Field reduction of ectomycorrhizal fungi has cascading effects on soil microbial communities and reduces the abundance of ectomycorrhizal symbiotic bacteria

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Abstract

Specific interactions between bacteria and ectomycorrhizal fungi (EcMF) can benefit plant health, and saprotrophic soil fungi represent a potentially antagonistic guild to these mutualisms. Yet there is little field-derived experimental evidence showing how the relationship among these three organismal groups manifests across time. To bridge this knowledge gap, we experimentally reduced EcMF in forest soils and monitored both bacterial and fungal soil communities over the course of a year. Our analyses demonstrate that soil trenching shifts the community composition of fungal communities toward a greater abundance of taxa with saprotrophic traits, and this shift is linked to a decrease in both EcMF and a common ectomycorrhizal helper bacterial genus, *Burkholderia*, in a time-dependent manner. These results not only reveal the temporal nature of a widespread tripartite symbiosis between bacteria, EcMF, and a shared host tree, but they also refine our understanding of the commonly referenced 'Gadgil effect' by illustrating the cascading effects of EcMF suppression and implicating soil saprotrophic fungi as potential antagonists on bacterial-EcMF interactions.

Introduction

Bacteria and fungi represent two of the most dominant microbial groups in forest soils (Anthony et al. 2023; Uroz et al. 2016), and their roles in forest microbiomes range from plant pathogen suppression (Tahat et al. 2010; Weller et al. 2002) to biogeochemical cycling (Zhour et al. 2018), plant growth promotion (Hayat et al. 2010; Mohammadi et al. 2011), and inter-microbial growth facilitation (Deveau et al. 2018). A major impediment to our understanding of forest microbiomes, however, is that much of our mechanistic knowledge comes from laboratory studies, which cannot capture the adaptive, complexity of forest soils (Tecon et al. 2019). Similarly, most efforts have focused on the soil dynamics of one or two single microbial guilds, such as bacteria, ectomycorrhizal fungi (EcMF), or saprotrophs (Awad et al. 2019; Hawkins et al. 2023; Rousk J and Bååth 2011). In other cases, the focus has been to highlight distinct community responses of soil bacteria and fungi, providing little insights into how these diverse communities coalesce to exhibit paralleled, dependent responses (Li et al. 2020). These studies have contributed key insights to our collective understanding of forest function, but they have been unable to reconcile the potential interactions that occur between and among multiple microbial guilds (Baldrian 2017).

One commonly referenced inter-guild interaction in forest soil ecology is the so-called 'Gadgil effect' (Gadgil and Gadgil 1971,1975). It encompasses a fundamental process in forest ecology (i.e., soil organic matter decomposition), whereby resource competition between EcMF and saprotrophic fungi, for example, can drive changes in the rate of decomposition and thus affect larger-scale soil biogeochemical cycles (Fernandez and Kennedy 2015). Efforts to understand the mechanisms that govern the 'Gadgil effect' have proven informative to both our conceptual and empirical models of how these microbial guilds coexist within forest microbiomes (Fernandez et al. 2020; Smith and Wan 2019). Reports, for instance, have shown that in some cases EcMF slow carbon cycling in forests by suppressing saprotrophic fungi (Averill and Hawkes 2016), while others have shown that EcMF can drive priming effects that increase the growth of soil saprotrophs (Choreño-Parra EM and Treseder 2024). Much research has therefore demonstrated how EcMF and soil saprotrohic fungi interact, but little is known about how bacteria factor into this equation (Lladó et al. 2017). Experiments primed to address the anticipated global reductions in EcMF (Arnolds 1991; Querejeta et al. 2021; Steidinger et al. 2020) and the subsequent cascading effects on microbial communities (Berrios, Bogar et al. 2024; Delgado-Baquerizo et al. 2020) across time will be fundamental for enhancing the accuracy of current Earth system models (Bonan and Doney 2018; Wieder et al. 2015).

Emerging evidence suggests that bacteria play a vital role in the success and maintenance of ectomycorrhizal symbioses (Berrios, Venturini et al. 2024; Frey-Klett et al. 2007). For instance, in a recent communication, we demonstrated that a small group of bacterial taxa can function as indicators of plant host and EcMF health (Berrios et al. 2023) and even more recently demonstrated that bacterial-EcMF interactions contribute to potential fungal pathogen suppression (Berrios, Bogar et al. 2024). It remains unclear in the literature, however, how the simultaneous reduction in EcMF and increase in soil saprotrophic fungi affect bacterial communities – particularly bacterial genera that function as positive indicators of EcMF and plant health. Moreover, how the potential interactions among these three microbial groups manifest across time remains equally uncertain. For instance, it is likely that a reduction in EcMF would allow soil saprotrophic fungi to predominate, since saprotrophs would then have less competition for space and nutrients. But whether these cascading effects may also weaken positive, synergistic interactions between EcMF and their helper bacteria over time remains untested. Therefore, the extent of bacteria-EcMF symbiotrophy, the potential role of fungal competitors, and the temporal scale that govern positive bacteria-EcMF interactions remain unclear. Efforts to address these uncertainties will not only reveal the spatiotemporal dynamics that govern a widespread tripartite symbiosis among bacteria, EcMF, and plants, but they will refine our concepts surrounding the 'Gadgil effect' and lend insights to the how a predicted global decline in EcMF may affect ecosystem functions (Steidinger et al. 2020; Willing et al. 2024).

To address these knowledge gaps, we conducted an experimental field manipulation in EcMF-dominated forests across a one-year period and investigated how reductions to EcMF in forest soils affected saprotrophic fungal communities and bacterial communities (with a targeted analysis on positive indicators of EcMF and plant health). Specifically, we used a common soil trenching approach to reduce the abundance of EcMF, which allowed us to test our hypotheses that a reduction in EcMF would (1) have cascading effects on saprotrophic fungal communities (particularly soil saprotrophs), (2) reduce the diversity of bacterial communities, and (3) alter the spatiotemporal dynamics of EcMF helper bacteria (i.e., those that were previously identified as positive indicators of EcMF abundance; see Berrios et al. 2023). Our data show that reducing EcMF in forest soils benefits saprotrophic fungi, decreases bacterial richness, and drives down the abundance of some EcMF helper bacteria in a time-dependent manner that also implicates soil saprotrophic fungi as potential competitors of these bacteria-EcMF mutualisms. These analyses therefore highlight the varying degrees of intimacy that exist between bacterial taxa, EcMF, and soil saprotrophs – which refines our understanding of a widespread, positive interaction among bacteria, EcMF, and plants and advances holistic concepts that surround the 'Gadgil effect' and bacterial-fungal interactions.

Methods

Field work and sampling strategy

Field sampling was carried out at Point Reyes National Seashore (PRNS) in Marin County, California, United States (38°04′N, 122°50′W) in October 2021, Janurary 2022, April 2022, and September 2022 (mean annual temperature = 16°C; mean annual precipitation = 91 cm). We established three local sites in monodominant Bishop pine forests (determined by local accessibility and forest structure) that were > 1 km apart to maximize spatial variability in fungal communities. To reduce the abundance of ectomycorrhizal fungi, we established 10 paired plots at each site (3 sites X 2 treatments X 10 replicates). Each plot pair consisted of two 1 m^2 plots with one trenched plot (to reduce EcMF abundance) and one control plot (i.e., untrenched). Trenching was initially done in June of 2021 to a depth of 40 cm using a large shovel to sever

root connections. These trenches were further maintained by retrenching with a handsaw once every two weeks throughout the course of one year. Plot pairs were located 1 m from each other and at least 5 m from any other plot pairs. Since severing roots can alter the influx of carbon (Averill and Hawkes 2016), we began sampling microbial communities in October 2021 (~ 4 months after trenches were established). From this time, soil cores were collected from each plot once every three months to capture seasonal variability and the temporal relationships among soil microbes (20 cores X 3 sites X 4 sampling periods = 240 cores total). Corers were surface sanitized between plots with 70% ethanol (EtOH), and soil washes were performed between each sample collection to limit carryover. Soil cores were also taken at the furthest point between paired trenched and untrenched plots to limit potential trenching effects on untrenched plots. Each core was placed into a separate, clean plastic bag and stored at 4°C within five hours of collection.

