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Long-term soil warming decreases fungal biomass and alters fungal but not bacterial communities in a temperate forest

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ABSTRACT

Long-term soil warming may alter microbial community structure and functioning in forest soils, thereby affecting carbon and nutrient cycling processes. We examined the effects of >14 years of soil warming (+4 °C during snow-free seasons) on the fungal biomass marker ergosterol, and on fungal and bacterial communities in a spruce dominated mountain forest in the Austrian Alps. Soil warming decreased ergosterol, and the ergosterol-to-microbial biomass carbon (MBC) ratio at 0-10 and 10-20 cm soil depth, with a stronger decline in ergosterol, indicating a higher sensitivity of fungi than bacteria to long-term warming. Warming also shifted the fungal community at both soil depths, favoring *Boletus luridus*, an ectomycorrhizal (ECM) fungus, which emerged as the dominant OTU in warmed plots. The dominance of ECM over saprotrophic fungi (SAP) under warming at topsoil likely resulted from increased fine root production and enhanced competition for substrates and nutrients. Bacterial abundance and community composition remained mostly unaffected at both depths, likely due to their greater resilience to elevated temperatures and their high taxonomic diversity. Our findings therefore suggest that long-term warming primarily affects fungal community composition and functional traits, thereby enhancing the contribution of ECM with fine roots to the carbon cycle in the calcareous forest soil.

1. Introduction

Soil microorganisms are essential drivers of organic matter decomposition, nutrient cycling, and ecosystem productivity in temperate forests (Bastida et al., 2019; Crowther et al., 2016; Sanaei et al., 2022). Ongoing climate change is projected to increase the global surface temperature by 1.4–4.4 °C by the end of this century (IPCC et al., 2021). In the European Alps, temperatures may rise by as much as 3.3 °C until 2100 (Gobiet et al., 2014; Warscher et al., 2019), potentially altering the community composition and activity of soil microorganisms in this region. Despite growing interest, the long-term impacts of global warming on soil microorganisms, particularly on bacteria and functionally distinct fungal guilds such as ectomycorrhiza (ECM) and saprotrophic (SAP) fungi, remain underexplored (Pec et al., 2021).

Biomarkers such as ergosterol, a lipid specific to fungal cell membranes, have been widely used to estimate fungal biomass in soils (Beni et al., 2014; Montgomery et al., 2000; Wallander et al., 2013). Ergosterol provides a reliable estimate of fungal biomass due to its specificity to fungi and minimal occurrence in other organisms (Beni et al., 2017; Nurika et al., 2018). However, the response of soil ergosterol to long-term warming has received limited attention. For example, a soil warming experiment in a subarctic heathland reported no change in

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ergosterol content despite a significant decrease in MBC, likely due to increased root biomass (Rinnan et al., 2007). Such findings suggest that fungal biomass and total microbial biomass may respond differently to soil warming.

The fungal community in temperate forest soils includes functionally distinct guilds, such as ECM and SAP fungi, which play interconnected roles in nutrient and C cycling (Awad et al., 2019; Shigyo and Hirao, 2021). ECM fungi form symbiotic relationships with plant roots, facilitating nutrient uptake in exchange for photosynthates (Fernandez et al., 2017, 2023; Smith and Read, 2010). Their extensive mycelial networks enhance nutrient and water acquisition beyond the rhizosphere, while some ECM species produce extracellular enzymes to decompose soil organic matter (SOM) and mobilize scarce nutrients (Averill et al., 2014; Frey, 2019). In contrast, SAP fungi and soil bacteria primarily decompose plant litter and soil organic matter, releasing and immobilizing C and nutrients essential for their metabolism (Baldrian et al., 2022). Bacteria and fungi, with their distinct resource utilization strategies, growth patterns, adaptability, and competitive abilities, often exhibit divergent responses to warming (Ali et al., 2018). Consequently, shifts in bacterial and fungal biomass and community composition may profoundly influence soil C and nutrient cycling over time. However, long-term warming may also have different effects on ECM and SAP fungi due to their distinct ecological roles and resource dependencies. Warming can stimulate plant root growth, increasing C allocation to ECM fungi and potentially enhancing their abundance (Kwatcho Kengdo et al., 2022; Leppälammi-Kujansuu et al., 2013). Conversely, SAP fungi, which rely on SOM and decomposing litter, may face increased competition with ECM fungi for diminishing soil resources — a phenomenon known as the “Gadgil effect” (Gadgil and Gadgil, 1971). Reduced nutrient availability in the soil can favor ECM fungi, which have several competitive advantages, such as the production of certain enzymes, the release of antibiotics, and most importantly C transfer from their host trees (Averill et al., 2014; Fernandez and Kennedy, 2016; Marañón-Jiménez et al., 2021). These dynamics could shift fungal community composition, with significant implications for forest nutrient cycling and C storage under warming.

Soil bacteria also play vital roles in C and nutrient cycling. The impacts of warming on bacterial communities are complex, with some studies reporting shifts in bacterial community composition due to changes in resource availability and quality (DeAngelis et al., 2015; Deslippe et al., 2012; Frey et al., 2008; Ricketts et al., 2020; Romero-Olivares et al., 2017). For example, soil warming experiments at the National Ecological Research Park in Oak Ridge, Tennessee, USA (+3 °C above ambient) demonstrated that in the absence of labile substrates, fungal communities were favored, whereas the addition of plant-derived labile C shifted microbial dominance toward bacteria (Castro et al., 2010). Similarly, a +5 °C increase in soil temperature altered the relative abundances of soil bacteria and increased the bacterial-to-fungal ratio in a temperate forest (DeAngelis et al., 2015). In contrast, other studies have found bacterial communities to be resilient, showing no significant changes under warming (Biasi et al., 2008; Fang et al., 2020). These discrepancies underscore the need for long-term studies exploring specific functional guilds within microbial communities, given their different ecological roles and possibly different sensitivities to rising temperatures. Adaptations of the microbial community may also rely on the duration of warming and associated changes in substrate availability in the soil.

Long-term experimental warming studies provide valuable insights into gradual changes in microbial dynamics and ecosystem processes. Here, we investigated the effects of long-term soil warming (+4 °C, >14 years) on ergosterol content, fungal and bacterial community composition, and the abundance of ECM and SAP fungi in the soil of a mountain forest in the European Alps, Achenkirch, Austria. A previous study found no change in the bacterial community composition after 4-5 years of warming (Kuffner et al., 2012). Further investigations in the Achenkirch soil warming experiment have shown an increase in fine root biomass and root tips (Kwatcho Kengdo et al., 2022), a reduction in phosphorus availability (Tian et al., 2023a), and a decrease in microbial biomass and necromass (Tian et al., 2023b; Liu et al., 2024). Despite a sustained sharp increase in soil CO_2 emissions, only a slight, non-significant decrease in soil organic carbon (SOC) stock has been detected to date (Schindlbacher et al., 2025). The near preservation of the SOC stock was attributed to increased root litter input and a simultaneous limitation of microbial process rates. However, it remains unclear to what extent the biomass and composition of fungal and bacterial taxa have responded to long-term soil warming.

