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# Afforestation induced shift in the microbial community explains enhanced decomposition of subsoil organic matter

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**Abstract**

Afforestation on former pastures is widely promoted due to its potential to increase soil organic carbon sequestration while reducing $CO_2$ emission. The establishment of a forest on a former pasture, however, might affect soil microbial community structure due to the alteration in substrate quality and thus impact carbon cycling in soils. To date, it still remains an open question if and how afforestation may alter the soil microbial community structure and related implications for soil organic matter stabilization. In addition, the majority of studies focuses on low altitude regions which results in uncertainties regarding the effects of afforestation on soil microbiology in mountainous regions. In this study, we aimed to investigate the consequences of afforestation of a subalpine pasture with Norway spruce (*Picea abies* L.) on the soil microbial community structure following 130 years of afforestation. We used a multi-proxy biomarker approach, including phospholipid fatty acids (PLFAs) and glycerol dialkyl glycerol tetraethers (GDGTs), to explore the shift in the microbial community structure following afforestation with increasing forest stand age. We found a significant increase in bacterial communities ($Gram^-$ and $Gram^+$ bacteria) with increasing forest stand age compared to the pasture. This trend, however, was reversed with increasing forest age when considering GDGT biomarkers. We thereby conclude that the microbial community in the pasture and forests of different forest stand ages utilize different carbon substrates as food resource, which is a direct consequence of the modification in litter input after the conversion of a pasture to forests. Our data further suggests that an increase in the soil organic matter decomposition results from the alteration in the microbial community structure, which is especially evident in the subsoil of the 130-year-old forest stand ages.

**Keywords**: Afforestation, subalpine ecosystem, Phospholipid fatty acids, Glycerol dialkyl glycerol tetraethers

## 1. Introduction

In the European Union, up to 40% of the land area is covered by forests (Pan et al., 2011). Afforestation of former pastures or grasslands has been particularly increasing over the recent years, driven by its potential to sequester $CO_2$ to mitigate climate change (Bethrong et al., 2009; Huang et al., 2011). In the European Alps especially, the abandonment of pastures that are no longer profitable for agriculture (Bolli et al. 2007) as well as the tree line upward shift contribute to forest expansion through the conversion of pastures to forests (Gellrich et al., 2007). Afforestation of former pastures does not only alter soil physical and chemical properties towards low soil pH values and high soil C:N ratios (Bethrong et al., 2009; Hiltbrunner et al., 2013). It also alters the litter composition and inputs (Gunina et al., 2017), with potential implications on the quality of soil organic matter and carbon

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sequestration in soils (De Deyn et al., 2008). This may also change the structure and diversity of soil microbial communities (Macdonald et al., 2009; Cavagnaro et al., 2016), which plays a crucial role in nutrient cycling, carbon sequestration and maintenance of soil fertility (Fierer, 2017). The litter inputs of coniferous tree species and the buildup of organic layers especially result in lower inputs of fresh organic matter from the organic horizons to the mineral ones, potentially leading to increased organic matter decomposition in deeper soil horizons (Speckert and Wiesenberg, 2023).

Woody plants are more abundant in forests than in pastures and are known to be chemically more recalcitrant compared to non woody plant species (Prescott, 2010; Moingt et al., 2016). The difference in the chemical composition of litter between pastures and forests suggests a difference in the functionality and composition of the soil microbial communities within these different land-uses (Crow et al., 2009; Gunina et al., 2017). Thus, fungal communities are often more abundant in coniferous forest soils than in pastures (Lauber et al., 2008) or deciduous forests (Frey et al., 2004). Most of the studies investigating the alteration of microbial communities in soils were restricted to either fungal communities (Kasel et al., 2008) or bacterial communities (Colloff et al., 2008). Phospholipid fatty acids (PLFAs) are key components of microbial cell membranes (Willers et al., 2015) and are classically used as biomarkers for bacterial and fungal communities in soils (Zhang et al., 2020). Additionally, the analysis of PLFAs has been frequently applied to explore the soil microbial composition (Willers et al., 2015) and microbial activity in soils (Pisani et al., 2016). This is possible, because of different performance of microbial groups in dependency of substrate quality (Wang et al., 2019). For example, fungi and $Gram^+$ bacteria are both able to decompose recalcitrant plant litter, while $Gram^-$ bacteria prefer carbon sources, which are generally more labile (Fanin et al., 2019). However, besides bacteria and fungi, archaea also play a major role in the soil microbial community, as their necromass might contribute to soil organic matter stabilization (Günther et al., 2014). The archaeal membrane is constituted of glycerol ether lipids rather than fatty acids. Therefore, the determination of the archaeal biomass requires the analysis of glycerol dialkyl glycerol tetraethers (GDGTs). Archaeal GDGTs are constituted of isoprenoid alkyl chains linked to glycerol by ether bonds (Schouten et al., 2013) and are referred as isoprenoid GDGTs (isoGDGTs). GDGTs can also be produced by some unknown bacteria, even though some of them belong to the phylum *Acidobacteria* (Sinninghe Damsté et al., 2014; Halamka et al., 2022). In contrast with archaeal GDGTs, bacterial GDGTs are constituted of branched alkyl chains instead of isoprenoid ones and are referred as branched GDGTs (brGDGTs), possibly feeding on root carbon sources (Huguet et al., 2012; Gocke et a., 2016). Both isoGDGTs and brGDGTs are ubiquitous in soils and sediments (Weijers et al., 2007; Huguet et al., 2013; Zhang et al., 2020). In a similar vein to the PLFA analysis, intact GDGTs still being attached to a polar headgroup are attributed to the living biomass, while core lipid GDGTs without any polar headgroup are attributed to the bacterial necromass (Gocke et al., 2017). Compared to the rapid turnover time (mean turnover time of 44 days in agricultural soils) of PLFAs (Gunina et al., 2017), GDGTs are characterized by a longer turnover time of a few decades (Weijers et al., 2010; Huguet et al., 2017), which provides a better time-integrated signal of soil microbial abundance than PLFAs.

To date, it is still underexplored to which extent afforestation of former pastures alters the soil microbial and fungal communities, especially in alpine and subalpine ecosystems. The majority of studies on fungal growth following afforestation are limited to low altitude regions (e.g., Högberg et al., 2007). In addition, little is known how time, e.g., forest age, impacts the alteration of the microbial and fungal communities following afforestation on former pastures. It is obvious that the adaptation of the microbial community to changes in land-use may take several

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years to become evident (Fierer, 2017) beyond the rapid changes occurring immediately after plantation due to the disturbance effect (Deng et al., 2016). Therefore, investigating afforestation sequences with a space-for-time approach can help to understand the long-term impact of afforestation on former pastures on the microbial communities and their connection to soil organic carbon dynamics. To the best to our knowledge, there is no other study yet using the combination of PLFA and GDGT molecular proxies to explore the shifts in microbial and fungal communities in the context of afforestation with coniferous tree species on a former pasture, especially in alpine and subalpine ecosystems.

In this study, we use a multi-proxy approach to explore the alteration in the soil microbial and fungal communities in a subalpine afforestation sequence (0 to 130 years) with Norway spruce (*Picea abies* L.) in Jaun, Switzerland. We further aimed to investigate if and to which extent the microbial community is altered after afforestation on a subalpine pasture and how this might affect soil organic matter stabilization and decomposition. Therefore, we combine PLFAs and core lipid GDGTs to investigate the alteration in the soil microbial community structure as a consequence of afforestation and in relation to increasing forest stand ages. Due to the already reported alteration in the litter quality towards more recalcitrant (high C:N ratio) litter input in this subalpine afforestation sequence (Hiltbrunner et al., 2013; Speckert et al., 2023), we hypothesize an increase in $Gram^+$ bacteria and in the fungal communities with increasing forest age in comparison to the pasture where less easily decomposable organic matter predominates. Moreover, we hypothesize a larger abundance in core lipid GDGTs in the pasture compared to the forest soils with a decline in their abundance with increasing forest stand ages due to the more easily decomposable organic matter (e.g., rhizodeposits) in soils under pasture than under forest use.