Since trenching can introduce unintended nutrient pulses, which could alter microbial activity in soils (Averill and Hawkes, 2016), we controlled for these possible disturbance effects by monitoring soil chemistry, soil water potential, and root ingrowth biomass at two control plots per site – in addition to sampling microbial communities ~ 4 months after trenches were established. These approaches together helped us (1) ensure that trenching soils did not cause significant changes to measured soil chemistry and water availability parameters between trenched and untrenched plots, (2) demonstrate the activity of ectomycorrhizal roots, and (3) establish adequate time for the impact of these potential nutrient pulses to dissipate. Though other trenching studies have tended to only investigate changes to water potential or total nitrogen (Averill and Hawkes, 2016; Hansson et al. 2018; Siira-Pietikäinen et al. 2003), we tested changes to 15 soil chemistry properties that include tests for changes to these typical disturbance-related metrics. Given that EcMF are known to liberate carbon, nitrogen, and phosphorus in most EcMF-dominated forests, our ability to disentangle whether soil communities change in response to increased nutrients that EcMF provide or from new colonizable structures (i.e., EcMF hyphae) is limited (See et al. 2022). Monitoring was done at only two plots per site to avoid disturbing the litter decomposition bags that were installed in the remaining eight plot pairs as part of a concurrent experiment. Soil chemistry was measured using Plant Root Simulator (PRS™) probes equipped with anion and cation exchange resins (Western Ag, Saskatoon, SK, Canada). Soil water content was monitored using a FieldScout TDR 150 Soil Moisture Meter (Spectrum Technologies, Inc.), and root ingrowth was measured by installing rigid mesh cores (20 cm depth) filled with pure sand. Root ingrowth cores were harvested at the end of the year, and the biomass of ectomycorrhizal rootlets was recorded on soil removed during core installation and in the core at the end of the experiment. All soil chemistry, water potential, and root ingrowth biomass data can be found in Fig. S1.

DNA extractions, library preparation, and amplicon sequencing

We characterized microbial communities by first weighing a 0.25 g subsample of collected soil material for DNA extractions, using quality controls appropriate for our system (Zinger et al. 2019). Specifically, our experiment included ten biological replicates sampled across two treatment conditions (n=2, trenched vs. untrenched) at each of three sites (n=3) and collected at four timepoint (n=4), for a total N = 240 (10 x 2 x 3 x 4). For each biological replicate we also included two technical DNA extraction replicates that were pooled prior to PCR. After initial collection in the field, 0.25 soil subsamples were stored at - 20°C until extractions were performed, and all extractions were performed within one week of sample collection. DNA extractions were conducted using a DNeasy PowerSoil Pro Kit according to the manufacturer's specifications (Qiagen, Carlsbad, CA, USA). Extraction controls (i.e., negative controls) were derived using Nanopure water as input extraction material. DNA extracts, including extraction negative controls, PCR negative controls, and positive control from a previously published synthetic mock community as a positive control for fungi (Palmer et al. 2018) and

ZymoBIOMICS Microbial Community Standard (ZYMO Research, USA) as a positive control for bacteria were used as PCR templates to determine both fungal and bacterial community composition. In a previous study we found that PCR replication had minimal effect on ecological inference in fungal community metabarcoding (Smith and Peay 2014). Thus, we chose to use a single technical PCR replicate to characterize each sample. First step PCR amplifications were performed by mixing 1 µl of template DNA, 5 µl of MyTaq^{TM} HS Red Mix, 3.2 µl of PCR-grade H_2O, and 0.4 µl of each primer. The 515f (GTGYCAGCMGCCGCGGTAA) / 806R (GGACTACNVGGGTWTCTAAT) primer pair was used to amplify the V4 hypervariable region of the bacterial 16S rRNA gene (Caporaso et al. 2011), and the KYO1 (CTHGGTCATTTAGAGGAASTAA) / KYO2 (TAGAGGAAGTAAAAGTCGTAA) primer pair was used to amplify the ITS1 subregion of the fungal rRNA operon (Toju et al. 2012). Extraction controls and positive controls (using mock bacterial and fungal communities) were also used as DNA templates for PCR amplifications. The PCR amplifications occurred in 96-well plates and consisted of 35 cycles with a denaturation temperature of 95°C, an annealing temperature of 50°C (52.5°C for fungi), and an extension temperature of 72°C. Indexed tags for Illumina sequencing were attached during a second step PCR amplification (Toju et al. 2016), which consisted of eight cycles with identical temperature settings as the first step PCR. Gel electrophoresis was used to confirm PCR products, and a magnetic bead purification method using Sera-Mag SpeedBeads (MilliporeSigma, Munich, Germany) was used to clean PCR products. A Qubit 3.0 fluorometer (Thermo Fisher, Waltham, MA, USA) was used to quantify cleaned DNA. Lastly, we pooled our samples into two libraries at equimolar concentrations and submitted them for 2 x 300 Illumina MiSeq sequencing at the Stanford Functional Genomics Facility. Raw sequences are available from NCBI Short Read Archive (BioProject: PRJNA1170419).

Bioinformatics and statistical analyses

We received 9,148,012 demultiplexed reads (avg. per sample = 52,274) for our bacterial dataset and 8,968,351 demultiplexed reads (avg. per sample = 49,541) for our fungal dataset. After filtering, denoising, merging forward and reverse reads, and removing chimeric sequences using the DADA2 workflow (Callahan et al. 2016), our bacterial dataset consisted of 3,697,587 reads (avg. per sample = 21,705), and our fungal dataset consisted of 5,211,759 reads (avg. per sample = 29,470). The DADA2 workflow accuracy was evaluated by inferring and matching mock community members (i.e., positive controls) to their expected sequences (residual error rate = 0%). Taxonomic classifications were assigned to bacteria and fungi using the SILVA (Quast et al. 2012) and UNITE (Nilsson et al. 2019) databases, respectively. Sequences assigned to 'chloroplast,' 'mitochondria,' and 'archaea' were removed from our bacterial dataset. For both our bacterial and fungal datasets, we removed taxa with less than 10 sequences, and samples were rarefied to a read depth of 5,000 using the rarefy_even_depth function in the *phyloseq* package in R (McMurdie and Holmes 2013). Since our negative controls contained less than these sequence number cutoffs, they were removed during sub-setting and rarefaction. After rarefaction, we retained 162 samples in our bacterial dataset and 159 samples in our fungal dataset. Our bacterial dataset consisted of 3,480 amplicon sequence variants (ASVs), and our fungal dataset consisted of 1,242 ASVs. Guild designations for fungi were assigned at the genus level using the FungalTraits database (Põlme et al. 2020).