We hypothesized that soil warming: (1) decreases total fungal biomass, as indicated by reduced ergosterol content and a lower ergosterol-to-MBC ratio, due to physiological stress and substrate depletion (Liu et al., 2024), with fungi being more sensitive than bacteria; (2) alters fungal community composition, favoring ECM fungi due to enhanced fine root production (Kwatcho Kengdo et al., 2022), while SAP fungi are being reduced due to increased competition with ECM fungi (Fernandez and Kennedy, 2016; Lindahl and Tunlid, 2015); and (3) does not significantly affect bacterial community composition, reflecting their resilience to temperature increases (Biasi et al., 2008; Fang et al., 2020). By addressing these hypotheses, this study aimed to provide novel insights into the long-term effects of soil warming and associated changes in fine root traits and soil parameters on microbial community composition.

2. Materials and methods

2.1. Study area and experimental setup

This research was conducted at the Achenkirch soil warming experiment site in the Austrian Limestone Alps (11°38′21″ E; 47°34′50″ N, 910 m a.s.l.). The site features a 140-year-old forest mainly consisting of Norway spruce (*Picea abies*), with smaller populations of European beech (*Fagus sylvatica*) and silver fir (*Abies alba*). The region's mean annual air temperature and precipitation from 1988 to 2017 were 7 °C and 1493 mm, respectively. The soil is classified as shallow Rendzic Leptosol, consisting of A and A/C horizons covered by a ∼1 2 cm thick organic layer. The experiment involved six blocks of paired 2 × 2 m plots, established in two phases (2004 and 2007). Each block had one control and one warmed plot. The warming treatment plots were heated using heating cables installed at 3 cm depth, spaced 7.5 cm apart, maintaining a temperature difference of 4 °C at 0-10 cm depth above that of the control plots during snow-free months (April–December). The temperature difference between the treatments was lower (∼3 °C) at 10-20 cm depth. Further site and experimental details are available in Schindlbacher et al. (2025).

2.2. Soil sampling

Soil sampling was conducted within a single day at the end of the growing season in October 2019. The samples were collected using soil cores measuring 5 cm in diameter and 20 cm in length. Six control plots and six warmed plots (heated since 2005) were sampled at two soil depths: 0−10 and 10−20 cm. In each plot, ten soil cores were randomly collected from each of these depth intervals. Due to the shallow soil and underlying dolomitic bedrock, the sampling depth was less than 20 cm at some points. During sieving (2 mm) of individual soil cores (n = 240), roots were sampled for determination of fine root biomass and morphology (Kwatcho Kengdo et al., 2022). The sieved soil samples were then pooled and mixed according to plot and depth, resulting in a total of 24 soil samples (*i.e.,* 12 plots, with 6 control and 6 warming plots × 2 soil depths). An aliquot of each soil sample was used for determination of microbial biomass carbon using the chloroform-fumigation-extraction method (Tian et al., 2023b). Another soil aliquot was frozen at −24 °C until the analysis of ergosterol and extraction of DNA for the microbial community composition was performed.

2.3. Ergosterol and fungal biomass

Soil fungal biomass was determined by measuring ergosterol concentrations, following the method of Djajakirana et al. (1996) with modifications by Enowashu et al. (2009). One gram of fresh soil was extracted in 25 ml of ethanol, shaken for 30 min at 200 rpm using a mechanical shaker, and centrifuged at 2888 g for 12 min. A 20 ml aliquot of the supernatant was evaporated using a rotary evaporator at 47 °C under vacuum. The dried extract was dissolved in 2 ml of HPLC-grade methanol in an ultrasonic bath and filtered through a 0.45 μm cellulose-acetate syringe filter. Quantitative analysis was performed using an HPLC system (Beckmann Coulter, System Gold 125 Solvent Module) with a 150 × 3 mm Spherisorb ODS II column and a UV detector (Beckmann Coulter, System Gold 166) set at 282 nm. Ergosterol content, used as a proxy for fungal biomass, varies among fungal species (Baldrian et al., 2013). Fungal biomass (FB) and fungal biomass carbon (FBC) were calculated described by Montgomery et al. (2000) as follows:

$$\text{FB}\ (\mu\text{g g}^{-1}\ \text{soil}) = \text{Ergosterol}\ (\mu\text{g g}^{-1}\ \text{soil}) \times \text{f} \times \text{Rf} \tag{1}$$

where f represents 250, indicating the biomass in mg per μg of ergosterol, and *Rf* is 1.61, which is a correction factor to account for an average recovery rate of 62% (1/0.62) for ergosterol extraction from soils. The fungal biomass (FB) can also be expressed in terms of its C content (FBC, Table S1), calculated as:

$$\text{FBC}=\text{FB} \times \text{C} \tag{2}$$

where C equals 0.43, reflecting the average carbon fraction in fungal species.

To calculate the ratio of ergosterol and MBC, we used published MBC data measured in the same samples and plots (Tian et al., 2023a). We also calculated bacterial biomass carbon (BBC, Table S1) using the equation:

$$\text{BBC} = \text{MBC} - \text{FBC} - \text{OBC} \tag{3}$$

where OBC represents the fraction of MBC attributed to archaea and protozoa, which is assumed to be 2% of MBC (Bardgett and Griffiths, 1997; Gattinger et al., 2002).

2.4. DNA extraction, sequencing and qPCR

DNA was extracted from the soil samples in duplicate (technical replicates per soil sample), using the NucleoSpin® Soil Kit (Macherey-Nagel, Düren, Germany). Amplicon libraries were prepared in two PCR steps. The first PCR (PCR1) targeted the V3–V4 regions of the bacterial 16S rRNA gene based on Amplicon libraries as recommended by Illumina (2013), and the ITS2 region of fungal DNA, using specific primers (gITS7 and modified ITS4g) with sample-specific barcodes. Reactions for PCR1 contained 370 nM of each primer, 2 μl of OneTaq® 2X Master Mix, and 0.2 μl of DNA extract, and were cycled 25 times with an initial denaturation at 95 °C for 2 min, followed by 20 s at 94 °C, 40 s at 45 °C, and 55 s at 68 °C, with a final extension at 68 °C for 7 min. Products of PCR1 were purified using a mixture of Exonuclease and Shrimp Alkaline Phosphatase. In PCR2, index sequences and Illumina adapters were appended in 5 cycles, using a similar PCR setup but with 270 nM primers and 2.5 μl of purified PCR1 product. The final amplicons were pooled and purified, and DNA concentration and size distribution were assessed using a Qubit® 2.0 fluorometer and Bioanalyzer 2100. Sequencing was performed on an Illumina MiSeq® platform with 2 × 300 bp paired end reads.

Sequence reads were initially sorted by the sequencer using dual index sequences, with each combination comprising a unique forward and reverse index. Sample assignment was conducted using QIIME version 1.9.0 (Caporaso et al., 2010), where only reads with at most one ambiguous base were retained. A quality filter (phred score threshold of 19) was applied, truncating reads after nine consecutive low-quality base calls and keeping those with at least 35% high-quality bases. Only R1 reads were further processed, with tag-sequence lengths adjusted using the FastX toolkit. *De novo* clustering into operational taxonomic units (OTUs) was performed using CD-HIT-OUT at 97% sequence similarity. Taxonomic assignments were made using QIIME and the UNITE database *v*8, with unassignable OTUs handled as per Peršoh et al. (2010). Using the 'rrarefy' function from the R package vegan (Oksanen et al., 2019), fungal OTUs were rarefied to 5400 reads and bacterial OTUs to 11,223 reads per sample. An OTU table comprising the rarefied read counts per OTU and sample was generated and standardized by the total number of reads per sample. Fungal OTUs were categorized into functional guilds according to their taxonomic affiliation, referencing databases such as 'Faces of Fungi' (Jayasiri et al., 2015) and 'FUNGuild' (Nguyen et al., 2016). For abundant and key OTUs, species-level identification or sequence comparison was conducted. We have prefixed the OTU numbers with the assigned taxonomic name to improve comprehensibility. Plant plastid sequences were removed from the bacterial dataset before stratification. For comprehensive details on the DNA extraction and sequencing methods, refer to Kwatcho Kengdo et al. (2022).