## 2. Materials and Methods

### 2.1. Study site, sampling and bulk elemental analysis

The study site is located near Jaun, a village in the Canton of Fribourg in Switzerland [7°15'54 E; 46°37'17 N]. It is an afforestation sequence with Norway spruce (*Picea abies* L.) with stand ages between 40 and 130 years on a former pasture on a south-exposed slope between 1450 and 1600m above sea level. More detailed information about the study site can be found in Hiltbrunner et al. (2013) and Speckert et al. (2023). The sampling campaign was conducted in July 2020. Five soil pits ($n$ = 5) in the pasture area (0-year-old forest) as well as three soil pits ($n$ = 3) for each forest stand age (40-, 55-, and 130-year-old forests) were prepared with dimensions of at least 100 cm length x 50 cm width on slope-parallel levels (more details; see Speckert et al., 2023). The mineral soil samples were taken with two volumetric steel cylinders (100 $cm^3$) on these slope-parallel levels that were incrementally increased by 5 cm to a maximum depth of 45 cm. Soil organic carbon and total nitrogen concentrations (Table S1) were determined using a Thermo Fisher Scientific Flash HT Elemental Analyzer, coupled to a Delta V Plus isotope ratio mass spectrometer via ConFlo IV (Thermo Fisher Scientific Bremen, Germany). Soil pH (Table S1) was measured using an ion sensitive electrode in calcium chloride solution ($CaCl_2$; 0.01M; soil to solution ratio 1:3).

### 2.2. Extraction and analysis of glycerol dialkyl glycerol tetraethers

Dried soil samples were extracted using Soxhlet extraction with a mixed solution of dichloromethane:methanol (DCM:MeOH; 93:7, v/v) and evaporated until constant weight (Wiesenberg and Gocke, 2017). Afterwards, the samples were separated by solid phase extraction with activated aluminium oxide ($Al_2O_3$) into two fractions using heptane:DCM (9:1, v/v) and DCM:MeOH (1:1, v/v), respectively. The latter was evaporated and re-dissolved in heptane and centrifuged for 1min and 7000 revolutions per minute. The supernatant was collected. The analysis

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and quantification of the GDGTs followed the guidelines of Huguet et al. (2019). Briefly, the GDGTs were separated using high-performance liquid chromatography coupled with mass spectrometry with an atmospheric pressure chemical ionization source (HPLC-APCI-MS; Shimadzu LCMS-2020 Corporation). The separation was achieved with two Hypersil Gold silica columns 150mm x 2.1mm, 1.9µm: Thermo Finnigan USA) by a controlled temperature of 40°C. The semi-quantification was performed by comparing the integrated signal of the respective compound with the signal of a $C_{46}$ synthesized internal standard (Huguet et al., 2006) assuming their response factors to be identical. The individual GDGT compounds were assigned according to De Jonge et al. (2014).

### 2.3. Extraction and analysis of phospholipid fatty acids

The PLFA analysis was performed following the guidelines by Zosso and Wiesenberg (2021) after Bligh and Dyer (1959) with some minor modifications. Based on the carbon content of the respective sample, we used 3g of the topsoil (0-10cm) and 5g of the deeper soil horizons (10- 45cm) of freeze-dried soil material. For the extraction, a solution of 1:2:0.8 (v/v/v) of trichloromethane ($CHCl_3$):MeOH:citric buffer was used, with the extraction being repeated four times and the extract being collected in a separation funnel. After adding $CHCl_3$ and citric buffer, the organic phase separated over night and was collected thereafter. The addition of $CHCl_3$ was repeated three more times and the organic phase was collected and combined. Afterwards, the extract was separated into neutral-, glycol-, and phospholipids using a solid phase extraction with activated silica gel (Zosso and Wiesenberg, 2021). The methylation of phospholipid fatty acids was performed according to Wiesenberg and Gocke (2017) with deuterated eicosanoic acid ($D_{39}C_{20}$) as internal standard for quantification to nmol per g soil material. Compound identification was done on a gas chromatograph (GC; 6890 Agilent Technologies, Inc.) coupled to a mass selective detector (MSD; 5973N Agilent Technologies, Inc.) equipped with a split/splitless injector. Compound quantification was performed using a GC (7890B Agilent Technologies, Inc.) equipped with a multimode inlet (MMI) and a flame ionization detector. The identification of the individual compounds was done by comparison of mass spectra with external standards as well as with the NIST mass spectra library. Both GC instruments were equipped with a J&W DB-5MS narrow-bore capillary column (50m x 0.2mm; 0.33µm film thickness) and a deactivated precolumn (1.5m) with helium as the carrier gas. The GC oven temperature started at 50°C (held for 4min) increased to 150°C with a rate of 10°C $min^{-1}$ and incrementally increased (2°C $min^{-1}$ to 160°C, 0.5°C $min^{-1}$ to 170°C, 0.2 °C $min^{-1}$ from 170 °C to 190°C and 2°C $min^{-1}$ from 190°C to 210°C) to 320°C (held for 15 min) (Wiesenberg and Gocke et al., 2017). The individual PLFA compounds were assigned according to Willers et al. (2015) into microbial community structure Gram$^+$ bacteria ($iC_{14:0}$, $aC_{14:0}$, $iC_{15:0}$, $iC_{16:0}$, $aC_{16:0}$, $iC_{17:0}$, and $aC_{17:0}$), Gram$^-$ bacteria ($C_{16\omega9c}$, $C_{16:1\ \omega7c}$, $C_{18:1\omega11c}$, $cyC_{17:0}$, and $cyC_{19:0}$), actinobacteria ($10MeC_{16:0}$ and $10MeC_{18:0}$), saprotrophic fungi ($C_{18:2\omega6c}$), and arbuscular mycorrhizal fungi (AMF: $C_{16:1\omega5c}$).

## 3. Data analysis

Data analysis was performed using the R Software v.4.3.2. (R core Team, 2020). To test whether there is a significant difference in the abundance of the individual molecular compounds (PLFAs and GDGTs) between pasture and forest areas with increasing forest age as well as with increasing soil depth, a two-way analysis of variance (ANOVA, $p < 0.05$) followed by a post-hoc Tukey HSD test ($p$ adj $< 0.95$) was applied. The PLFA analysis was performed in duplicates ($n = 2$) and the GDGT analysis was performed with one replicate ($n = 1$) for each stand age (0 to 130 years). Correlations were tested by Pearson correlation ($p < 0.95$). A Principal Component Analysis (PCA) was performed using the *FactoMineR* package to determine correlations between individual

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compounds (PLFAs and GDGTs) and chemical soil properties (soil pH, soil organic carbon, and nitrogen concentration) and fine root biomass. Additionally, the analysis of the PCA was further used to identify if there was a difference in correlations in relation to different forest stand ages (0 to 130 years ). The PCA of the PLFAs comprised 153 data points for the pasture, 144 data points for the 40-year-old forest, 153 data points for the 55-year-old forest, and 162 data points for the 130-year-old forest. The PCA of the GDGTs comprised 189 data points for the pasture and all forest stand ages. All variables were standardized before PCA analysis.

### 3.1. Calculations of the phospholipid fatty acid indices

The Fungi:Bacteria (F:B) ratio is known to be a good indicator to assess environmental changes in the soil as bacteria react less sensitively to environmental changes than the fungal community (Zhong et al., 2020). The F:B ratio was calculated by PLFA-derived bacterial biomass (Gram$^+$, Gram$^-$ bacteria and actinobacteria) divided by PLFA-derived fungal biomass (saprotrophic fungi and AMF).