Statistical analyses were performed in R studio version 4.2.1 (Team RC). The *phyloseq* package was used to derive alpha (α) and beta (β) diversity estimates (McMurdie and Homles 2013). The *microbiome* package was used to relativize our amplicon sequence abundance data (Lahti and Shetty 2017). We tested for differences in bacterial (n=162) and fungal (n=159) community composition between conditions (i.e., trenched versus untrenched soils) and as a function of seasonal variation using permutational analyses of variance (PERMANOVA) as implemented in the adonis2 function from the *vegan* package (Oksanen et al. 2007). The

betadisper function was used to test for homogeneity of variances. To investigate interactions between bacteria and fungi, we used linear regression models to examine correlations between the abundance of both bacteria and EcM fungi and bacteria and soil saprotrophic fungi from our amplicon datasets. Since soil chemistry analyses demonstrated that trenching had no statistically significant effect on soil moisture and only one of sixteen analyzed soil chemicals, and our root ingrowth data demonstrated that trenching significantly restricted host roots for EcMF (Fig. S1), the effects from trenching that we observed in our models are likely to be primarily the result of biotic interactions. Our statistical models included trenching treatment, sampling timepoint (i.e., seasonal variation), site location, and the interaction between each of these categorical variables and the quantitative variables in question as predictor variables. For models that sought to identify the statistical significance of tripartite interactions, we used sequence abundance ratios since this approach integrates the direct relationship between fungal guilds and bacterial sequences within and between samples by default. Each model was generated with the lm function in the *stats* R package (Team RC). For partial regression plots, we plotted the residuals of response variables (y-axis) by the residuals of all the co-variates that contribute to the main predictor variable (x-axis). We determined the relative importance of predictor variables in the final models as the r^2 contribution averaged over orderings among regressors (the 'lmg' option in calc.relimpo from the relaimpo package; Grömping 2006). All plots were generated using either base R or *ggplot2* (Wickham and Wickham 2007). The code used to analyze these datasets can be found at https://github.com/LouisBerrios/Gadgil_Project.

Results

The effects of soil trenching and seasonality on fungal community composition

Since trenching can introduce unintended soil disturbance (Averill and Hawkes 2016), we first tested that our control plots did not differ substantially between trenching treatments. When we analyzed soil chemistry, we found phosphorus concentrations were significantly elevated in untrenched plots (relative to trenched plots) but that trenching had no significant effect on soil moisture content or on the remaining 15 chemicals that were analyzed ($p > 0.05$; Fig. S1A-Q). We also measured ectomycorrhizal root ingrowth masses and found that trenching effectively reduced the amount of ectomycorrhizal colonized rootlets and had no significant effect on the mass of other plant roots (Fig. S1R). Therefore, these control plot data suggest that detected changes to microbial communities are likely not an artefact of our experimental set-up and that our trenching approach effectively decreased the abundance of ectomycorrhizal fungi (EcMF).

To identify how soil trenching, seasonality, and the interaction between the two affected the composition of soil fungal communities, we first derived α and β diversity estimates for whole fungal communities. α-diversity (i.e., observed richness) estimates were marginally affected by trenching ($F_{1,157} = 3.24$; $p = 0.073$) and statistically unaffected by season ($F_{3,155} = 1.78$; $p = 0.154$), and interactions between trenching and season were not observed (Table S1; Fig. S2). The β-diversity of whole fungal communities was significantly changed by both trenching (PERMANOVA: $F_{1,157} = 2.41$; $p = 0.004$) and seasonal variation (PERMANOVA: $F_{3,155} = 1.65$; $p = 0.004$), but no significant interaction between the two was observed (Table S2; Fig. S3A). When we assessed individual changes to EcMF and saprotrophic fungi communities, however, we found that trenching had a marginally significant effect on EcMF communities (PERMANOVA: $F_{1,157} = 1.50$; $p = 0.068$; Table S3), with no seasonal effects detected (PERMANOVA: $F_{3,155} = 0.904$; $p = 0.68$), whereas both trenching (PERMANOVA: $F_{1,157} = 3.16$; $p = 0.003$) and season (PERMANOVA: $F_{3,155} = 2.41$; $p = 0.001$) significantly changed saprotrophic communities (Table S4; Fig. S3). Like whole fungal communities, no interaction between trenching treatment and season was detected for either ectomycorrhizal or saprotrophic fungal communities. In addition to these observed mean effects on composition, we also found significant differences in compositional variance (i.e., sample to sample variability within

treatments) for whole fungal communities and saprotrophic fungal communities, whereby heterogenous dispersions across trenching treatment and season indicated that untrenched treatments and winter and spring seasons had the most compositional variance. In contrast, EcMF communities were homogenously dispersed (Fig. S4). While PERMANOVA is relatively unaffected by heterogeneity in dispersion (Anderson and Walsh 2013), the observed treatment effects are likely due to changes in both mean and dispersion.

Next, we sought to understand how the relative abundance and taxonomic composition of ectomycorrhizal and saprotrophic fungal communities changed, since soil trenching should have significant effects on their abundance. On average, soil trenching decreased the relative abundance of EcMF and increased the relative abundance of saprotrophic fungi (Fig. 1). Analysis of variance (ANOVA) models further demonstrated that trenching and season had significant effects on the abundance of these fungal guilds (Fig. 1B-C). However, like many of our findings reported above, an interaction between trenching and season was not observed for EcM (ANOVA: $F_{3,155} = 0.544$; $p = 0.653$) or saprotrophic fungal communities (ANOVA: $F_{3,155} = 0.803$; $p = 0.494$). In terms of taxonomy, a total of 30 EcM genera and 114 saprotrophic fungal genera (i.e., dung, litter, soil, wood, and unspecified) were detected (Fig. S5; Table S5). We observed variable inter-genus compositional shifts for EcMF communities (Fig. S4). Some genera (e.g., *Hydnum* and *Inocybe*), for instance, were consistently suppressed in trenched soils, while trenching increased the relative abundance of other genera (e.g., *Amanita* and *Polyozellus*). For saprotrophic fungi, we focused our taxonomic analysis on soil saprotrophs since these were the most abundant and trench-responsive saprotrophs in our dataset (Fig. 1A; Table S5). Like EcMF genera, soil saprotrophs also displayed differing responses to trenching at the genus-level (Fig. S5). The genus *Geminibasidium*, for example, remained more abundant in trenched plots across each timepoint compared to untrenched plots, whereas *Saitozyma* – the most dominant soil saprotrophic genus – was continually suppressed by soil trenching. Collectively, these fungal community sequence data demonstrate that our soil trenching methods effectively decreased the relative abundance of EcMF and increased the relative abundance of saprotrophic fungi – though inter-guild and inter-genus variations were observed.

Bacterial community compositional changes caused by soil trenching and seasonality

We derived estimates of bacterial richness (i.e., α-diversity) and β-diversity to understand how soil trenching, seasonality, and the interaction between these two variables impacted bacterial community structure. Both trenching and season had significant effects on bacterial richness, whereby richness was typically highest in untrenched soils (Fig. 2A; Table S6) and followed a sinusoidal curve over time on average, with peaks in winter and summer (Fig. S6). When we assessed changes to β-diversity, we found that trenching (PERMANOVA: $F_{1,160} = 1.82$; $p = 0.019$) and season (PERMANOVA: $F_{3,158} = 2.88$; $p = 0.001$) had significant effects – though no interaction between these variables was detected (PERMANOVA: $F_{3,158} = 0.916$; $p = 0.667$). A beta dispersion tests suggested that the communities compared between trenching conditions were dispersed homogeneously (Fig. S7A), whereas communities across seasons were dispersed heterogeneously with the greatest distance to centroids occurring during winter and spring ($F_{3,158} = 4.98$; $p = 0.002$; Fig. S7; Table S7B).