For absolute quantification of selected barcoding genes by qPCR, taxon-specific primers (Table S2) were designed for OTUs commonly found by the metabarcoding approach in all samples, *i.e.,* the fungal OTU6 (*Helotiales sp*. 6) and the bacterial OTU4 (*Nitrospirota*). The DNA fragments targeted by the primer combinations were 348 bp and 332 bp in length and synthesized as gene standards (gBlocks™) by Integrated DNA Technologies (Leuven, Belgium). Dilution series from 10^3 to 10^7 copies of the gene standards were co-processed for calibration. Each qPCR reaction included the primers (0.25 μM, each), 9 μl of the Luna® Universal qPCR Master Mix (New England Biolabs GmbH, Frankfurt am Main), and 0.5 μl of DNA extract in a total volume of 18 μl. Reactions were run on a qPCR instrument (CFX96, Bio-Rad, Ca, USA) and included an initial denaturation (95 °C for 60 s) followed by 40 amplification cycles (95 °C for 15 s, 60 °C for 30 s + plate read) and melting curve assessment.

The absolute abundance of bacterial and fungal OTUs in a single sample was calculated as a proportional share of all samples per soil depth ($n = 12$). This calculation allowed us to identify shifts of individual taxa between the two treatments for each soil depth and includes following steps:

$$RA_{OTU} = \frac{Ns}{Nt} \times 100 \tag{4}$$

where RA_{OTU} is the relative abundance of a specific OTU (%), *Ns* is the read counts of a specific OTU, and *Nt* is the read counts of all OTUs in a single sample, determined by NGS metabarcoding.

$$TNS = \frac{TN_{OTUx}}{RA_{OTUx}} \tag{5}$$

where *TNS* is the total number of gene copies of all bacterial or fungal OTUs in a single sample, TN_{OTUx} is the gene copy number determined by qPCR for *Nitrospirota* (OTU4, bacteria) or *Helotiales sp*. 6 (OTU6, fungi) and RA_{OTUx} is the relative abundance of OTU4 or OTU6, respectively, in a single sample.

$$TN_{OTU} = \frac{RA_{OTU} \times TNS}{100} \tag{6}$$

where TN_{OTU} is the total gene copy number of a specific OTU in a single sample.

$$PS = \frac{TN_{OTU}}{TN} \times 100 \tag{7}$$

where *PS* is the proportional share of the gene copies of a specific OTU in a single sample (%) relative to the total of bacterial or fungal gene copies in all samples (TN) of the corresponding depth ($n = 12$). Hereafter, *PS* is referred to as 'absolute abundance'.

We used Fisher's alpha, Pielou's evenness, and Shannon–Wiener indices to characterize the diversity of fungal and bacterial communities at the two soil depths and treatments as detailed by Clarke et al. (2014).

2.5. Statistical analysis

Normality of all data was checked using the Shapiro-Wilk test, conducted with the shapiro.test function from the 'stats' package, and if normality was not given, the data was transformed before statistical analysis. All statistical analyses and graphs were performed using R *v.* 4.3.1 (R Core Team, 2023). We used linear mixed-effects models to test the effect of soil depth and treatment on ergosterol, ergosterol-to-MBC ratio, abundance (%) of ectomycorrhizal and saprotrophic fungi, fungal phyla (Ascomycota and Basidiomycota), fungal and bacterial diversity indices (Fisher's alpha, Pielou's Evenness Shannon-Wiener) using the 'lme4' package (Bates et al., 2015). Treatment and soil depth were included as fixed factors, while plot was included as a random factor. The models were fitted using the lmer function, and P values for fixed effects were obtained using the 'lmerTest' package (Kuznetsova et al., 2017). The effects of treatment and soil depth on parameters were considered statistically significant when $P \leq 0.05$.

To test the effects of soil depth and treatment on fungal and bacterial communities, we used permutational multivariate analysis of variance (PERMANOVA) based on the Bray–Curtis dissimilarity implemented in the adonis function of the 'vegan' package (Oksanen et al., 2019). The contribution of individual OTUs to the dissimilarity between control and warming treatments was assessed using the Similarity Percentages (SIMPER) analysis, performed with the simper function in the 'vegan' package. We applied the CLAM (Classification Method) function from the 'vegan' package to assess whether specific OTUs exhibited a preference for control or warming treatments. This statistical approach, based on a multinomial model, classifies OTUs as either generalists—those evenly distributed across both treatments—or specialists—those showing a significant preference for one treatment. The classification was determined by comparing the read counts of OTUs between treatments (Chazdon et al., 2011).

Given the positive correlation between ECM and both fine root biomass and root tip density (Kwatcho Kengdo et al., 2022), we conducted a principal component analysis (PCA) to examine the relationships between the abundance of ECM OTUs—specifically those contributing more than 1% to the dissimilarity shown in Figs. 2c and 3c—and various soil biological properties and root traits. These properties included ergosterol, microbial biomass carbon (MBC) (data from Tian et al., 2023a), soil respiration (Schindlbacher et al., 2025), fine root biomass, and root tip density (Kwatcho Kengdo et al., 2022). ECM, ergosterol, MBC and fine root parameters were determined from the same soil samples in October 2019. The PCA was performed using the R packages 'FactoMineR' (Lê et al., 2008) and 'factoextra' (Kassambara, 2017). The R packages 'ggplot2' (Wickham, 2016) and 'gridExtra' (Auguie, 2017) were used for data visualization.

3. Results

3.1. Impact of soil warming on ergosterol, and ergosterol-to-MBC ratio

Soil warming decreased the mean ergosterol content from 10.9 to 6.1 μg g^{-1} soil ($P < 0.001$) at 0–10 cm depth (Fig. 1a) and from 3.7 to 1.6 μg g^{-1} soil ($P = 0.04$) at 10–20 cm depth (Fig. 1b). Additionally, soil warming reduced the ergosterol-to-MBC ratio by 37% ($P < 0.001$) at 0–10 cm depth (Fig. 1c) and and 38% ($P = 0.03$) at 10–20 cm depth (Fig. 1d), indicating stronger reduction in fungal biomass in relation to total microbial biomass. Both, ergosterol content and the ergosterol-to-MBC ratio differed significantly ($P < 0.001$) between the two soil depths (Fig. 1). Microbial biomass (MBC), fungal biomass (FBC), and bacterial biomass (BBC) across treatments and depths are presented in Table S1. While FBC decreased by 37%, there was an increase in BBC of 56% at 0-10 cm in the warming plots. In addition, the ratio of FBC:BBC decreased from 2.9 in the controls to 1.2 in the warmed plots at a depth of 0–10 cm. Both FBC and BBC decreased by 50% and 26%, respectively, at 10-20 cm by soil warming.