### 3.2. Calculation of the glycerol dialkyl glycerol tetraether indices

The CBT (Cyclisation ratio of Branched Tetraethers) captures the degree of cyclisation of brGDGTs and is correlated with soil pH (Weijers et al., 2007). The CBT was further modified by De Jonge et al. (2014) after improvement of the GDGT analysis allowing the separation of 5 methyl- and 6-methyl brGDGT isomers. The $CBT_{5Me}$ is defined as follows (Eq. 1):

$$CBT_{5Me} = {}^{-10}\log\left(\frac{Ib+IIb}{Ia+IIa}\right) \quad (1)$$

The relative abundance of 6-methyl over 5-methyl brGDGTs was calculated according to De Jonge et al.(2014; Eq. 2), which compares the relative abundance of 6- and 5-methyl homologues among brGDGT-II and brGDGT-III groups.

$$IR_{6Me} = \left(\frac{IIa' + IIIa'}{IIa + IIIa + IIa' + IIIa'}\right) \quad (2)$$

The MBT (Methylation index of Branched Tetraethers) was shown to correlate with mean annual air temperature (Weijers et al., 2007). The MBT was further modified by De Jonge et al. (2014) and defined as follows (Eq. 3):

$$MBT'_{5Me} = \frac{Ia + Ib + Ic}{Ia + Ib + Ic + IIa + IIb + IIc + IIIa} \quad (3)$$

The roman numbers correspond the structures defined in De Jonge et al. (2014).

## 4. Results

### 4.1. *Phospholipid fatty acid composition*

A significant increase ($p < 0.01$) in the concentrations of Gram$^+$ bacteria was observed from pasture to forest soils with increasing forest age (Fig. 01a). Peak values were observed in the topsoil (0 – 5 cm) of the 130-year-old forest (150.9 ± 20.4 nmol g$^{-1}$) followed by the 40-year-old forest, 55-year-old forest, and by the pasture, respectively (Table S2). A similar trend was observed for the concentrations of Gram$^-$ bacteria and actinobacteria with the highest abundance in the upper soil depths and always in the 130-year-old forest (Fig. 01b and c). Gram$^-$ bacteria were more abundant than Gram$^+$ bacteria in both, pasture and forest stand ages with decreasing values with increasing soil depth (Table S2). The largest concentration ($p < 0.001$) of AMF was observed in the 130-year-old

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forest in the upper 10 cm (0-5 cm: 25.9 ± 5.2 nmol $g^{-1}$ ; 5-10 cm: 26.3 ± 12.4 nmol $g^{-1}$), while the lowest concentration of AMF was observed in the pasture (0 – 5 cm: 3.6 ± 1.5 nmol $g^{-1}$) in comparison to the forest stand ages (Fig. 1d;Table S2). The concentration of saprotrophic fungi was highest ($p$ = 0.18) in the topsoil (0 – 5 cm) in the 40-year-old forest, followed by the 130-year-old forest, pasture, and finally by the 55-year-old forest (Fig. 01e). In comparison to the pasture, the F:B ratio significantly decreased ($p$ = 0.01) after 40 years of afforestation but increased afterwards with increasing forest stand age (Fig. 02; Table S3). The F:B ratio also showed a positive correlation with the C:N ratio in the 55-year-old ($r$ = 0.18) and in the 130-year-old forest ($r$ = 0.39), which was neither observed in the pasture nor in the 40-year-old forest (Fig. S1).

### 4.2. Concentrations of phospholipid fatty acids and soil environmental factors

The organic carbon and nitrogen concentrations were positively correlated with $Gram^+$ bacteria, $Gram^-$ bacteria and AMF in the pasture (explaining 44.8% of the variance; Fig. 03a; Fig. S2 and S3) as well as in the 55-year-old forest (explaining 72.8% of the variance, Fig. 03c). Additionally, in the 55-year-old forest the fine root biomass strongly correlated ($r$ = 0.75;Fig. S4) with the abundance of AMF (Fig. 03c). With increasing forest stand ages, the correlation between organic carbon as well as nitrogen concentration and individual PLFAs became stronger (Fig. 03b to c; Fig. S2 and S3). Highest correlations were observed between $Gram^+$ bacteria and the nitrogen concentration in the 55-year-old ($r$ = 0.79), between $Gram^+$ bacteria and the organic carbon concentration in the 130-year-old forest ($r$ = 0.76) and between AMF and the organic carbon as well as the nitrogen concentration in the older forest stand ages (55-year-old and 130-year-old forest; Fig. S2 and S3). In contrast to the older forest stand ages, there was a weak positive correlation between the organic carbon and the nitrogen concentration and all individual PLFAs in the pasture (Fig. 03a) and in the 40-year-old forest (Fig. 03b). The fine root biomass correlated strongly with all individual PLFA compounds with the exception of the actinobacteria in the 55-year-old forest (Fig. S4a to e), which was not observed in the pasture, the 40-year-old or 130-year-old forest. Common to all investigated areas is the negative correlation between soil pH and all individual PLFAs, most prominent with $Gram^+$ ($r$ = -0.74) and $Gram^-$ bacteria ($r$ = -0.67; Fig. S5a and b, respectively) and in the 40-year-old forest (explaining 57.5% of the variance, Fig. 03b). Additionally, the pasture was the only site with a positive correlation ($r$ = 0.13; Fig. S5c) between fungi and soil pH (Fig. 03a).

### 4.3. Glycerol dialkyl glycerol tetraether concentration and distribution

The largest concentrations of brGDGTs were observed in the topsoil (0 – 5 cm, 5 – 10 cm) in the pasture (4040.7 ng $g^{-1}$) and forest stand ages ($forest_{40}$ = 3980.8 ng $g^{-1}$; $forest_{55}$ = 3775.9 ng $g^{-1}$; $forest_{130}$ = 2991.2 ng $g^{-1}$) with declining concentrations ($p$ < 0.001) with increasing soil depth, except in the 130-year-old forest (Figure 04a; Table S4). There were two maxima in the brGDGT concentrations in deeper soil horizons between 5 – 10 cm and 15 – 20 cm (Fig. 04a), especially for the predominant brGDGTs Ia (Fig. 04b) and IIa (Fig. 04c). The brGDGTs Ia, IIb, and Ib represented altogether approximately 50% of all brGDGTs in all soils. In the 130-year-old forest, the brGDGTs Ia, IIb, and Ib altogether account for 60 – 70% of all brGDGTs (Table S4). The concentration of the 6-methyl brGDGTs (IIa', IIIa', IIb', IIIb', IIc', IIIc') was generally lower compared to their 5-methyl counterparts (IIa, IIIa, IIb, IIIb, IIc, IIIc; Table S3). Both, 5-methyl and 6-methyl brGDGTs declined in their concentration with increasing soil depth in the 55-year-old and 130-year-old forest (Table S4). In the 40-year-old forest, the concentration of 6-methyl brGDGTs (IIb', IIIb') increased, particularly in the deeper soil horizons (35 – 40 cm) compared to the 5-methyl counterparts. The opposite was observed for the pasture with a decline in the concentration of the 6-methyl brGDGTs while the 5-methyl brGDGT concentration increased, especially for the

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individual brGDGTs IIIc', IIIc, respectively (Table S4). Generally, the pasture soil was characterized by a higher proportion of brGDGT with cyclopentane moieties compared to forest areas. The pasture and the 40-year-old forest were characterized by significant higher ($p < 0.001$; Table S5) $CBT_{5Me}$ values compared to the 55-year-old and 130-year-old forests (Fig. 05a). The same trend was observed for the $IR_{6Me}$ ratio with a significant lower ($p <$ 0.001) ratio in the older forest stands (55-year-old and 130-year-old forests) compared to the pasture and the 40-year-old forest (Fig. 05b). The $MBT_{5Me}$ ratio was significantly ($p < 0.001$) lower in the 130-year-old forest in comparison to the younger forest stand ages as well as to the pasture (Fig. 05c; Table S5).The isoGDGT concentrations were significantly higher ($p < 0.001$) in the pasture soil compared to forest soils, with highest concentrations in the topsoil (pasture = 131.2 ng $g^{-1}$; $forest_{40}$ = 22.9 ng $g^{-1}$ ; $forest_{55}$ = 9.2 ng $g^{-1}$; $forest_{130}$ = 2.2 ng $g^{-1}$; Fig. 06a). Among the isoGDGTs, isoGDGT-0 and crenarchaeol were the most dominant (Table S6) with a significant higher concentration ($p < 0.001$) in the pasture compared to all forest stand ages (Fig. 06b and c). The concentration of the individual isoGDGT-0 ranged between 94.2 and 11.9 ng $g^{-1}$ in the pasture and between 4.3 and 0.09 ng $g^{-1}$ in the 55-year-old forest ($forest_{40}$ = 11.05 to 0.13 ng $g^{-1}$; $forest_{130}$ = 5.98 to 0.61 ng $g^{-1}$). The concentration of crenarchaeol ranged between 32.1 ng $g^{-1}$ and 2.1 ng $g^{-1}$ in the pasture (Fig. 06b). In forested areas, the concentration of crenarchaeol ranged between 4.2 and 0.02 ng $g^{-1}$ in the 55-year-old forest ($forest_{40}$ = 5.65 to 3.89 ng $g^{-1}$ ; $forest_{130}$ = 1.45 to 0.17 ng $g^{-1}$ ; Fig. 06b; Table S6).