Taxonomically, trends at the phyla-level and finer hierarchical levels were evident. For instance, the phylum Acidobacteria was suppressed by soil trenching, whereas – relative to untrenched plots – the phylum Verrucomicrobiota was elevated (Fig. 2C). Other phyla exhibited seasonal trends but exhibited no obvious changes due to trenching (e.g., Acinobacteriota elevated in Fall and Summer sampling periods and Bacteroidota elevated in Winter and Spring). At the genus level, we observed 349 bacterial genera in our dataset. To narrow our genus-level investigation, we targeted 11 bacterial genera that can be indicators of EcMF relative abundance (Berrios et al. 2023). Most of these genera, however, did not show strong responses to soil trenching and seasonal variation, but a few genera appeared to be either depleted (i.e.,

Burkholderia), enriched (i.e., *Acidibacter*), or seasonally affected (i.e., *Puia*) due to trenching (Fig. S8). Together, these data converge on the notion that reducing EcMF in forest soils decreases bacterial richness and alters the dynamics of a small group of bacteria.

Relationships between EcMF, soil saprotrophs, bacterial richness, and Burkholderia species

Because soil trenching had significant effects on EcMF, saprotrophic fungi (particularly soil saprotrophs), and bacterial communities (Fig. 1 and Fig. 2), we wanted to further investigate the links between these microbes. To these ends, we first constructed linear regression models that used EcMF relative sequence abundance as predictor variables for bacterial richness. We found that EcMF sequence abundance correlated positively with bacterial richness and functioned as significant predictors of bacterial richness ($F_{1,80} = 4.88$; $p = 0.03$), though trenching alone explained much of the predictive strength in our model ($F_{1,80} = 7.51$; $p = 0.008$; Fig. 3A; Table S8). In addition, we detected an interaction between EcMF abundance and season/sampling timepoint ($F_{3,78} = 5.06$; $p = 0.003$). These observations, paired with the fact that (1) soil saprotrophs and EcMF compete for resources and resultantly drive each other's spatiotemporal dynamics (Fernandez and Kennedy 2016) and (2) soil saprotrophs were both the most abundant saprotrophic fungal guild in our dataset and were most sensitive to seasonal and trenching effects (Fig. 1A), led us to investigate whether soil saprotroph abundance could predict bacterial richness. Our linear regression analysis demonstrated that soil saprotrophic fungi correlated negatively with bacterial richness and that both saprotroph sequence abundance and the effect of trenching were significant predictors of bacterial richness (Fig. 3B). In each of our models (Fig. 3), season also appeared to have a significant effect, with detectable interactions between season and EcMF abundance (Table S8) and between season and soil saprotroph abundance (Table S9). Site location was not a significant factor for either model. Because relative abundance data can present some interdependence between measured variables, we next used the ratio of EcMF and soil saprotrophic fungi relative abundances as predictors of bacterial richness. Our regression model (Table S10) demonstrated that indeed the ratio of EcMF and soil saprotrophs functioned as a significant predictor of bacterial richness ($F_{1,80} = 14.37$; $p = 0.0003$), and the individual r^2 contributions of season ($r^2 = 11\%$), EcMF:soil saprotroph ratios ($r^2 = 7.5\%$), and EcMF:soil saptroph ratios as a function of time ($r^2 = 11\%$) totaled roughly that of what we observed in our regression models without ratios.

To understand how soil trenching and season alter the relationships between EcMF and bacteria in the genus *Burkholderia* – which we have found to be a positive indicator of tree seedling and EcMF health in this system (Berrios et al. 2023) – we first examined the degree to which significant decreases in the relative abundance of *Burkholderia* species were attributable to soil trenching and season. We found that a significant decrease in *Burkholderia* species manifested after one year post trench establishment (Fig. 4A). To further enhance our understanding of these relationships, we constructed a linear regression model (predictor variables = *Burkholderia* relative sequence abundance, trenching treatment, sampling season, and sampling site; response variable = EcMF relative sequence abundance). Though site location was a significant predictor of EcMF sequence abundance, our model indicated that *Burkholderia* sequence abundance was also a significant predictor (Fig. 4B). However, since the effect of trenching has potentially cascading effects on the abundance of soil saprotrophic fungi and *Burkholderia* sequences over time (Fig. 3), we built in soil saprotroph, sampling timepoint/season, and sampling site interaction terms (Table S11). Indeed, this refinement improved overall model strength by 14% (i.e., increased r^2 from 35% to 49%), detected a statistical interaction between *Burkholderia* and soil saprotrophs ($F_{1,80} = 14.10$; $p = 0.0003$), and reiterated the time-dependent response we observed (Fig. 4A) for the potential interactions between *Burkholderia* and EcMF ($F_{3,78} = 4.10$; $p = 0.009$). Therefore, these data sum to illustrate that a reduction in EcMF alters the spatiotemporal dynamics of soil saprotrophs, which has

varied effects on bacterial communities and elicits a time-dependent response for a common bacterial symbiont of EcMF and pine trees.

Discussion

How we have come to understand the complexity of forest microbiomes has been derived primarily through the observed interactions of one or two microbial guilds – an approach that has both answered and left open many questions. Multiple lines of evidence, for instance, have shown that competition between EcMF and saprotrophic fungi can lead to changes in soil nutrient economies (Averill and Hawkes 2016; Bending 2003; Gadgil and Gadgil 1971,1975). This phenomenon, commonly called the 'Gadgil effect,' can have substantial impacts on forest functions (Fernandez and Kennedy 2016; Simard et al. 1997), but no experimental field evidence has shown how the competition between EcMF and saprotrophic fungi may impact the spatiotemporal dynamics of soil bacteria – particularly bacteria that support EcMF and plant health. With this knowledge gap in mind, we conducted a year-long trenching experiment in a tractable forest system (Peay and Bruns 2014; Peay et al. 2010) to understand how a reduction in EcMF alters the spatiotemporal dynamics of bacteria, EcMF, and saprotrophic fungi. Our data demonstrate that a reduction in EcMF not only alters the community composition of bacteria and fungi, but this reduction increases soil saprotrophic fungi and significantly reduces the relative abundance of ectomycorrhizal helper bacteria in the genus *Burkholderia* (Berrios et al. 2023) after about one year (see Fig. 5 for a conceptual diagram). This longitudinal field study therefore refines our view of a long-standing phenomenon that occurs in EcMF-dominated forest soils (i.e., the 'Gadgil effect'), providing a nuanced understanding of the factors that contribute to symbioses between bacteria and fungi.

Consistent with previous findings (Fernandez et al. 2020), our data show that soil trenching drives down the relative abundance of EcMF and enriches for soil saprotrophic fungi (Fig. 1; Fig. S1). How this occurs has received considerable attention (Lang et al. 2021; Malik 2019; Sterkenberg et al. 2010), and the degree to which soil disturbance, changes in soil moisture content, the release of organic substrates post-trenching, and direct interactions between EcMF and soil saprotrophs influence the 'Gadgil effect' have been difficult parameters to untangle in complex systems (Averill and Hawkes 2016; Brzostek et al. 2015; Lindahl et al. 2010). Although our analyses cannot separate the magnitude and direction for each of these potential variables, our trenching controls suggest that the observed shifts in community structure are not simply artefacts of treatment induced changes in the soil environment (Fig. S1), and our regression models likewise capture the effects of trenching as a function of seasonal variability (Fig. 1; Fig. S3-S5; Table S1-S4). The detection of seasonal variability is key here because it suggests that we detected changes in microbial activity and not simply relic DNA. In addition, experiments primed to address the disturbance effects of soil trenching – perhaps the largest potential confounder of these experiments – have shown that the often-used alternative method (i.e., using mesh bags to exclude EcMF) generates similar results to those of soil trenching experiments (Averill and Hawkes 2016). Therefore, it is unlikely that the observations we report herein are simply artefacts of soil disturbance – though decreased soil phosphorus availability in trenched plots cannot be omitted as a potential contributor. In line with this notion is the fact that our analyses are unable to detect whether and to what degree organic matter that EcMF liberate in soils, hyphal biomass as a new colonizable niche, or a combination of the two facilitated the changes to microbial communities (See et al. 2022). Further, the mechanisms that drive the standard view of the 'Gadgil effect' are largely out of the scope of this communication. For example, it is likely that the inter-genus compositional changes of EcMF are linked to competition-colonization trade-offs within the EcMF guild (Fig. S5), and these interactions could play a role in how soil saprotrophic fungi respond to a reduction in EcMF. Nonetheless, we

instead discuss the intra- and inter-guild changes of fungal communities only to the extent that allows us to situate our primary focus (i.e., the spatiotemporal dynamics of bacterial communities) in the context of inter-guild fungal competition and bacterial-fungal interactions.