3.2. Warming effects on soil fungal community

Combining metabarcoding and qPCR data, a total of 16,243,417 fungal gene copy numbers were present across both soil depths in the control and warming plots, which were assigned to 586 fungal OTUs. Of these, 16% were classified as ECM fungi, and 12% as SAP fungi, while 69% were not assignable, and 3% belong to other categories (*e.g.,* pathogens, root-associated, lichenicolous). Soil warming altered the fungal community composition ($P = 0.014$, Table 1) and abundance distribution (Fisher's Alpha, $P = 0.049$, Table S6). According to the CLAM classification method, soil warming affected fungal taxa differently across depths (Figs. 2 and 3). The proportional absolute abundance of the OTUs Boletus-2, Saitozyma-19, Inocybe-20, Tricholoma-21, and several unidentified fungi increased whereas Inocybe-15 and Inocybe-107 decreased at 0–10 cm depth in the warmed plots (Fig. 2). Soil warming increased the proportional absolute abundance of Boletus-2, Inocybe-34, Inocybe-20, Mortierella-63, Boletopsis-77, Ramaria-97 and Inocybe-75 at 10–20 cm soil depth (Fig. 3). Only the proportional absolute abundance of Inocybe-15 decreased at 10-20 cm depth in the warmed plots. At both soil depths, Boletus-2 contributed 9.1% (0-10 cm) and 7.5% (10-20 cm) to the Bray-Curtis dissimilarity between the fungal communities of the control and warmed soils (Figs. 2c and 3c, Table S3).

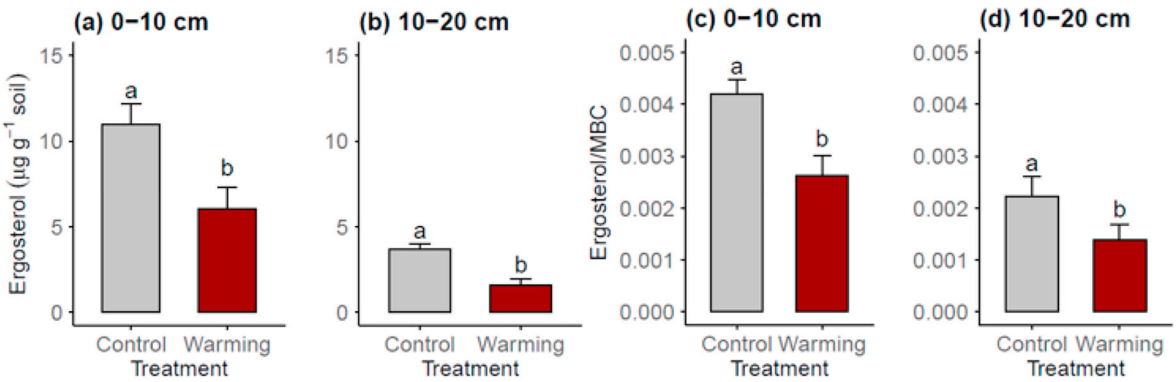

Fig. 1. Effects of soil warming on ergosterol, and ergosterol-to-MBC at two soil depths (0–10 and 10–20 cm). Significant differences between the control and warming treatment are indicated by different letters.

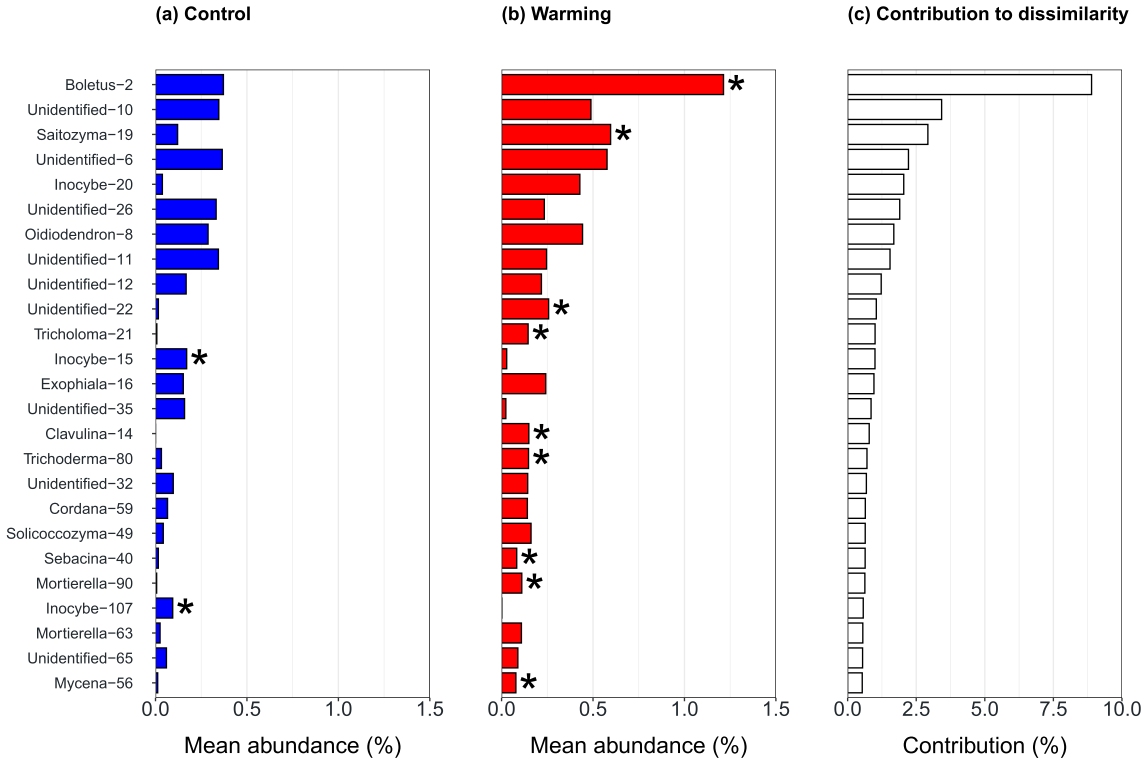

Fig. 2. Mean proportional absolute abundance of 25 most abundant fungal OTUs in control (a) and warmed (b) plots at 0–10 cm soil depth and their contribution to dissimilarity (c) as determined by SIMPER analysis. The numbers behind fungal genera are OTU numbers. Asterisks denote significantly higher abundances according to CLAM.