### 4.4. Glycerol dialkyl glycerol tetraether composition and soil environmental factors

In the pasture, the brGDGTs IIc, IIIc, and IIIc' showed no clear correlation with the organic carbon or nitrogen concentrations (Fig. 07a). The identified main brGDGTs Ia, IIa, and Ib, however, strongly correlated with the organic carbon and nitrogen concentrations in the pasture and in the forest stands, with the exception of the 55-year-old forest (Fig. S6 and S7). Crenarchaeol positively correlated with the organic carbon concentration in the pasture ($r$ = 0.96) and in the 40-year-old forest ($r$ = 0.82), but negatively ($r$ = -0.68) in the 130-year-old forest ( Fig. S6). The GDGT-0 showed a strong positive correlation with the organic carbon and nitrogen concentrations, particularly in the pasture area (Fig. 07a), which was observed in a weaker correlation in the forest stand ages ( Fig. S6 and S7). Fine root biomass showed a strong correlation with brGDGTs in the pasture and in the 55-year-old forest (Fig. 07a and c; Figure S8). Opposing trends were observed in the 40-year-old and 130-year-old forests with a negative correlation between fine root biomass and brGDGTs, particularly with 6-methyl brGDGTs (IIb', IIIb', IIc', IIIc'; Fig. 07b to d). Common to both, the pasture and the forest stand ages, was the negative correlation between br- and isoGDGTs and soil pH ( Fig. S9), with two exceptions: The brGDGT-IIIc in the pasture (Fig. 07a) and the crenarchaeol in the 130-year-old forest (Fig. 07d).

## 5. Discussion

### 5.1. Pasture soil microbiology

The decline in the abundance of bacterial and fungal PLFA biomarkers with increasing soil depth (Fig. 01a to e) is consistent with other studies reporting a decrease, particularly in bacterial biomarkers with increasing soil depth as a consequence of a lower organic carbon content in deeper soil horizons (Soong and Nielsen, 2016; Fierer, 2017). This dependence on either soil organic carbon and/or nitrogen is also reflected in the strong correlation of particular $Gram^+$ and $Gram^-$ bacteria with the organic carbon and nitrogen concentration. The observed higher proportion of $Gram^-$ bacteria relatively to $Gram^+$ bacteria in this pasture area is in line with results reported by Hiltbrunner et al. (2012), who reported a higher concentration of PLFA-derived $Gram^-$ bacteria (157 nmol $g^{-1}$ dry

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soil) over Gram$^+$ bacteria (124.0 nmol g$^{-1}$ dry soil) in the same subalpine pasture. However, our results were opposite to the results reported by Francisco et. al (2016), who observed a high proportion of Gram$^+$ bacteria relatively to the concentration of Gram$^-$ bacteria in alpine grasslands across Europe. Other recent studies reported that Gram$^-$ bacteria are common in environments rich in organic matter (Cui et al., 2019). The investigated pasture has relatively high soil organic carbon stocks of 13.4 ± 1.2 kg m$^{-2}$ (Hiltbrunner et al., 2013) and of 11.5 ± 0.5 kg m$^{-2}$ (Speckert et al., 2023) compared to other studies on alpine grasslands in Switzerland (5.5 to 10.2 kg m$^{-2}$ in Budge et al. (2011); 4 to 8 kg m$^{-2}$ Zeeman et al. (2010)). This might partially explain the observed higher proportion of Gram$^-$ over Gram$^+$ bacteria. Another explanation might be the labile litter produced by grass species, as Gram$^-$ bacteria rely on more labile plant-derived carbon as food resources compared to Gram$^+$ bacteria (Fanin et al., 2019; Cheng et al., 2024). Additionally, the root exudates such as sugars and organic acids represent the main energy source for microorganisms (Gunina and Kuzyakov, 2015) and are easily degradable, which could be another explanation for the observed higher proportion of Gram$^-$ than Gram$^+$ bacteria. This is further supported by the positive correlation ($r$ = 0.34) between Gram$^-$ bacteria and fine root biomass in this pasture, which was weaker for the Gram$^+$ bacteria ($r$ = 0.27). Surprisingly, we found a lower concentration of AMF than saprotrophic fungi in the investigated pasture, although AMF are often abundant in soil under pasture as they are associated with grass roots (Terrer et al., 2021). This might suggest a possible competition between AMF and saprotrophic fungi or Gram$^-$ bacteria for root carbon as food resource. Also, the sampling period during the dry summer months might be a possible reason, as a reduced amount of root exudates (Moore-Kucera and Dick, 2008) and a low soil water availability are limiting factors for the microbial activity (Hiltbrunner et al., 2013). Another explanation could also be the cattle grazing (during summer period), which can negatively affect the growth of mycorrhizal fungi (Olsson et al., 2010).

The strong correlation between fine root biomass and brGDGTs in the investigated pasture (Fig. 03a) is consistent with other studies (e.g., Ayari et al., 2013; Huguet et al., 2013), who reported a strong association of bacterial derived brGDGTs with roots. The cyclic brGDGT Ib was identified as one of the main brGDGTs with a significant higher concentration in the pasture than in forest stands (Fig. 04d) and also strongly correlated (r = 0.84) with fine root biomass. Such a high concentration of brGDGTs of one or more cyclopentane rings (e.g., Ib, IIb', Ic, IIc') in pasture soils is in good agreements with the results obtained by Naeher et al. (2014), who described a higher abundance in brGDGTs with one or two cyclopentane moieties in alpine grasslands compared to soils under deciduous trees and shrubs. Additionally, there was a negative correlation between both, cyclic and acyclic brGDGTs and soil pH (Fig. 07a), which is in line with the findings reported by Véquaud et al. (2021) with a strong correlation between cyclic brGDGTs and soil pH in grasslands in the French Alps. The shared increase in isoGDGT-0 and crenarchaeol in the pasture can be explained by the fact that both compounds are produced by Nitrososphaera, known as ammonia oxidizer (Nicol et al., 2008). Thereby, the additional ammonia input by grazing cattle excretion (Mutschlechner et al., 2018) might favour the growth of this group of bacteria.

### 5.2. Forest soil microbiology and its alteration with increasing forest stand age

From the 40-year-old to the 130-year-old forest stand age, we observed a significant increase in the quantity of the bacterial and fungal communities while a decrease was observed in the 55-year-old forest (Fig. 01a to d). Generally, with increasing forest stand age, the substrate quality (e.g., difference in vegetation cover, soil pH, organic carbon concentration, C:N ratio etc.) changed resulting in an alteration of the soil microbial and fungal communities (Macdonald et al., 2009; Pollierer et al., 2015; Gunina et al., 2017; He et al., 2017; Xu et al., 2021). However,