Though seasonality affected the composition of whole fungal communities and saprotrophic fungal communities, observed shifts in EcMF communities were less evident (Table S2-S4; Fig. S3-S4). Some reports have noted significant seasonal effects on EcMF – both in terms of mycelial biomass in soils (Wallander et al. 2001) and host root colonization (Soudzilovskaia et al. 2015; Swaty et al. 1998). Others, however, have observed marginal seasonal effects on EcMF communities, noting instead strong effects of season on saprotrophic fungal communities (Gorfer et al. 2021) – such as those that we observed during the spring for soil saprotrophic fungi, which may correspond to a concomitant shift to warmer, wetter weather (Fig. 1). These discrepancies are likely a product of local, climatic and edaphic factors (Beidler et al. 2023; Gong et al. 2022), and the lack of detectable seasonal effects on EcMF communities in our system likely reflects the relatively consistent seasonal nature of the environment in an evergreen, coastal Mediterranean ecosystem. The significant degree of temporal shifts that we observe for other fungal guilds, however, may be a result of comparatively fast growth rates (i.e., slower for EcMF and higher for other guilds), which may also factor into the potential competitive play between EcMF and other fungal guilds. Therefore, it is not too surprising that we do not observe large, seasonal shifts of EcMF composition. But why EcMF abundances were greater in colder months than warmer months (Fig. 1A) is likely due to the greater physiological activity of Bishop pine trees during the colder months in California, which may be caused, in part, by greater precipitation. It's also known that EcMF communities can have species-specific responses to temperature (Koizumi and Nara 2020), and our data illustrate that EcMF also respond differentially at the genus level (e.g., *Cortinarius* exclusively detected during fall and winter; see Fig. S5). These intra-guild dynamics are also likely driven my similar intra-guild dynamics that shape saprotrophic fungi, which together ultimately shape the inter-guild changes that we observed. Nevertheless, a closer examination of how seasonality impacts competition-colonization tradeoffs within EcMF communities could help clarify these presumably context-dependent observations (Kennedy et al. 2011; Moeller and Peay 2016; Smith et al. 2018).

When we investigated how soil trenching and seasonality affected bacterial communities, we found that these factors had variable effects (Fig. 2; Fig. S6; Fig. S7). Trenching decreased bacterial richness, and seasonal effects were observed, but we did not observe a detectable interaction between the two factors (Fig. 2A; Table S6). These observations are consistent with recent field (Berrios et al. 2023) and greenhouse (Berrios, Bogar et al. 2024) studies, and the absence of an interaction between soil trenching and sampling season suggests that seasonal variability neither intensifies nor suppresses the effects we observed from trenching. In terms of bacterial β-diversity, seasonal variation was the strongest and most significant driver, and – like our observations on bacterial richness – we did not detect an interaction between soil trenching and sampling season (Fig. 2B; Fig. S7; Table S7). Previous reports have shown that EcMF enrich a small group of bacterial taxa (Berrios et al. 2023; Berrios, Bogar et al. 2024; Bowen GD and Theodorou 1979; Nguyen and Bruns 2015), so it is therefore not surprising that only small, community-wide differences in bacterial composition were detected (Fig. 2; Fig. S7). In line with this notion, taxonomic shifts in bacterial communities were also moderate (Fig. 2C). On average, the effect of trenching decreased the relative abundance of acidophilic (e.g., *Acidibacter*) or acidotolerant (e.g., *Burkholderia*) bacterial taxa and elevated the relative abundance of Verrucomicrobiota (Fig. 2C; Fig.S8). These results are likely associated with a decrease in EcMF and an increase in saprotrophic fungi in trenched plots (Fig. 1), since EcMF tend to foster a lower pH, and saprotrophic fungi favor a relatively more neutral pH (Yamanaka

2003). However, they do not fully capture the inter-phyla dynamics of bacteria since some Verrucomicrobiota are also acidophilic (Dunfield et al. 2007). In addition, many previously reported indicator bacterial taxa of EcMF relative abundance (Berrios et al. 2023; Berrios, Bogar et al. 2024; Nguyen and Bruns 2015) were relatively unaffected by soil trenching (Fig. S8), suggesting that (1) many of these taxa may thrive on the necromass of EcMF (Maillard et al. 2024); (2) there was still sufficient EcMF available for them to find a niche, and (3) soil saprotrophs play a significant role in the spatiotemporal dynamics of these bacterial-EcMF interactions (Fig. 3).

The bacterial genus *Burkholderia* has been linked to enhanced ectomycorrhizal fungal colonization and pine seedling growth (Berrios et al. 2023), and it has been a temporally stable bacterium in Bishop pine forests for decades (Nguyen and Bruns 2015). Therefore, we sought to uncover how decreasing EcMF in forest soils affected the abundance of this genus over time. Our data demonstrated that soil trenching decreased the relative abundance of *Burkholderia* over time (Fig. 4A) and that *Burkholderia* sequence abundance functioned as a positive predictor of EcMF sequence abundance (Fig. 4B), which aligns with previous field and greenhouse observations (Berrios et al. 2023; Nguyen and Bruns 2015). Past analyses, however, were unable to reconcile the spatiotemporal dynamics of this ectomycorrhizal helper bacteria and moreover had not situated these findings in a longitudinal study within the complex, adaptive environment of forest soils. Our analyses here not only demonstrate that the reduction of EcMF (and likely active, healthy plant host roots) drives down the abundance of *Burkholderia* species (Fig. 4), but they implicate soil fungal saprotrophs as a potentially antagonistic factor for the proliferation of these species ($F_{1,80}$ = 14.10; p = 0.0003; Table S8). Why it took a year before trenching effects significantly impacted these bacteria may be explained by the fact that our soil trenching methods can be viewed as a 'knock-down' rather than a 'knock-out' of EcMF in this system. The most parsimonious explanation is therefore that *Burkholderia* species were still deriving benefits from EcMF in trenched plots, though direct necromass uptake is unlikely for this genus given a lack of detectable chitinase activity in a previous transcriptomic study (Berrios et al. 2023) and a general lack of genes that encode chitinases in many publicly available *Burkholderia* genomes (Berrios 2022a). In contrast, a less conservative explanation is that the suppression of EcMF in trenched plots derepressed soil saprotrophic fungi, which resultantly attenuated the tight association between EcMF and *Burkholderia* through resource competition. The exact mechanism for this bacterial-fungal competition is unclear, but a recent analysis demonstrated that soil fungi are up to four times more efficient at obtaining complex compounds in the soil (Wang and Kuzyakov 2024). Though direct biotic warfare (e.g., antibiotic production) may also explain these observations. Nevertheless, these findings do clarify the temporal relationships among a previously reported widespread tripartite relationship among bacteria, EcMF, and a shared plant host (Berrios et al. 2023), but further studies that use additional manipulative and quantitative approaches would help strengthen these observations.