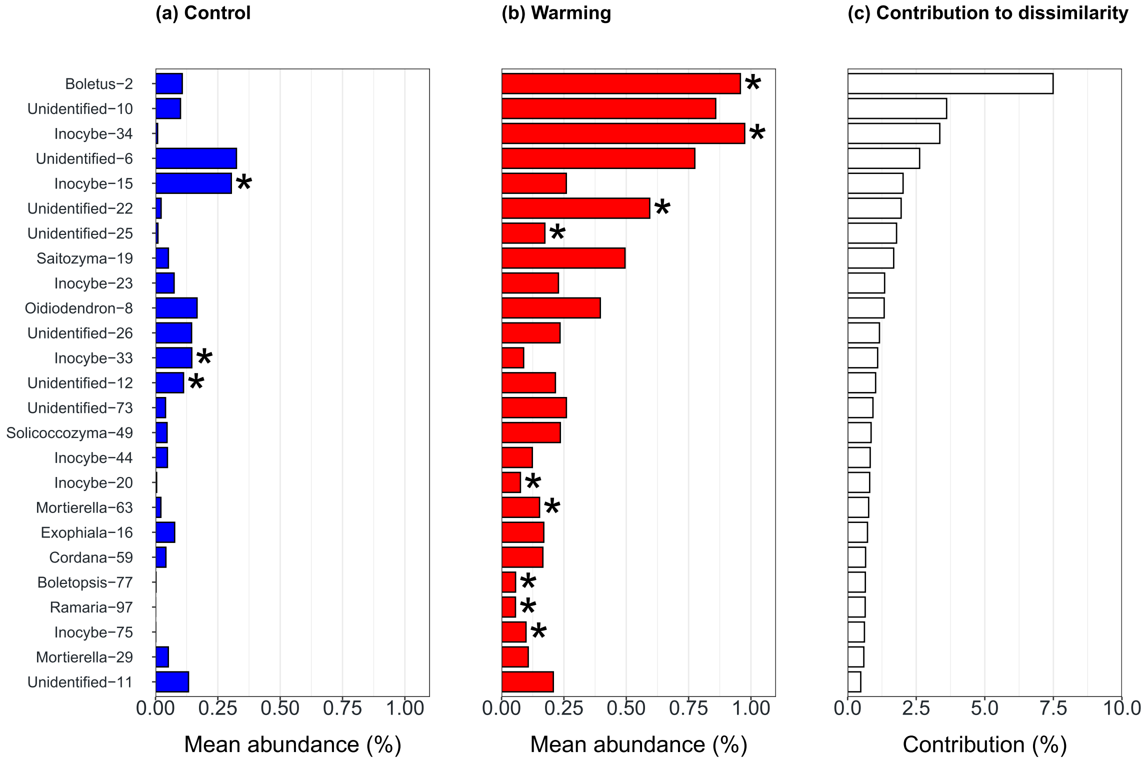

Fig. 3. Mean proportional absolute abundance of 25 most abundant fungal OTUs in control (a) and warmed (b) plots at 10–20 cm soil depth and their contribution to dissimilarity (c) as determined by SIMPER analysis. The numbers behind fungal genera are OTU numbers. Asterisks denote significantly higher abundances according to CLAM.

The other most abundant fungal OTUs contributed 4.2% (ECM) and 3.8% (SAP) to the dissimilarity at 0–10 cm, and 8.0% (ECM) and 7.0%

Table 1

Results of PERMANOVA based on Bray-Curtis dissimilarity analysis showing the effects of warming treatment (T: control and warming treatments) and soil depth (D: 0–10 and 10–20 cm soil depth) on the absolute abundance (%) of fungal and bacterial communities. Abbreviations: df – degrees of freedom; SS – the sum of squares; MS – the mean sum of squares; Pseudo-F – F value by permutation; P (perm) – P values based on more than 9000 permutations; and % Var – the percentage of variation explained.

Source	Fungal community					Bacterial community				
	df	SS	Pseudo-F	P (perm)	% Var	df	SS	Pseudo-F	P (perm)	% Var
T	1	0.526	2.37	**0.0014**	9.63	1	0.083	0.864	0.277	3.78
D	1	0.299	1.31	0.0607	5.29	1	0.186	1.952	**0.016**	8.54
T × D	1	0.122	0.54	0.799	2.24	1	0.038	0.385	0.880	1.74
Residual	20	4.528			82.84	19	1.873			85.75
Total	23	5.466				22	2.184			

(SAP) at 10–20 cm depth.

The first and second principal components (PC1 and PC2) explained 33.9% and 24.0% of the variance in 0–10 cm soil depth (Fig. 4a). None of the ECM fungi showed a positive correlation with fine root biomass (FRB), root tip density (RTID), ergosterol, microbial biomass (MBC) and soil respiration. Furthermore, the four most common ECM fungi showed no correlation, except for Inocybe-20 and Tricholoma-21. For 10–20 cm soil depth, PC1 and PC2 explained 36.9% and 28.9% of the variance (Fig. 4b). Except for Inocybe-15, ECM fungi were not correlated with the root parameters. A positive relationship was only found between Inocybe-15 and fine root biomass and root tip density.

At 0−10 cm soil depth, ECM fungi were more abundant (27%, $P = 0.051$) in the warming plots compared to the control plots (14%, Fig. 5a). At 10–20 cm, the absolute abundance of ECM fungi was not different between the treatments. The absolute abundance of SAP fungi was slightly higher in the warming treatment (20% at 0−10 cm, 21% at 10−20 cm), but the differences to the control were not significant (Fig. 5b). The change in the fungal community composition at 0-10 cm did not affect the absolute abundance of Basidiomycota (58% at 0-10 cm and 56% at 10-20 cm) and Ascomycota (42% at 10 cm and 44% at 10-20 cm) in the warming treatment (Fig. S1).

3.3. *Bacterial community responses to soil warming*

Combining metabarcoding and qPCR data, a total of 2,732,851,851 bacterial gene copies, were found across both treatments and depths, which were assigned to 2879 bacterial OTUs. Although slightly more bacterial OTUs were found in the warming plots, the differences in the distribution of bacterial OTUs between treatments was not significant (Table 1). In contrast, bacterial communities differed between the two soil depths regardless of the treatment. The most abundant bacterial OTUs included phyla of Pseudomonadota (OTUs 1, 3, 5, 11, 16, 22, 31), Actinomycetota (OTUs 6, 7, 9), Verrucomicrobiota (OTU2), and Nitrospirota (OTU4) (Figs. 6 and 7) and summed up to 4.5% at 0-10 cm and 3.7% at 10-20 cm across both treatments. Methyloceanibacter-1, Terrimicrobium-2, Bradyrhizobium-3, Nitrospirota-4 and Agromyces-7 were commonly present across both depths and treatments (Figs. 6 and 7), together contributing 5.4% and 3.2% to the dissimilarity at the 0–10 cm and 10–20 cm depths, respectively (Table S4). The control plots showed higher Pielou's Evenness (Table S7), indicating a more balanced distribution of bacterial species across the plots.