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neither soil pH (Table S1) nor climate (e.g., soil temperature; Hiltbrunner et al., 2013) significantly differed between the different forest stand ages. One possible explanation for the observed lower soil bacterial and fungal community in the 55-year-old forest could be the significant lower soil organic carbon and total nitrogen stock in comparison to the 40-year-old and 130-year-old forest stand ages (Speckert et al., 2023). This might suggest a low C:N ratio as well as a lower carbon and nitrogen availability, which might explain the constantly lower bacterial and fungal abundance in the 55-year-old forest compared to the 40-year-old and 130-year-old forest. The key role of organic carbon and particularly nitrogen for soil bacterial abundance was observed in numerous studies (Soong and Nielsen, 2016; Fierer, 2017) and was further supported by the positive correlation between organic carbon and nitrogen concentrations in older forest stand ages in this study (Fig. S2 and S3). In contrast to the 55-year-old forest, the 40-year-old and the 130-year-old forests had equal soil organic carbon and total nitrogen stocks (Speckert et al., 2023), although they differ significantly in the proportion of $Gram^+$ $Gram^-$ bacteria, actinobacteria, as well as AMF (Fig. 01a to d). Thus, another reason for the observed alteration of the proportion of soil microorganisms with increasing forest stand ages might be the alteration in litter quality resulting from differences in the vegetation cover. While the 40-year-old forest was characterized by spruce needle- and also moss- and grass-derived organic matter input, the 130-year-old forest was dominated mainly by spruce needle-derived organic matter input (Speckert and Wiesenberg, 2023). This alteration in litter quality towards more recalcitrant organic matter input with a higher C:N ratio might explain the observed pattern of a larger abundance of $Gram^+$ bacteria and actinobacteria, which are both able to decompose recalcitrant organic material (Dang et al., 2017; Fanin et al., 2019). Specifically, $Gram^+$ bacteria prefer feeding on old organic matter (Kramer and Gleixner, 2008). This could be indicative for an increased decomposition of old organic matter in the 130-year-old forest, which is consistent to previous studies on the same study site which observed carbon loss in the subsoil of this afforestation sequence with increasing stand age (Hiltbrunner et al., 2013; Speckert and Wiesenberg, 2023). Further, the high proportion of needle-litter input and the higher C:N ratio in the 130-year-old over the 40-year-old forest stand ages might also be responsible for the increased AFM and saprotrophic fungi concentration due to several lignin-decomposing fungi (Macdonald et al., 2009), which are rather expected in the 130-year-old forest with spruce needles as the dominant source of soil organic matter (Speckert and Wiesenberg, 2023). This expected increase in fungal communities with increasing forest stand ages is also supported by the increasing F:B ratio with forest age in this afforestation sequence (Fig. 02).

Soil pH is assumed to be one of the controlling factors for the abundance of brGDGTs with higher concentrations with lower soil pH (Weijers et al., 2007). This is supported by the observed negative correlation in this study with the abundance of individual brGDGTs and soil pH in all investigated areas (Fig. 04a to d). This is consistent with results obtained globally (De Jonge et al., 2014). However, the largest difference in the abundance of brGDGTs (Ia, IIa, Ib) was observed in the topsoil (0 – 5 cm) between the different forest stand ages, with lowest concentrations in the 130-year-old forest (Fig. 07a to d). There was, nevertheless, no significant difference in the soil pH values between the different forest stand ages, which thereby contradicts that soil pH is the only controlling factor for the abundance of brGDGTs in this afforestation sequence. A possible explanation might be the preference of brGDGTs to feed on root carbon (Ayari et al., 2013; Huguet et al., 2013). The 55-year-old forest had the highest fine root biomass in the topsoil (0 – 5 cm) among the investigated forest stands with a rapid decline with increasing soil depth (Speckert et al., 2023). The same pattern was observed in the concentrations of individual brGDGTs in the 55-year-old forest (Fig. 04a to d). Among the different forest stands, also the litter quality changed with the potential to alter the soil microbial community. These shifts in the soil microbial community can be reflected in

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the $CBT_{5Me}$ ratio (Eq. 1; Naafs et al., 2021; Halamka et al., 2022). The $CBT_{5Me}$ ratio was significantly less negative in the 40-year-old forest compared to the 55-year-old and 130-year-old forest (Fig. 05a). This thereby suggests a rather similar soil microbial community between the old forest stand ages, and a different soil microbial community compared to the 40-year-old forest. Also, the $IR_{6Me}$ ratio (Eq. 2) was in the same range value range for the pasture and the 40-year-old forest and significantly higher compared to the 55-year-old and 130-year-old forest. These shifts between young and older forest stand ages might be related to the alteration in the litter quality towards more recalcitrant litter input with increasing forest stand age in this afforestation sequence.

### 5.3. Shift in the soil microbial community structure following afforestation on a subalpine pasture assessed by the combination of PLFA and GDGT markers

Land-use change, such as afforestation of a former pasture, can have a substantial impact on the structure and composition of soil microorganisms due to alterations in quality and quantity of above- and belowground plant-derived organic matter input (De Deyn et al., 2008). In this afforestation sequence, the litter quality decreased with increasing forest age towards more needle-derived organic matter with a high C:N ratio in comparison to the pasture (Hiltbrunner et al., 2013; Speckert et al., 2023). The alteration from labile grass-derived organic matter (low C:N) towards needle-derived organic matter (high C:N) with increasing forest stand age on the one hand caused the increase in $Gram^+$ bacteria and actinobacteria from the pasture to the 130-year-old forest and on the other hand, resulted in an increased F:B ratio with increasing forest stand age (Fig. 05). The observed increased F:B ratio parallels not only the increased C:N ratio with increasing forest age, but it might also be an indicator for a lower carbon availability with increasing forest age. Fungal species, particularly mycorrhizal fungi, have a better carbon use efficiency than bacteria and have the ability of mining carbon from soil organic matter and thereby also prime organic matter decomposition (Clemmensen et al., 2014; Friggens et al., 2020). To conclude, the alteration in the organic matter input and the associated decrease in nutrient availability with increasing forest age might be key factors responsible for the observed alteration in the soil microbial community structure and composition captured by the PLFA composition.

A shift in the composition of the soil microbial community as a result of the alteration in the vegetation cover was also captured by the br- and isoGDGT composition, but mainly due to the alteration in the root biomass following afforestation. Given the fact that previous studies already showed that root carbon is an important food resource for brGDGT producing bacteria (Huguet et al., 2013), the strongly rooted topsoil (0 – 5 cm; 5 – 10 cm) showed the largest concentration in brGDGTs, particularly brGDGT-Ib in the pasture area. Interestingly, while the brGDGT concentration decreased with increasing soil depth in the pasture area, the brGDGT concentration in forested areas showed several additional maxima in deeper soil depths (Fig. 04a to d). This might be due to the needle-litter organic matter becoming less prominent with increasing soil depth (Speckert and Wiesenberg, 2023) and root carbon as a potential food resource is taking over. Another possibility for the overall higher brGDGT concentration in the topsoil of the pasture as well as in the 40-year-old and 55-year-old forests might be the difference in the microenvironment. A previous study by Häggi et al. (2023) found significantly higher $MBT'_{5Me}$ values in grasslands compared to forest soils as a lack of shading in the grassland area which results in higher surface soil temperatures. Additionally, Hiltbrunner et al. (2013) reported a temperature difference of an average 5°C in the upper soil horizons (0 – 5 cm) between the pasture and the different forest stand ages in the investigated afforestation sequence. Therefore, the difference in the soil surface temperature between pasture and forested areas could be another possible explanation for the observed difference in the concentrations of individual brGDGTs,

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which is supported by the higher $MBT'_{5Me}$ values in the pasture compared to the 130-year-old forest (Fig. 05c). However, the 40-year-old and 55-year-old forests showed higher concentrations of Ia and IIa, particularly in the topsoil, compared to the pasture, which is also reflected in a higher $MBT'_{5Me}$ ratio in those forests compared to the pasture. This argues for a warmer soil surface temperature, especially in the 55-year-old forest compared to the pasture. This could also be due to the lack of shading, as the 55-year-old forest was characterized by a lower canopy density than the 130-year-old forest (Speckert et al., 2023). The significant lower $CBT_{5Me}$ ratio in the old forest stand ages (55-year-old and 130-year-old) compared to the pasture and the 40-year-old forest reveals a different bacterial community towards bacteria who preferentially synthesize brGDGT with more cyclopentane moieties. Additionally, the $IR_{6ME}$ ratio was significantly higher in the pasture and in the 40-year-old forest compared to the older forest stand ages (Fig. 05b) indicating a higher relative abundance of 6-methyl over 5-methyl brGDGTs (De Jonge et al., 2024). The significant higher concentration of isoGDGT-0 in the pasture compared to the forest stand ages is in good agreement with other studies reporting a dominance of isoGDGT-0 in various grasslands (e.g., Häggi et al., 2023).