The field of bacterial-fungal interactions continues to emerge as an important yet neglected sub-discipline in microbial ecology and holds promise for both fundamental and applied research in forest ecology (Artursson et al. 2006; Berrios 2022b; Berrios, Venturini et al. 2024; Frey-Klett et al. 2011; Manfredini et al. 2021). These observations, coupled with the critical role of EcMF for terrestrial carbon cycling (Hawkins et al. 2023) and the anticipated global warming-fueled rise in plant pathogenic soil saprotrophs (Delgado-Baquerizo et al. 2020), suggest that more efforts to understand the interactions between bacteria, EcMF, and saprotrophs are warranted and timely. In this study, we leverage a tractable forest system to understand the spatiotemporal response of bacterial communities and previously reported ectomycorrhizal helper bacteria to a reduction of EcMF. Our data demonstrate that a reduction in EcMF has cascading effects on saprotrophic fungal communities and contributes to a time-dependent reduction in the genus *Burkholderia* –

bacteria that function as positive indicators of EcMF and tree health (Berrios et al. 2023). With predictions that point to EcMF diversity loss (Arnolds 1991; Steidinger et al. 2020), a predominance of saprotrophic fungi (Delgado-Baquerizo et al. 2020), and climate-related mismatches between soil microbiota and their plant hosts (Rudgers et al. 2020), it will become even more essential to understand the temporal dynamics that govern multipartite relationships between plants and microbes (Berrios and Rentsch 2022; Gschwend et al. 2021). Our data collectively highlight that a loss of EcMF will not only cause a loss of bacterial biodiversity, but it will also cause a loss of bacteria that benefit plant hosts and symbiotic fungi – which may further exacerbate competition between EcMF and soil saprotrophic fungi. These observations therefore begin to clarify the temporal dynamics of common EcMF-bacteria interactions and contribute a nuanced view of how bacterial communities factor into the potential interactions surrounding the so-called 'Gadgil effect.'

It will be essential moving forward to understand how bacteria – especially mycorrhizal helper bacteria – both facilitate and interfere with competition between EcMF and saprotrophic fungi. From our data, it is clear that the degree of symbiotrophy between bacteria and EcMF differ across space, time, and taxonomic designation (Fig. 2-4), which could potentially impact the magnitude, direction, type, and outcome of interactions among various bacterial, fungal, and plant groups. A major consideration therefore is the focal metric or proxy being measured and what those system-wide outcomes might be at varying scales. In our system, EcMF are key for the establishment and maintenance of the host tree, and we have identified bacterial taxa that support ecological interactions of ectomycorrhizal symbiosis. But how these multipartite relationships control processes that link carbon budgets, fire ecology, and climate resilience, and how they can be applied to further conservation efforts remains less apparent (Bisbing et al. 2023; Taylor et al. 2020). Though our data cannot identify, for example, changes to soil respiration or carbon deposition rates, efforts to gauge these responses should be undertaken because they could serve as important metrics to determine system-wide outcomes of multipartite relationships (Averill and Hawkes, 2016). Given the innumerable context dependencies that shape plant-soil interactions, it is likely that the level of 'benefit' and 'detriment' that soil microbial communities provide and incur, respectively, changes over time and with respect to community context (Berrios 2022b; Fukami 2015). Experiments that investigate the eco-evolutionary relationships among bacteria, EcMF, saprotrophs, and host trees will help organize these context dependencies and clarify our predictions of how forests may change in the future. How we then integrate this information into Earth system models (Bonan and Doney 2018) and leverage them for forest conservation efforts remains a primary research agenda that could reveal key insights into soil nutrient economies (Wieder et al. 2015), atmospheric exchange processes (Sutton et al. 2007), and land management strategies (Pongratz et al. 2018) as we traverse further into the Anthropocene.

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Author Contributions: LB designed the experiments, collected and processed the samples, analyzed the data, and wrote the manuscript. KGP established the soil trenches and maintained them throughout the experiment with the help of LB. KGP and LB edited the manuscript.

Figures and Legends

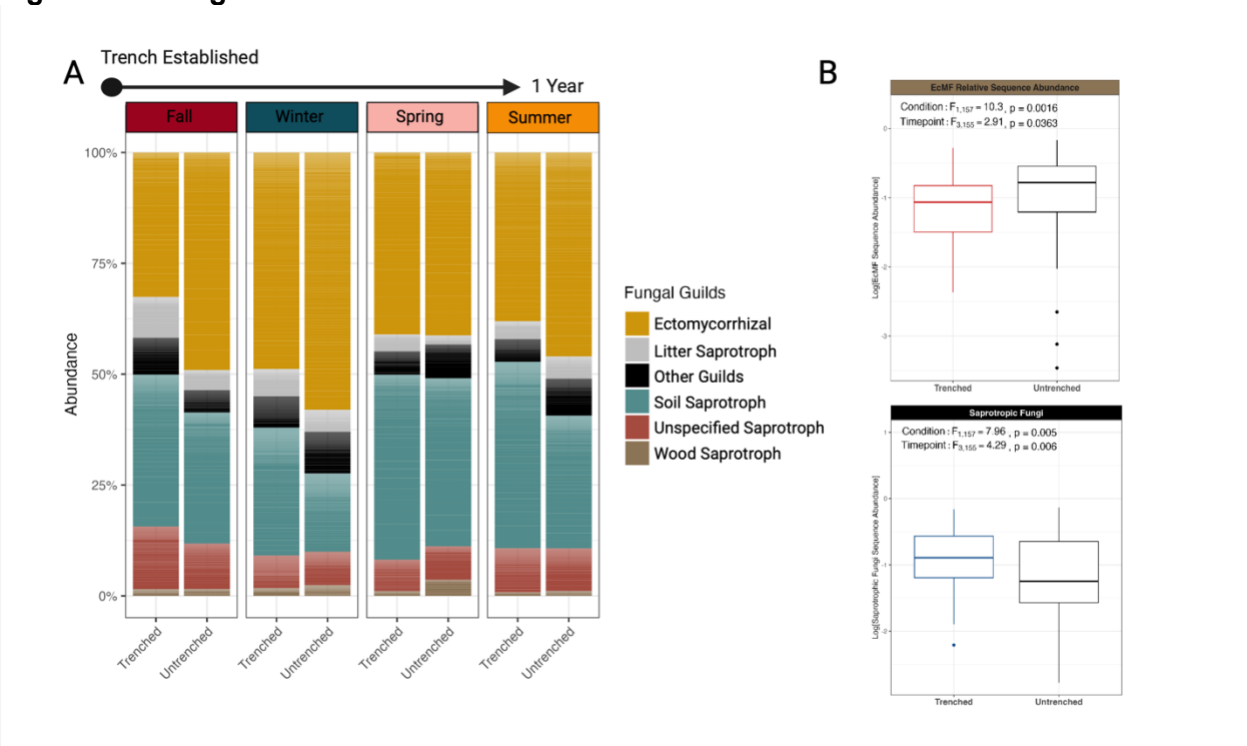

Fig. 1. Compositional changes of fungal taxa. (A) Stacked bar plot showing the compositional changes of fungal guilds across time. The plot is faceted by season (timepoint of sampling) and grouped by condition (i.e., trenched or untrenched). **(B)** Ectomycorrhizal fungi (EcMF) relative sequence abundance (top) and saprotrophic fungi relative sequence abundance (bottom) comparisons between trenched and untrenched soils. ANOVA model statistic outputs are shown in each panel.