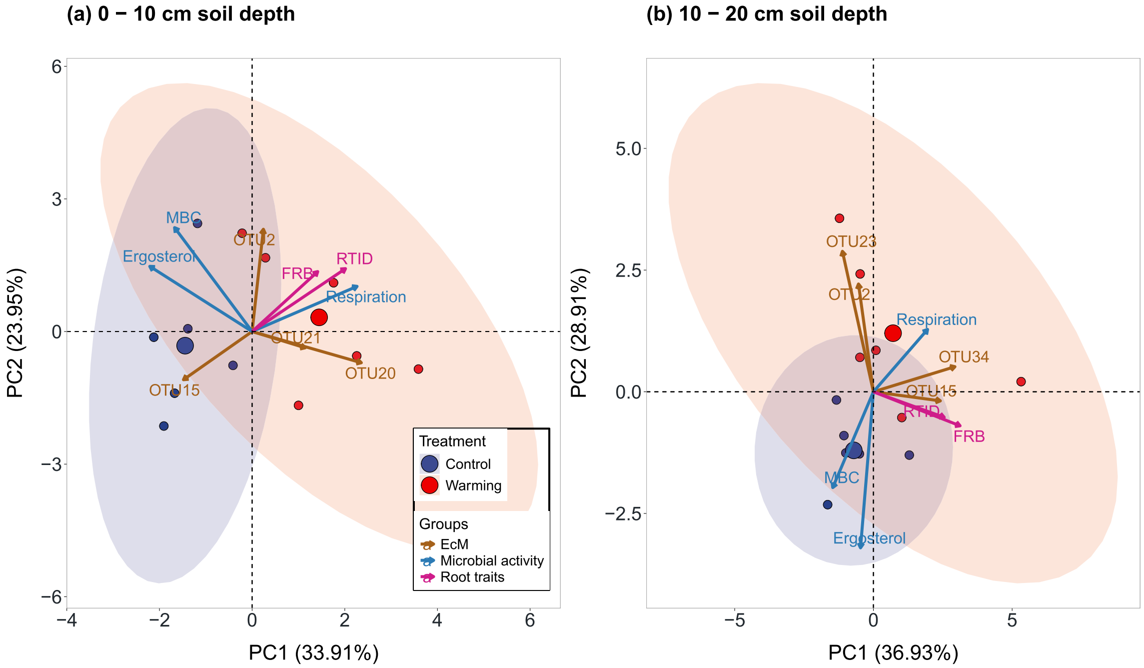

Fig. 4. Principal Component Analysis (PCA) of selected ectomycorrhizal fungi ("ECM OTUs", contributing >1% to dissimilarity in Figs. 2c and 3c), microbial activity, and fine root traits at two soil depths: 0–10 cm (a) and 10–20 cm (b). Symbols (blue and red circles) represent sample scores for each treatment, while arrows indicate variable loadings. Brown arrows indicate ECM OTUs; blue arrows represent microbial activity (MBC: microbial biomass carbon, ergosterol, and soil respiration); and red arrows denote fine root traits (FRB: fine root biomass, RTID: root tip density). 95% confidence ellipses are shown.

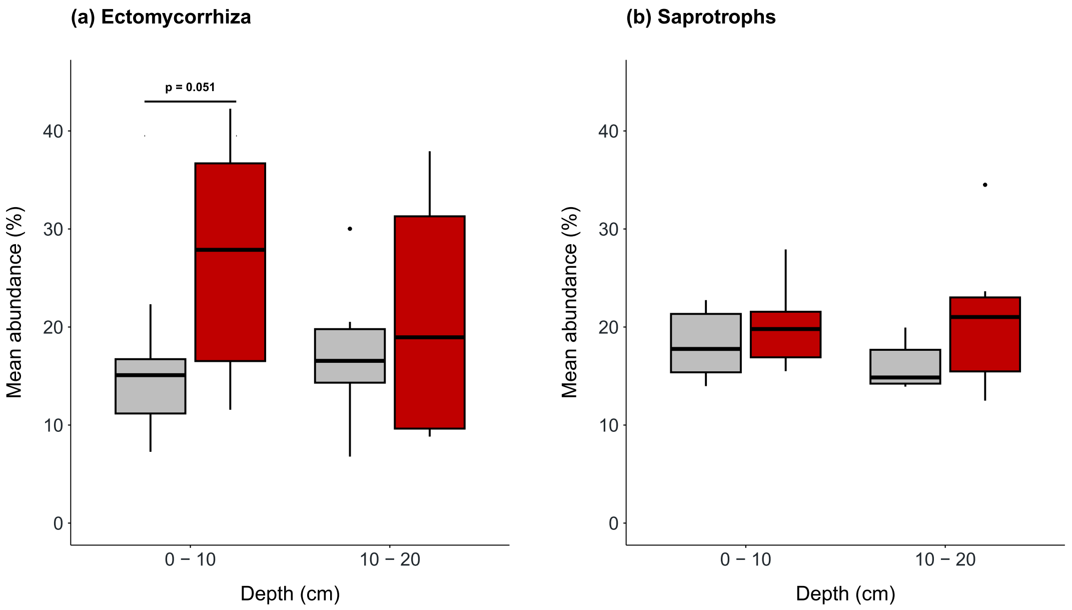

Fig. 5. Impact of soil warming on the mean abundance (%) of ectomycorrhizal and saprotrophic fungi in two soil depths (0–10, and 10–20 cm). Dots above bars represent outliers.

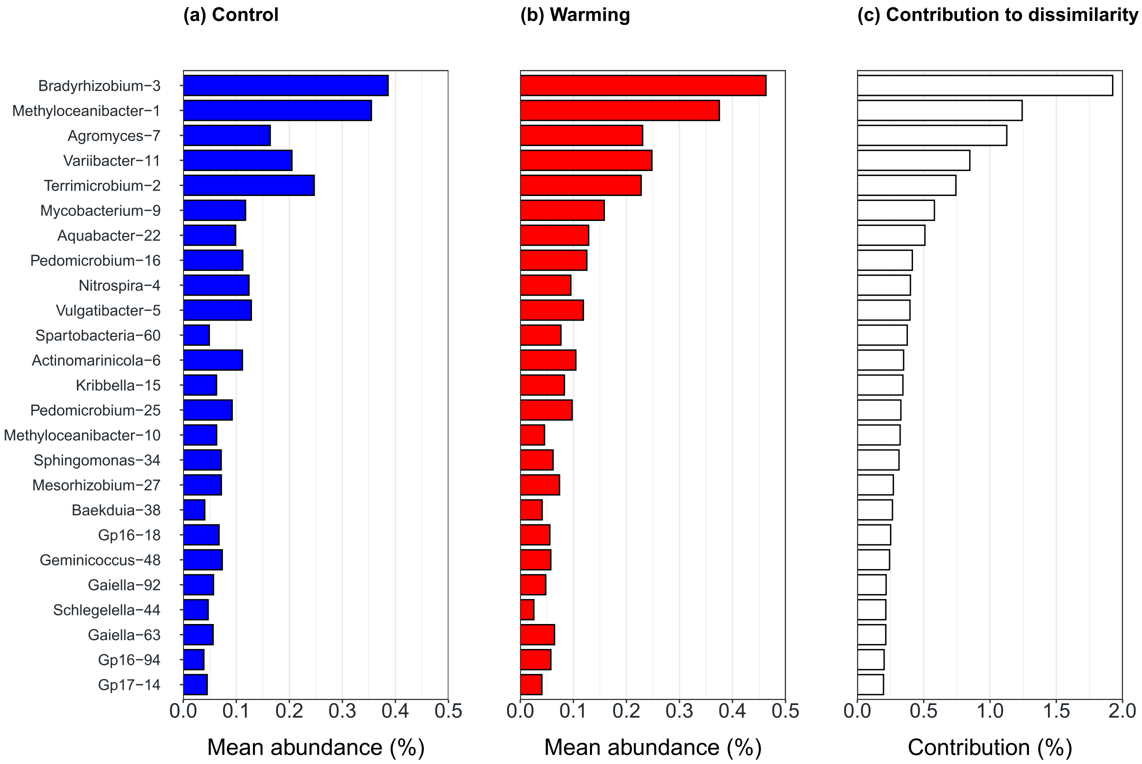

Fig. 6. Mean proportional absolute abundance of 25 most abundant bacterial OTUs in control (a) and warmed (b) plots at 0-10 cm soil depth and their contribution to dissimilarity (c) as determined by SIMPER analysis. The numbers behind bacterial genera indicate OTU numbers. None of the OTUs occurred at significantly higher abundances in either treatment according to CLAM.