Overall, the analysis of two different substance classes (PLFAs and GDGTs) to identify potential shifts in the microbial community structure and composition following afforestation on a subalpine pasture has been proven useful to uncover the different microbial community between two different land-use types (pasture vs. forests). Thereby, the microbial community in the pasture is characterized by bacteria and archaea who preferentially feed on root carbon while the all forest stand ages are characterized by fungi and bacteria that are able to feed on recalcitrant plant-litter as the predominant carbon source. In addition, the PLFAs provide a rather short-term view of the soil microbial communities while the GDGTs showed a longer time-integrated signal. Particularly the GDGT indices have been proven useful to detect shifts in the microbial community over time with similar communities in the pasture and the 40-year-old forest. After 40 years of afforestation, the microbial community changes most likely as an adaption to the alteration in organic matter input. Thus, the change in the soil microbial community following afforestation on a subalpine pasture is a direct consequence of the alteration in organic matter input – root exudates *vs.* needle-dominated litter – and chemical composition – labile C *vs.* more complex C compounds.

## 6. Conclusion

The current study investigated the effect of a long-term afforestation of Norway spruce on a sub-alpine pasture on the soil microbial community structure. The concentrations of $Gram^+$ bacteria and the arbuscular mycorrhizal fungi increased with increasing forest age compared to the pasture. An opposite trend was observed for brGDGT and isoGDGT concentrations. These changes in molecular biomarker concentrations with afforestation reflect a shift in the microbial community structure. This shift in the microbial community is directly related to the alteration in the quality of litter input (high C:N ratio) following afforestation. Improved understanding of shifts in soil microbial communities following afforestation is essential to better understand whether afforestation might contribute to soil organic carbon sequestration or not on a decadal to centennial time scale. The observed shift in the soil microbial community following afforestation suggests that soil organic matter might react sensitively to environmental changes. In addition, the trend towards a bacterial and fungal community several decades after afforestation argues for an adaptation of the microbial community to decompose rather old and complex organic matter in the coniferous forest. As a consequence, also preserved complex soil organic matter can be decomposed by the altered microbial community in the long-term. The shift in the microbial community structure is therefore a key to better understand long-term consequences of afforestation on soil organic matter stabilization on a decadal

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to centennial timescale. However, as the high pH value and high soil organic carbon stocks as well as the subalpine climate argue for a comparatively slow response of the soil microenvironment in the chosen afforestation chronosequence, longer afforestation sequences as well as further in-depth assessments of molecular changes, e.g., monitoring throughout growing seasons, might provide further insights into afforestation influencing soil microbial community structures in sensitive alpine and subalpine settings.

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**Competing interests**

The contact author has declared that none of the authors has any competing interests.

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## Figures

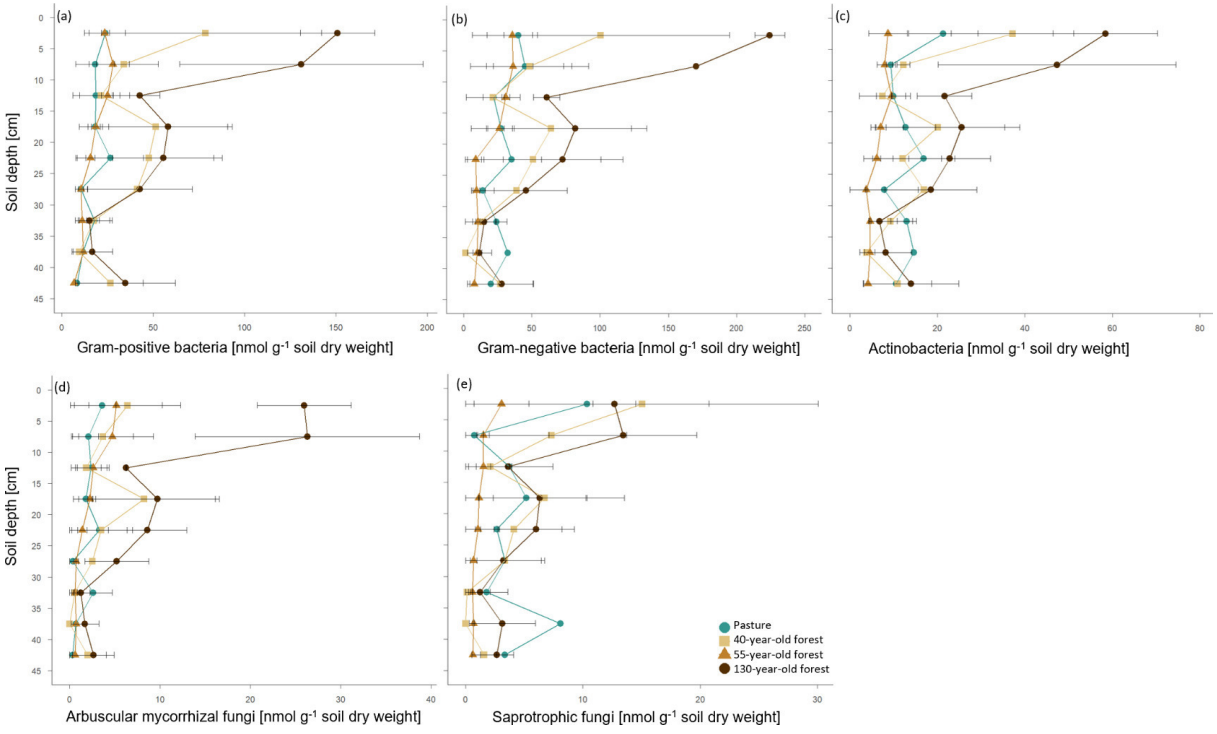


*Figure 01.* Concentrations of individual phospholipid fatty acids: (a) Gram$^+$ bacteria, (b) Gram$^-$ bacteria, (c) actinobacteria, (d) arbuscular mycorrhizal fungi, and (e) saprotrophic fungi of the pasture and different forest ages in relation to soil depth (0 – 45 cm). Error bars indicate standard deviation of 2 replicates.

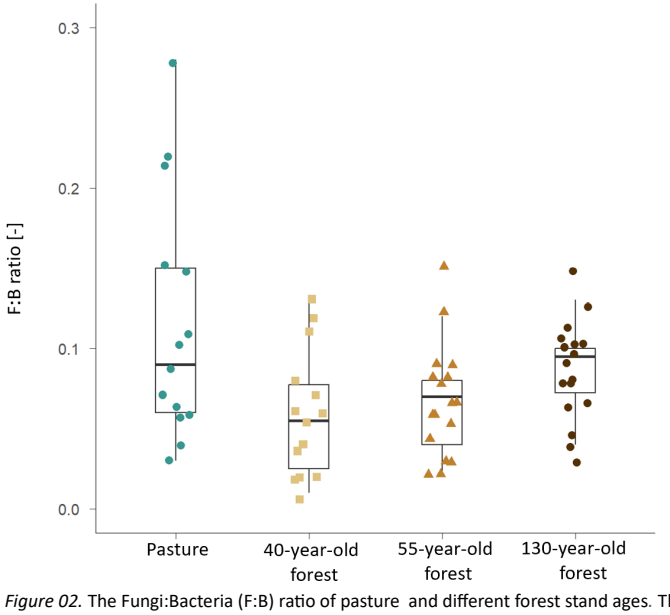


*Figure 02.* The Fungi:Bacteria (F:B) ratio of pasture and different forest stand ages. The individual sample points represent the mineral soil samples of 0 to 45 cm soil depth.