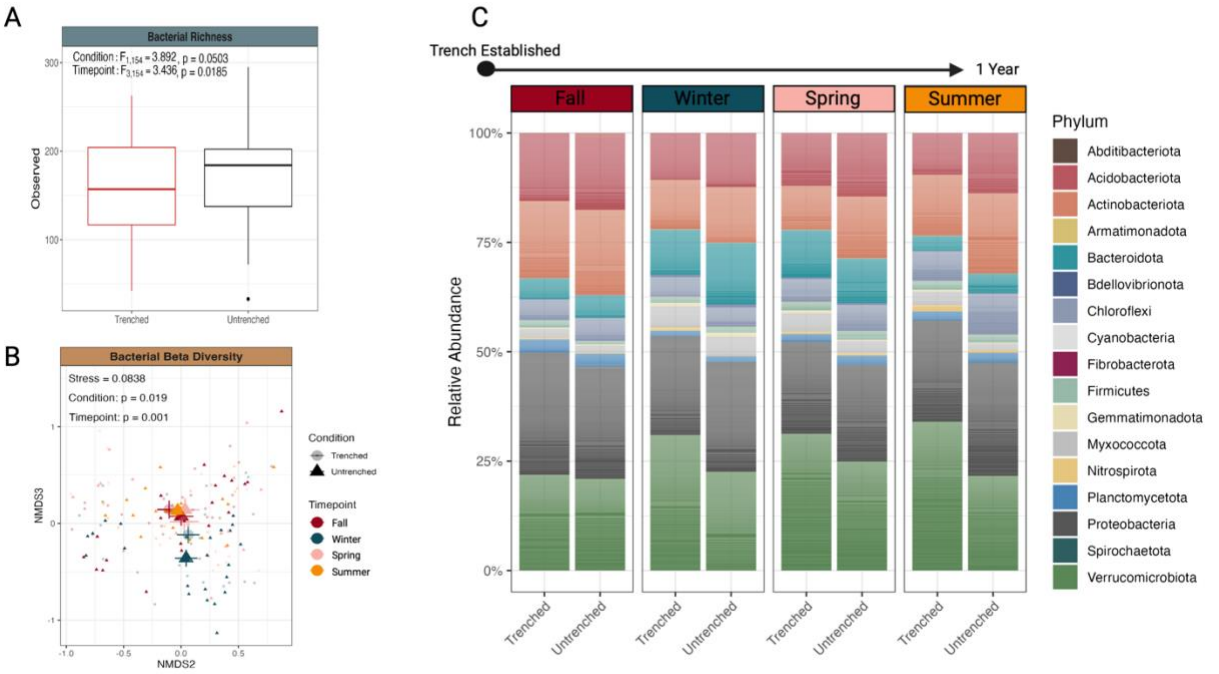

Fig. 2. Compositional changes of bacterial taxa. (A) Observed bacterial richness (i.e., α-diversity) comparisons between trenched and untrenched soils. ANOVA model outputs are shown for the effect of condition (i.e., trenched or untrenched soils) and sampling timepoint (i.e., season). See Table S6 for the complete model output. **(B)** Non-metric multidimensional scaling (NMDS) analysis that illustrates the changes of bacterial β-diversity as a function of condition and sampling timepoint. Centroids for each condition are shown with emanating standard error lines, and PERMANOVA model statistics are shown in the top left corner. See Table S7 for the complete model output. **(C)** Stacked bar plot showing the compositional changes of bacterial phyla across time. The plot is faceted by season (timepoint of sampling) and grouped by condition. Note that the focal bacterial genus in our analysis hereafter (i.e., *Burkholderia*) belongs to the phylum Proteobacteria.

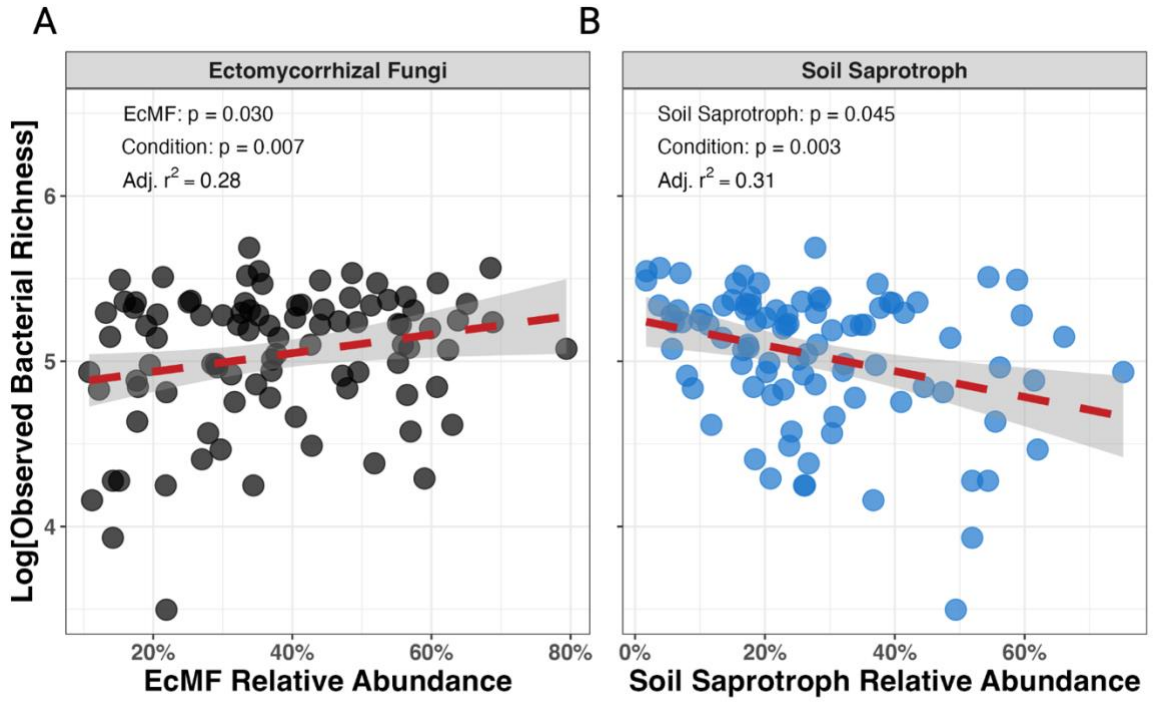

Fig. 3. Relationships between ectomycorrhizal fungi (EcMF), soil saprotrophs, and bacterial richness. (A) Correlation between observed bacterial richness and EcMF relative sequence abundance. Linear regression model statistics are shown. **(B)** Correlation between observed bacterial richness and soil saprotrophs relative sequence abundance. The 95% confidence interval is shown in gray, and linear regression model statistics are shown. Full model outputs are shown in Table S8 and Table S9.

