mature forests resulted in higher competition with microbes for nutrients than the young forest treatments (Kuzyakov and Xu, 2013). On the other hand, it is also possible that as young tree plantations mature into forests, the changes in soil community composition (Kang et al., 2018) exert a negative influence on the symbiosis between *F. alni* and *A. glutinosa*, as seen by the decreased nodule density. This could be related to other microbes from the mature forest soils inhibiting this symbiosis as previous research has suggested that this can happen as early as 25 years after tree planting (Georgopoulos et al., 2024). Despite this, *A. glutinosa* still manages to form this symbiosis. Thus, the relevance of *Frankia* for *A. glutinosa* performance in the context of microbial communities of mature forests is likely not the same as in the early stages where *Frankia* may play a more important role in the growth of *A. glutinosa* as it is considered a pioneer species (Fremstad, 1983).

Despite non-significant effects of forest development stage or size fraction on CO_2 and N_2O fluxes, contrary to our hypothesis, CO_2 emissions in the pots inoculated with the largest community size fractions from both forest development stages were lower than those from the smaller community size fractions. This could be attributed to the fact that reducing community complexity can lower carbon sequestration (Wagg et al., 2014). The overall high amount of variation and lack of significant differences in GHG net fluxes reveals that despite the alterations in the community composition among the mesocosms inoculated with young and mature forest size fractions, the apparent microbial functioning remains unaltered suggesting a possible functional redundancy between those different communities (Yang et al., 2018). This could indicate that there is a microbial community that is very adaptable to the absence/presence of other microbial groups. The lack of apparent differences in CH_4 fluxes could be due to a possible inactivity of methanotrophs in our mesocosms. Since methanotrophs consist of a very specialized microbial community which is easily disturbed and has a low growth rate (Kumaresan et al., 2011), the disturbance caused by the wet sieving in this experimental setup could have led to methanotroph inactivity. This is further supported by in-situ forest studies which show higher CH_4 fluxes in undisturbed ecosystems (Covey and Megonigal, 2019). Although our results show credible correlations between microbes and tree growth, the link to microbial functioning, expressed as GHG fluxes could not be reliably detected. In our explorative approach, we attempted to relate individual soil animal, bacterial and fungal taxa with GHG fluxes. However, to identify the active part of the microbial community involved in these processes, RNA sequencing is preferred in order assess the effects of soil and root microbiomes on C and N cycling processes (Regan et al., 2011; Brenzinger et al., 2017). Nevertheless, our study suggests that several root-associated bacterial and fungal taxa may be linked to CO_2 and N_2O fluxes.

5. Conclusion

In conclusion, our study reveals that *A. glutinosa* strongly selects the root-associated community composition, and that the identity of those communities differs depending on the ecosystem developmental stage with consequences for tree performance. Filtering out larger organisms leads to an increase in biomass production presumably due to the absence of soil animals or reduced competition between plants and microbes for nutrients. The lower root nodulation in mature forest treatments, despite the higher relative abundance of Frankiales, suggests that soil biota in mature forests potentially inhibit the symbiosis of *A. glutinosa* with its prime beneficial symbionts. The lack of significant differences in GHG net fluxes indicates potential functional redundancy among microbial communities and possible methanotroph inactivity, underscoring the need for further investigation using RNA-based approaches to better understand microbial effects on GHG fluxes.

CRediT authorship contribution statement

Konstantinos Georgopoulos: Writing – review & editing, Writing – original draft, Visualization, Software, Methodology, Investigation, Formal analysis, Data curation, Conceptualization. **T Martijn Bezemer:** Writing – review & editing, Writing – original draft, Validation, Supervision, Resources, Project administration, Methodology, Investigation, Conceptualization. **Jesper Riis Christiansen:** Writing – review & editing, Validation, Supervision, Software, Resources, Methodology, Investigation, Data curation. **Klaus Steenberg Larsen:** Writing – review & editing, Validation, Software, Methodology, Investigation, Data curation. **Gina Moerman:** Writing – review & editing, Methodology, Data curation. **Roos Vermeulen:** Writing – review & editing, Methodology, Data curation. **Sten Anslan:** Writing – review & editing, Software, Investigation, Formal analysis, Data curation. **Leho Tedersoo:** Writing – review & editing, Software, Formal analysis, Data curation. **Sofia IF. Gomes:** Writing – review & editing, Writing – original draft, Supervision, Project administration, Methodology, Investigation, Data curation, Conceptualization.

Data availability

The data is deposited in Dryad under (DOI): https://doi.org/10.5061/dryad.dfn2z35bg.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.soilbio.2024.109643.

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