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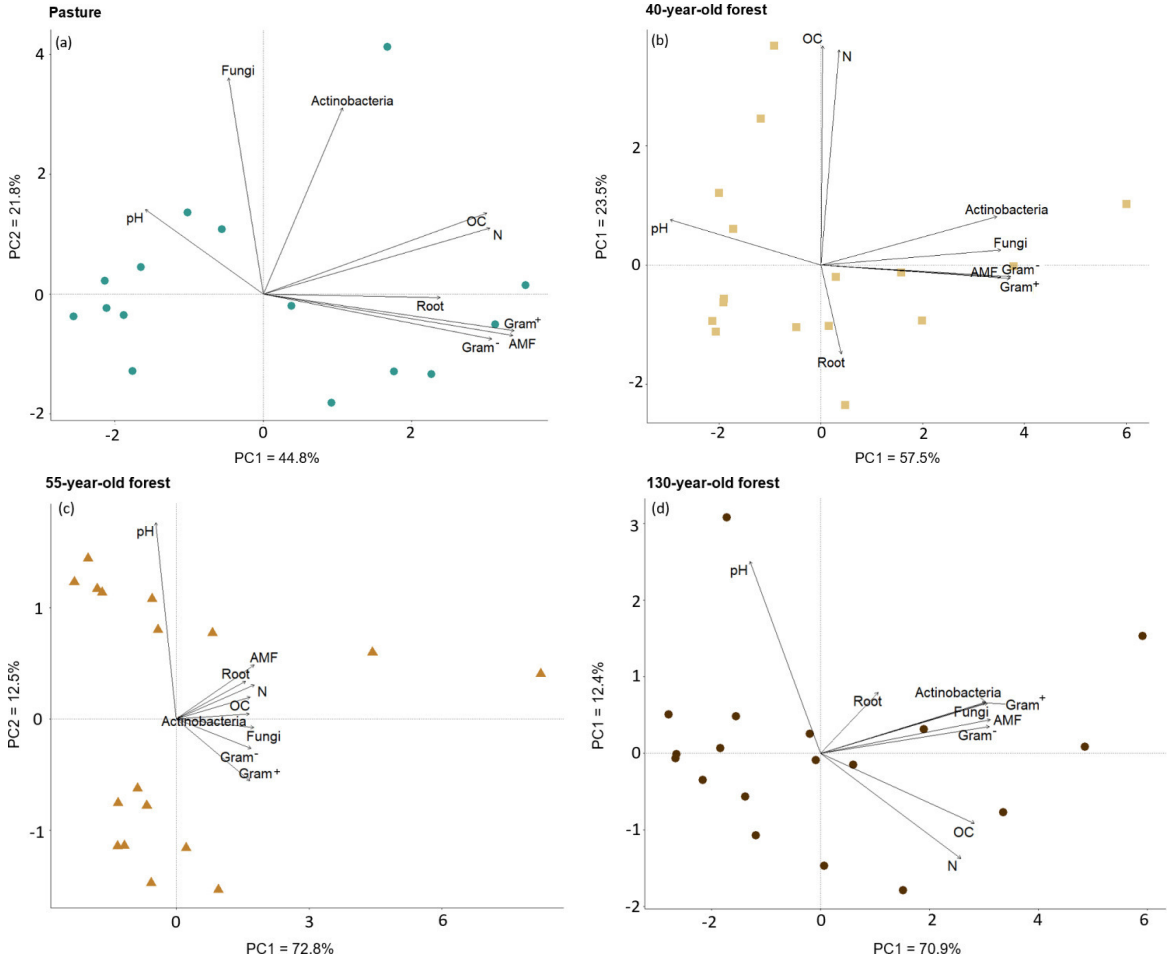


*Figure 03.* Principial Component Analysis (PCA) of individual PLFA compounds of the pasture (a) and different forest stand ages (b to d). PC1 and PC2 explain the variance of the data. AMF = Arbuscular mycorrhizal fungi, Fungi = Saprotrophic fungi, $Gram^+$ = $Gram^+$ bacteria, $Gram^-$ = $Gram^-$ bacteria, OC = organic carbon concentration, N = nitrogen concentration; Root = Fine root biomass.

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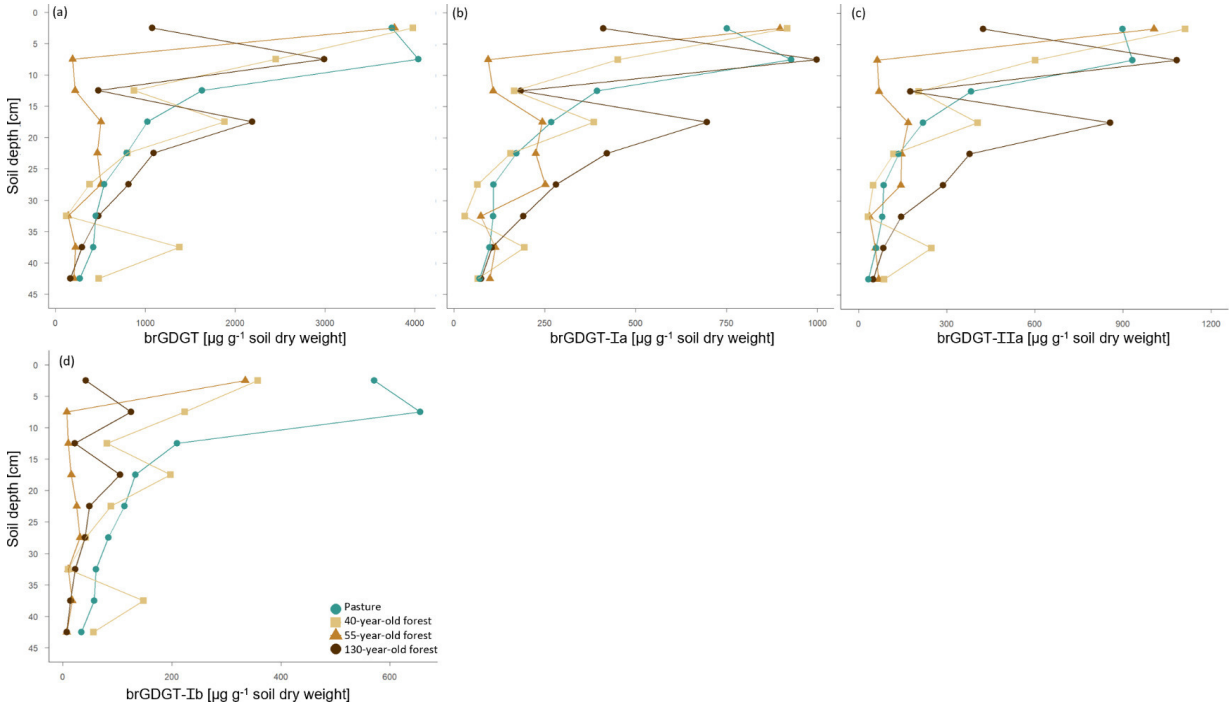


*Figure 04.* Concentrations of the total brGDGTs (a) and the identified main brGDGTs Ia (b), IIa(c), and Ib (d) of the pasture and the different forest stand ages in relation to soil depth (0 – 45 cm).