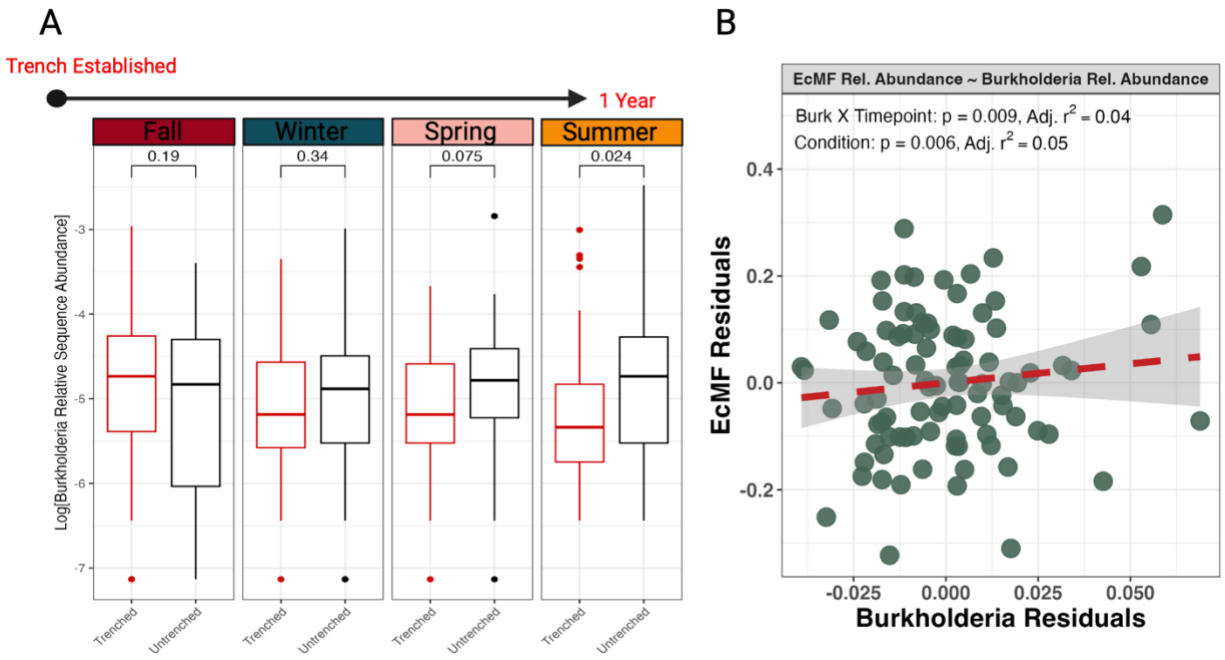

Fig. 4. Relationships between ectomycorrhizal fungi and the bacterial genus *Burkholderia*. (A) Log-transformed relative sequence abundance of amplicon sequence variants (ASVs) matched to the genus *Burkholderia*. Abundance values are grouped by soil condition (i.e., trenched and untrenched) and further faceted by sampling season (as observed in Marin County, CA, USA). P values for t-test statistics are shown above each pairwise comparison. Statistics were performed in R using the stat_compare_means() function in the *ggpubr* package (Kassambara 2023). **(B)** Linear regression analysis investigating the relationship between EcMF relative sequence abundance and *Burkholderia* sequence abundance. Residuals from linear regression outputs are shown. Partial regression r^2 values were derived using the *relaimpo* package in R and are shown for each listed predictor value. The 95% confidence interval is shown in gray, and model output statistics are shown in the panel. See Table S10 for the complete model output statistics.

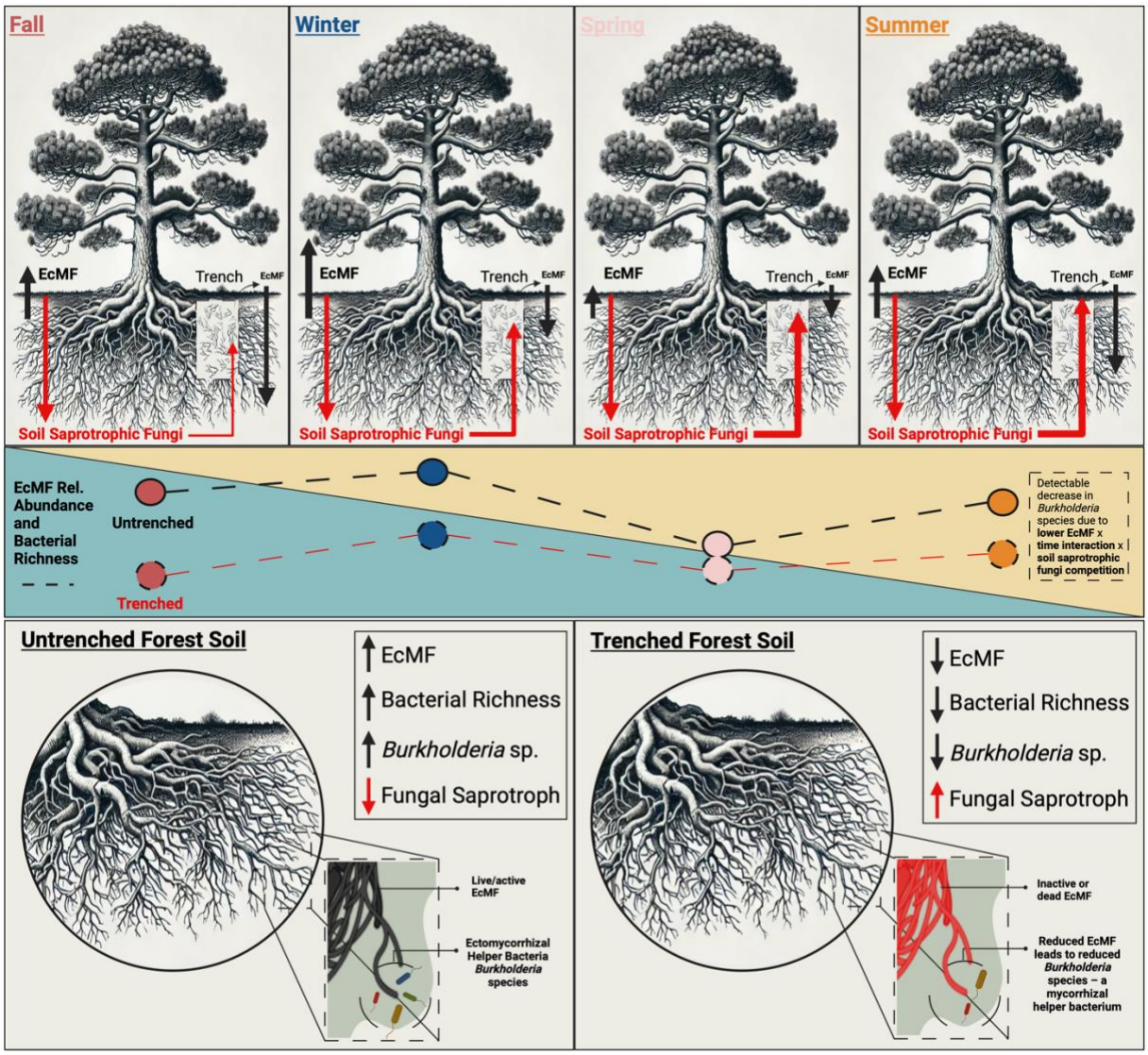

Fig. 5. Conceptual diagram of the spatiotemporal dynamics of bacteria-fungi interactions. The relative increases and degrees of EcMF, soil saprotrophic fungi, and EcMF helper bacteria (i.e., *Burkholderia* species) across time and with respect to forest soil trenching are illustrated. Black arrows correspond to EcMF, and red arrow correspond to soil saprotrophic fungi. In general, we see that EcMF relative abundances are increased in untrenched plots compared to trenched plots and that soil saprotrophic fungi relative abundances are decreased in untrenched plots (relative to trenched plots). The thickness and direction of arrows correspond to the relative change in abundance (i.e., thicker arrows = stronger relative change and thinner arrows = weaker, less detectable relative change). For example, the relative abundance of EcMF in untrenched plots was observed to increase strongly in the winter, whereas a moderate rise in soil saprotrophic fungi was observed in the winter, followed by a strong increase in the spring. We also illustrate the coupled fluctuations of EcMF relative abundance, bacterial richness, and amplicon sequence variants (ASVs) matched to *Burkholderia* species throughout the seasons (shown by the line-connected dots), where dots higher on our scale correspond to greater relative abundance and vice versa. Lastly, the general pattern of microbial fluctuations is shown to illustrate the differences in soil microbiota (particularly the changes to the mycorrhizal helper

bacterial genus, *Burkholderia*) between untrenched and trenched forest soils. Raw data for this visual summary can be found throughout the main text and supplementary data, and the depictions represented here function solely to capture the primary results from our analyses.

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