4. Discussion

4.1. Soil warming decreases ergosterol and ergosterol/MBC

Confirming hypothesis (1), decreases in fungal ergosterol and ergosterol-to-MBC ratios point towards a constrained ability of fungi to compete with bacteria in the long-term warmed forest soil. The observed reduction in ergosterol and ergosterol-to-MBC ratios can be related to longer-term changes in soil properties and fine root dynamics (see 4.2.). In the shorter-term (after 4-5 years), repeated phospholipid fatty acid (PLFA) analysis did not indicate a change in the fungi to bacteria ratios by warming (Schindlbacher et al., 2011). In the Achenkirch experiment, longer-term warming effects include a decline in soil C and N concentrations (Tian et al., 2023a), and a reduced P availability for the microbial community (Tian et al., 2023b). Consequently, MBC (Tian et al., 2023a) and microbial necromass C (Liu et al., 2024) both declined in the

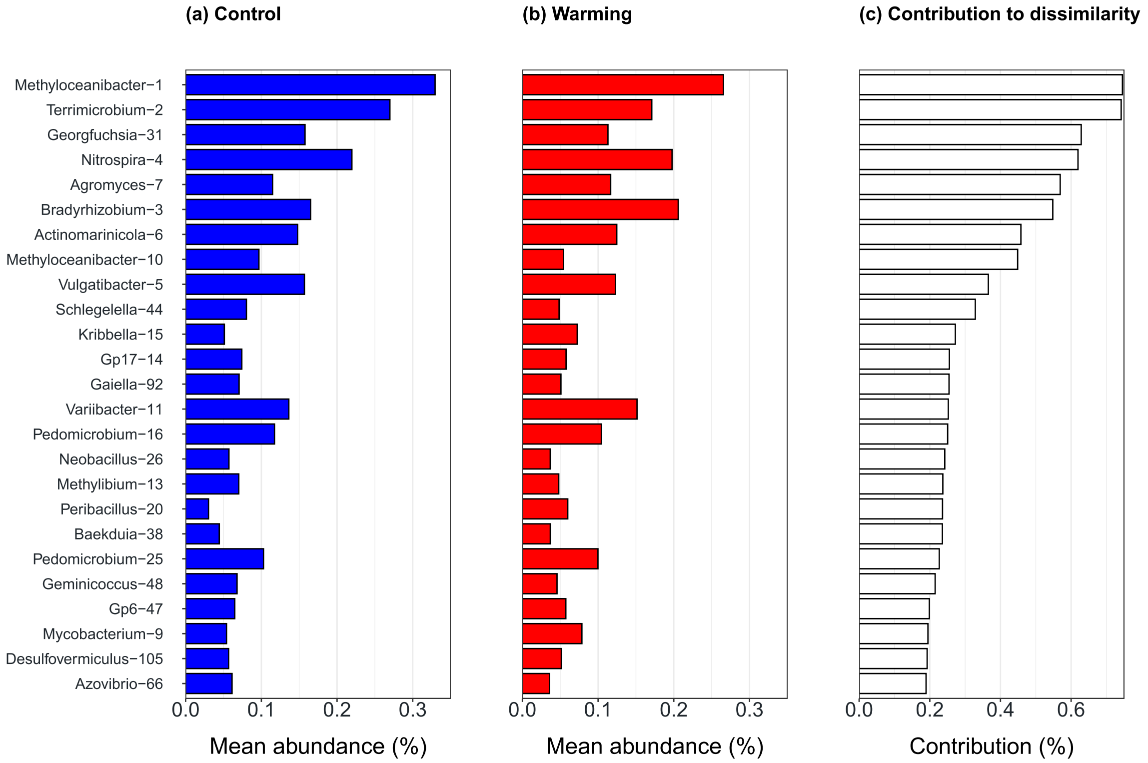

Fig. 7. Mean proportional absolute abundance of 25 most abundant bacterial OTUs in control (a) and warmed (b) plots at 10-20 cm soil depth and their contribution to dissimilarity (c) as determined by SIMPER analysis. The numbers behind bacterial genera indicate OTU numbers. None of the OTUs occurred at significantly higher abundances in either treatment according to CLAM.

long-term warmed soil, while reduced microbial carbon use efficiency (CUE) also points to physiological stress due to long-term warming (Liu et al., 2024). Thus, under elevated temperatures, fungi (and less so bacteria) may have allocated more C to respiration and maintenance, leaving less C for growth and biomass production (Allison et al., 2010; Schindlbacher et al., 2015; Tian et al., 2023a). In the warmed soil, elevated microbial activity and extracellular enzyme production, such as β-glucosidase and N-acetylglucosaminidase, increased the decomposition of soil organic matter (Tian et al., 2023a), depleting easily degradable C sources like amino acids and carbohydrates (Frey et al., 2008; Liu et al., 2024). The initial increase in labile substrates *via* plants due to warming favors bacteria, which utilize low molecular compounds (Castro et al., 2010). However, as these substrates become depleted, microbial competition intensifies, potentially leading to shifts in community structure and functions (Lladó et al., 2017).

4.2. *Warming shifted the fungal community toward ECM over SAP*

Contrary to earlier findings that suggested no impact of warming on fungal community composition in Achenkirch (Kuffner et al., 2012; Schindlbacher et al., 2011), our results demonstrate significant long-term changes (Table 1), favoring the dominance of specific fungal taxa, including ECM and SAP fungi (Figs. 2 and 3), which support our second hypothesis. Similar shifts in fungal communities have been observed in other ecosystems subjected to long-term warming (e.g., D'Alò et al., 2024; Melillo et al., 2017). For instance, Solly et al. (2017) reported that experimental soil warming at the alpine tree line altered fungal community composition, leading to an increased dominance of ECM fungi adapted to higher N availability. This finding aligns with our observation that long-term warming can favor specific fungal taxa. Similarly, Pec et al. (2021) demonstrated that long-term warming (5 and 20 years) at the Harvard Forest resulted in compositional shifts in soil fungal communities, particularly within ECM and SAP, with consequences for soil organic matter chemistry, as demonstrated by the significant loss of lignin. Beyond community composition, warming-induced microbial shifts have been linked to changes in soil C processes. For instance, Wang et al. (2022) found that short-term warming primarily affects bacterial communities and labile C pools, whereas long-term warming drives fungal-mediated decomposition of recalcitrant C, contributing to soil C loss.

The reduction in fungal biomass alongside an increase in fine root biomass and root tip density under soil warming (Kwatcho Kengdo et al., 2022) is surprising. Based on the rise in fine root tips, we anticipated an increase in ECM fungal biomass in the soil (Kwatcho Kengdo et al., 2022). However, many ECM fungi are categorized as contact, short- or medium-distance exploration types, with hyphal networks typically only a few millimeters long (Agerer, 2001). These fine hyphae may have been largely removed from the soil samples during root extraction. In contrast, *Boletus luridus*-2 (Basidiomycota), which forms long-distance hyphae, showed an increase under warming conditions. This pattern aligns with observations from the ECM community on root tips, where *Boletus luridus*-2 was the second most abundant species in the warmed plots (26%) but was absent from control plots (Kwatcho Kengdo et al., 2022). These findings highlight adaptive strategies of root-associated fungi that support root development and enhance nutrient acquisition by trees under elevated temperatures.