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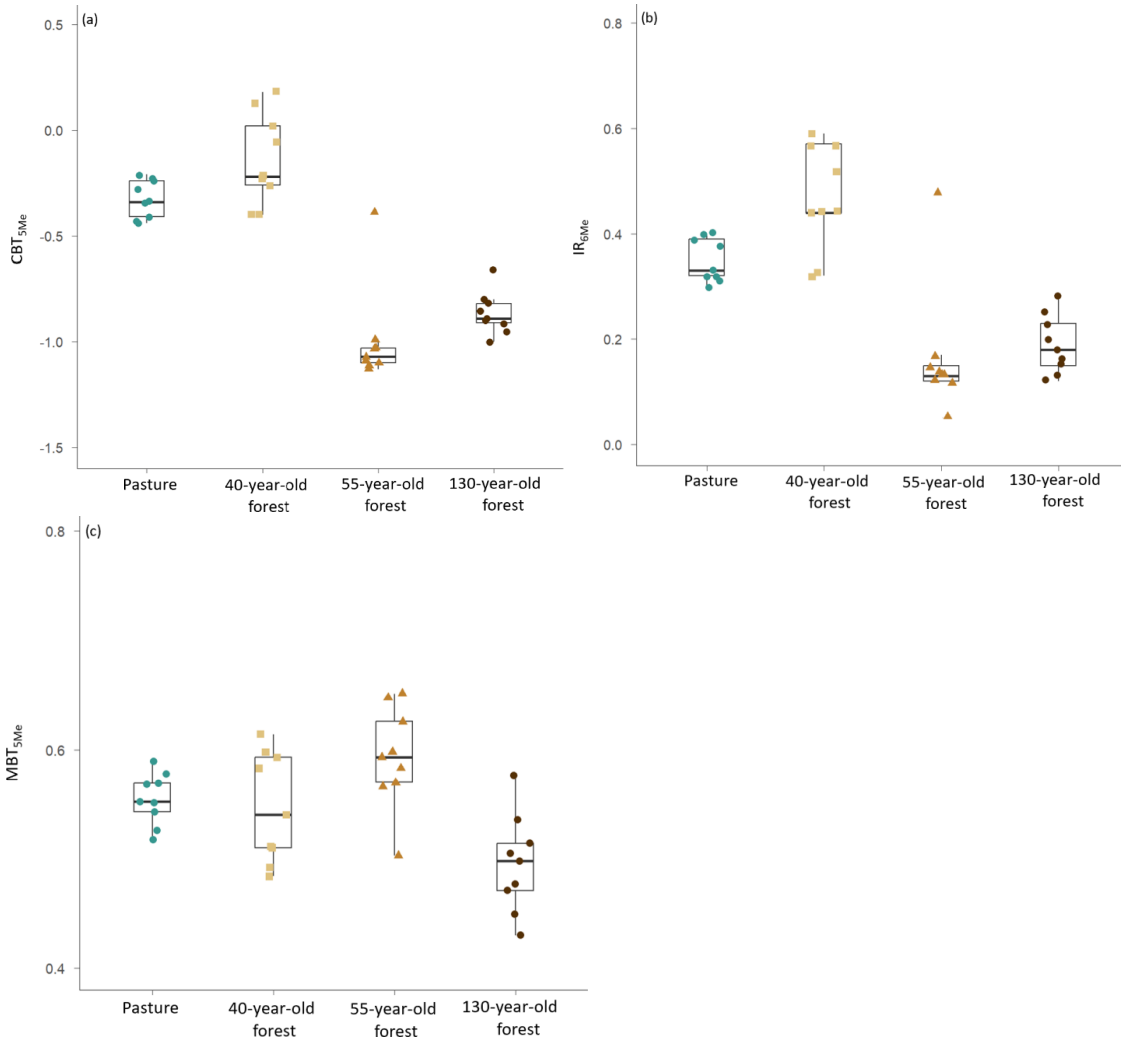


*Figure 05*. (a) $CTB_{5ME}$ ratio (Cyclisation ratio of Branched Tetraethers; Eq. 1), (b) $IR_{6ME}$ ratio (Isomer Ratio; Eq. 2) and (c) $MBT_{5ME}$ ratio (Methylation index of Branched Tetraethers; Eq. 3) of the pasture and the different forest stand ages. The individual sample points represent the mineral soil samples between 0 and 45 cm soil depth.

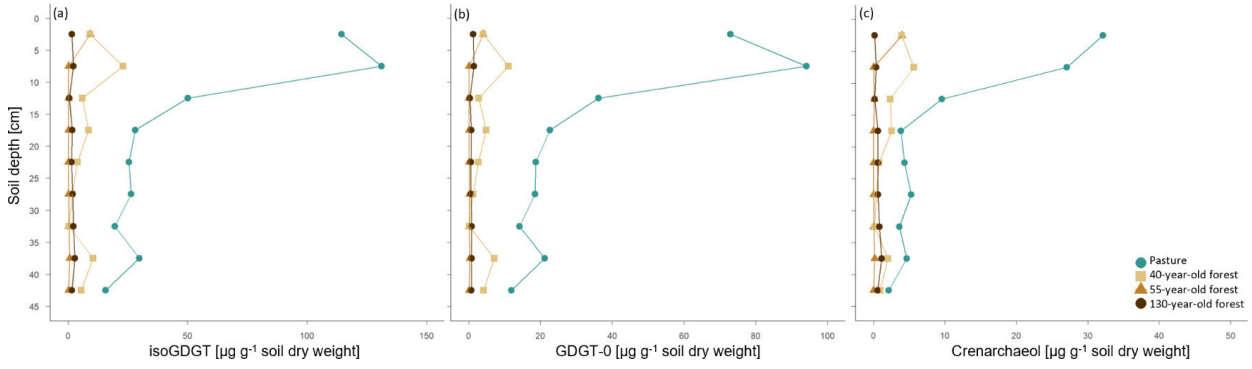


*Figure 06.* Concentration of total isoGDGT (a) and the main compounds GDGT-0 (b) and Crenarchaeol (c) of the pasture and the different forest stand ages in relation to soil depth (0 – 45 cm).

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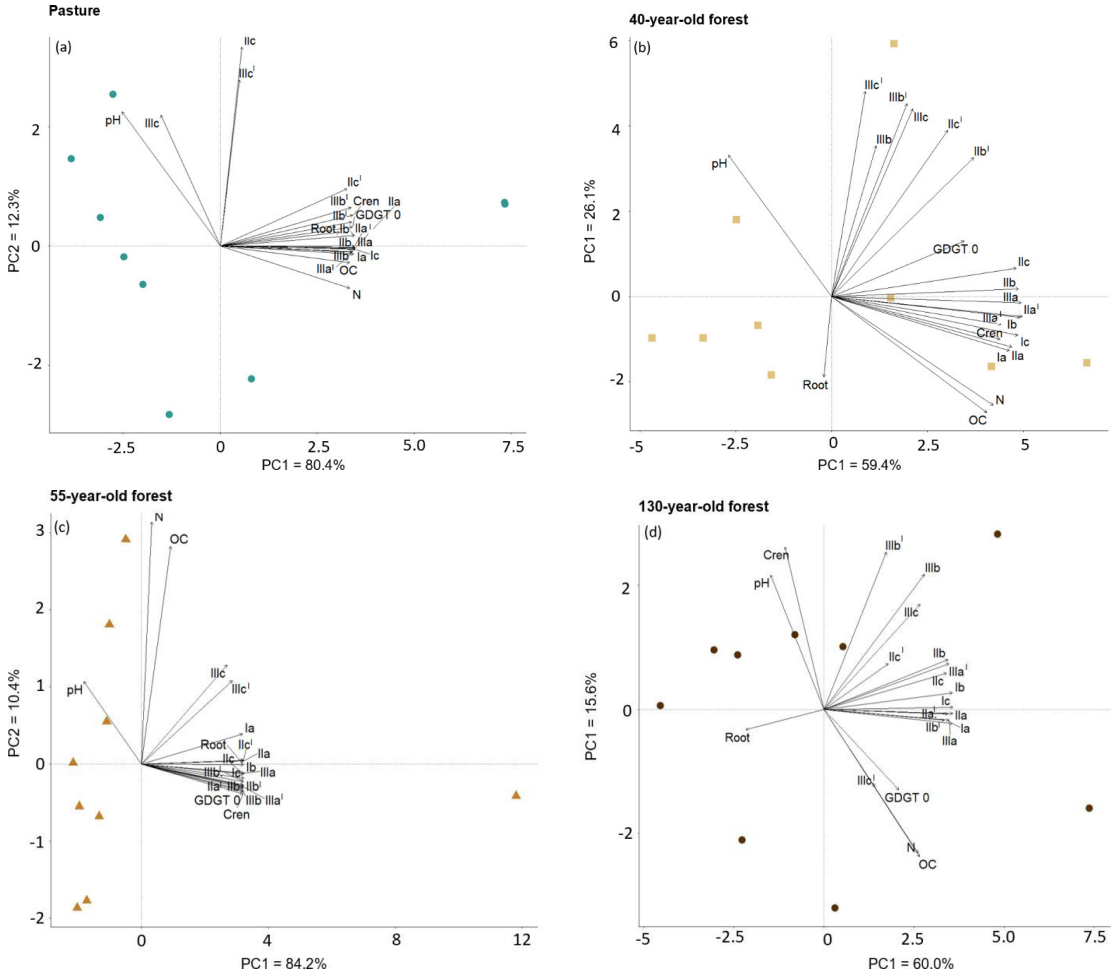


*Figure 07.* Principial Component Analysis (PCA) of individual brGDGTs and isoGDGTs of the pasture (a) and different forest stand ages (b to d). PC1 and PC2 explain the variance of the data. Cren = Crenarchaeol, OC = organic carbon concentration, N = nitrogen concentration, Root = fine root biomass.