The increase in root-associated parameters likely serves as a tree-compensatory mechanism to maintain nutrient cycling and mitigate the effects of warming-induced changes in soil conditions. As nutrient availability has decreased in the warming plots, nutrient requirements of trees can be compensated for, at least in part, by more fine roots and ECM fungi in the subsoil. Increasing absolute abundances of ECM fungi, such as *Boletus luridus* and some species of the genus *Inocybe*, suggest that soil nutrients are being utilized more efficiently at 10-20 cm soil depth. These results underscore the role of warming in driving functional changes in fungal dynamics and plant-root interactions across soil depths. Overall, the findings demonstrate that soil warming not only alters microbial composition but also transforms fungal functional dynamics and their interactions with plants and soil processes.

Considering our second hypothesis, the greater increase in the abundance of ECM compared to SAP fungi under warming in the upper 10 cm of soil (Fig. 5a) can be attributed to their competitive advantages over SAP and as a response to enhanced fine root production. ECM form mutualistic associations with host plants, benefiting from increased tree belowground C allocation (Hawkins et al., 2023), particularly under elevated temperatures (Clemmensen et al., 2006; Kwatcho Kengdo et al., 2022, 2023). Compared to SAP, ECM are not C limited through photosynthate transfer from their host trees and therefore can allocate more C to mobilize limiting nutrients using exoenzymes. ECM fungi therefore likely outcompete SAP for organic matter and nutrients (Averill et al., 2014; Pec et al., 2021), especially in surface soils where root activity is higher or when nutrients are scarce (Kwatcho Kengdo et al., 2022). However, several other studies examining ECM responses to soil warming have found small and/or absent effects in boreal and temperate forests (Fernandez et al., 2017; Mucha et al., 2018; Parrent et al., 2006; Tu et al., 2015). Furthermore, the decline in SAP abundance observed in this study is potentially linked to reductions in SOC. Pec et al. (2021) similarly reported that 20 years of soil warming resulted in compositional shifts in the soil fungal community, particularly in the SAP fungi, which were associated with declines in SOC concentrations, and reductions in lignin content. In line with this observation, overall lower concentrations of carbohydrates and lignin were found at 0 to 20 cm soil depth at our study site in Achenkirch (Schnecker et al., 2016). The absence of a significant increase in ECM abundance at the 10–20 cm depth in the warming plots may reflect depth-specific constraints, such as reduced fine root biomass and limited availability of organic substrates (Kwatcho Kengdo et al., 2022; Parts et al., 2019). This finding supports prior research indicating that soil warming can differentially affect microbial communities and their interactions in soil horizons, potentially altering C and nutrient cycling processes in forest ecosystems.

4.3. Bacterial community responses to soil warming

We did not observe significant alterations in bacterial community composition by long-term soil warming which supports our third hypothesis. This stability may be attributed to two key factors: the inherent adaptability of bacterial communities to elevated temperatures (Stuble et al., 2019; Feng et al., 2017) and their acclimation potential to decreased nutrient availability, *e.g.,* low phosphorous availability (Tian et al., 2023b). Consistent with these findings, previous studies at the Achenkirch site have reported similar patterns of stability in bacterial diversity and composition under warming. For example, Kwatcho Kengdo et al. (2022) observed increased root-associated bacterial diversity without significant shifts in community composition after 14 years of warming, while Kuffner et al. (2012) reported no significant differences in bacterial diversity between control and warmed soils in the short-term. In contrast, Seaton et al. (2022) documented significant community changes in a peaty podzol after 18 years of warming, likely driven by changes in soil pH. In the Achenkirch warming experiment (Table S5), bacteria typically dominate the microbial community due to the near-neutral pH, maintained by the high carbonate contents and calcareous bedrock (Shi et al., 2023). This bacterial dominance in calcareous soils aligns with the findings of Pastore et al. (2022), who reported that phosphorus-solubilizing bacteria in temperate forest soils with calcareous bedrock enhances bedrock weathering and microbial carbonate dissolution. Our findings revealed a higher absolute abundance of *Pseudomonadota* (Methyloceanibacter-1, Bradyrhizobium-3) and the persistence of rare phyla like *Nitrospirota* and *Verrucomicrobiota*, underscoring microbial stability in C and N cycling under sustained warming. These results suggest that in calcareous temperate forest soils, bacterial communities can remain functionally stable despite prolonged warming, possibly buffering ecosystem functions such as nutrient cycling under future climate change.

5. Conclusions

Long-term soil warming shifted fungal community composition while bacterial communities remained mostly unaffected. Warming led to a notable decline in fungal biomass, as evidenced by the reduction in ergosterol, which consequently contributed to the decrease in total MBC. This highlights the greater sensitivity of fungal communities to long-term soil warming compared to bacteria. Our findings reveal that long-term warming promotes ECM over SAP fungi by increasing fine root production and fine root biomass. The relative increase in ECM fungi suggests enhanced plant-fungal nutrient exchange under warming conditions, whereas the decline in SAP fungi coincides with the decrease in microbial carbon use efficiency and decreasing availability of substrates. Together, these changes highlight how long-term warming reshaped microbial community structure and may thereby modulate biogeochemical cycling processes. However, a better mechanistic understanding of these interactions requires explicitly linking not only microbial community composition, but also the activity of specific community members, with process-based carbon flux measurements. Future research should therefore integrate advanced molecular tools, such as metagenomics and metaproteomics, to identify the active taxa of microbial communities and elucidate their functional roles in soil carbon cycling under continued warming.

CRediT authorship contribution statement

Mohammad Rahmat Ullah: Writing – review & editing, Writing – original draft, Visualization, Software, Formal analysis, Data curation. **Steve Kwatcho Kengdo:** Writing – review & editing, Writing – original draft, Software, Investigation, Formal analysis, Data curation. **Derek Peršoh:** Writing – review & editing, Writing – original draft, Validation, Software, Methodology, Investigation, Formal analysis, Data curation. **Ye Tian:** Writing – review & editing, Investigation. **Jakob Heinzle:** Writing – review & editing, Investigation. **Carolina Urbina Malo:** Writing – review & editing, Investigation. **Chupei Shi:** Writing – review & editing, Investigation. **Tillmann Lueders:** Writing – review & editing, Validation, Methodology. **Christian Poll:** Writing – review & editing, Validation, Methodology, Investigation. **Wolfgang Wanek:** Writing – review & editing, Project administration, Funding acquisition, Conceptualization. **Andreas Schindlbacher:** Writing – review & editing, Project administration, Funding acquisition, Conceptualization. **Werner Borken:** Writing – review & editing, Supervision, Resources, Methodology, Investigation, Funding acquisition, Conceptualization.

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.2026.110120.

Data availability

All raw sequence reads of bacteria and fungi are available in the European Nucleotide Archive (http://www.ebi.ac.uk/ena) under the accession number PRJEB48843. Read counts, relative abundances, qPCR gene copy numbers and share of absolute abundances of bacteria and fungi are available in the repository of the University of Bayreuth: https://doi.org/10.57880/rdspace-ubt-49.

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